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Application Number: 15589968

Document Date: 05/08/2017

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- Sequence Listing

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I hereby certify that this paper is being transmitted via the Office electronic filing system in accordance with 37 CFR § 1.6(a)(4).

Dated: May 8, 2017

Electronic Signature for Conchita Picazo-Mejia: /Conchita Picazo-Mejia/

Docket No.: 680132000106
(PATENT)

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Patent Application of:
Klaus GIESE et al.

Application No.: Not Yet Assigned

Confirmation No.: Not Yet Assigned

Filed: Concurrently Herewith

Art Unit: Not Yet Assigned

For: INTERFERING RNA MOLECULES

Examiner: Not Yet Assigned

FIRST PRELIMINARY AMENDMENT UNDER 37 C.F.R. 1.115

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Dear Sir:

INTRODUCTORY COMMENTS

Prior to examination on the merits, Applicant respectfully requests entry of this Preliminary Amendment for the above-captioned patent application.

Amendments to the Specification begin on page 2 of this paper.

Remarks/Arguments begin on page 3 of this paper.

Submitted herewith is an Information Disclosure Statement (IDS).

AMENDMENTS TO THE SPECIFICATION

Please amend the first paragraph after the title as follows:

CROSS REFERENCE TO RELATED APPLICATIONS

This application is a continuation of U.S. Patent Application, 14/977,710, filed December 22, 2015, which is a continuation of U.S. Patent Application 14/578,636, filed on December 22, 2014, now U.S. Patent No. 9,222,092, issued December 29, 2015, which is a continuation of U.S. Patent Application 13/692,178, filed on December 3, 2012, now U.S. Patent No. 8,933,215, issued January 13, 2015, which is a continuation of U.S. Patent Application 12/986,389, filed on January 7, 2011, now U.S. Patent No. 8,324,370, issued December 4, 2012, which is a continuation of U.S. Patent Application 12/200,296, filed on August 28, 2008, now U.S. Patent 7,893,245, issued February 22, 2011, which is a continuation of U.S. Patent Application No. 10/633,630, filed on August 5, 2003, now U.S. Patent No. 7,452,987, issued November 18, 2008, which claims the benefit of U.S. Provisional Application No. 60/402,541, filed August 12, 2002, which claims priority to European application No. 02017601.2, filed August 5, 2002[[.]], and European application No. 03008383.6, filed April 10, 2003. Each of these applications are incorporated herein by reference in their entirety, including all figures, tables and amino acid or nucleic acid sequences.

~~The Sequence Listing for this application is labeled "Seq-List.txt" which was created on August 28, 2008 and is 84 KB. The entire contents of the sequence listing is incorporated herein by reference in its entirety.~~

SUBMISSION OF SEQUENCE LISTING ON ASCII TEXT FILE

The content of the following submission on ASCII text file is incorporated herein by reference in its entirety: a computer readable form (CRF) of the Sequence Listing (file name: 680132000106seqlist.txt, date recorded: May 1, 2017, size: 85 KB).

REMARKS**Regarding the Specification Amendment**

The “Cross Reference to Related Applications” paragraph has been added to reflect the related applications and, as such, does not constitute new matter.

Also, the specification has been amended to refer to submission of a Sequence Listing on an ASCII text file in the present application. Applicant requests consideration and entry of the Sequence Listing. No new matter has been added by these amendments.

If it is determined that a telephone conference would expedite the prosecution of this application, the Examiner is invited to telephone the undersigned at the number given below.

In the event the U.S. Patent and Trademark Office determines that an extension and/or other relief is required, applicant petitions for any required relief including extensions of time and authorizes the Commissioner to charge the cost of such petitions and/or other fees due in connection with the filing of this document to **Deposit Account No. 03-1952** referencing *docket no. 68013200106*. However, the Commissioner is not authorized to charge the cost of the issue fee to the Deposit Account.

Dated: May 8, 2017

Respectfully submitted,

Electronic signature: /Jian Xiao/
Jian Xiao

Registration No.: 55,748
MORRISON & FOERSTER LLP
755 Page Mill Road
Palo Alto, California 94304-1018
(650) 813-5736

**DECLARATION (37 CFR 1.63) FOR UTILITY OR DESIGN APPLICATION USING AN
APPLICATION DATA SHEET (37 CFR 1.76)**

Title of Invention	Interfering RNA Molecules	COPY
<p>As the below named inventor, I hereby declare that:</p> <p>This declaration is directed to: <input type="checkbox"/> The attached application, or <input checked="" type="checkbox"/> United States application or PCT international application number <u>13/692,178</u> filed on <u>December 3, 2012</u></p> <p>The above-identified application was made or authorized to be made by me.</p> <p>I believe that I am the original inventor or an original joint inventor of a claimed invention in the application.</p> <p>I hereby acknowledge that any willful false statement made in this declaration is punishable under 18 U.S.C. 1001 by fine or imprisonment of not more than five (5) years, or both.</p> <p>WARNING:</p> <p>Petitioner/applicant is cautioned to avoid submitting personal information in documents filed in a patent application that may contribute to identity theft. Personal information such as social security numbers, bank account numbers, or credit card numbers (other than a check or credit card authorization form PTO-2038 submitted for payment purposes) is never required by the USPTO to support a petition or an application. If this type of personal information is included in documents submitted to the USPTO, petitioners/applicants should consider redacting such personal information from the documents before submitting them to the USPTO. Petitioner/applicant is advised that the record of a patent application is available to the public after publication of the application (unless a non-publication request in compliance with 37 CFR 1.213(a) is made in the application) or issuance of a patent. Furthermore, the record from an abandoned application may also be available to the public if the application is referenced in a published application or an issued patent (see 37 CFR 1.14). Checks and credit card authorization forms PTO-2038 submitted for payment purposes are not retained in the application file and therefore are not publicly available.</p>		
<p>LEGAL NAME OF INVENTOR</p> <p>Inventor: <u>KLAUS GIESE</u> Date (Optional): <u>08/12/2012</u></p> <p>Signature: <u>[Signature]</u></p>		
<p>Note: An application data sheet (PTO/SB/14 or equivalent), including naming the entire inventive entity, must accompany this form. Use an additional PTO/SB/AIA01 form for each additional inventor.</p>		

This collection of information is required by 35 U.S.C. 115 and 37 CFR 1.63. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.11 and 1.14. This collection is estimated to take 1 minute to complete, including gathering, preparing, and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, VA 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. SEND TO: Commissioner for Patents, P.O. Box 1460, Alexandria, VA 22313-1460.

If you need assistance in completing the form, call 1-800-PTO-9199 and select option 2.

Under the Paperwork Reduction Act of 1995, no persons are required to respond to a collection of information unless it displays a valid OMB control number.

DECLARATION (37 CFR 1.63) FOR UTILITY OR DESIGN APPLICATION USING AN APPLICATION DATA SHEET (37 CFR 1.76)

Title of Invention	Interfering RNA Molecules
--------------------	---------------------------

As the below named inventor, I hereby declare that:

This declaration is directed to: ☐ The attached application, or ☒ United States application or PCT international application number 13/692,178 filed on December 3, 2012

The above-identified application was made or authorized to be made by me.

I believe that I am the original inventor or an original joint inventor of a claimed invention in the application.

I hereby acknowledge that any willful false statement made in this declaration is punishable under 18 U.S.C. 1001 by fine or imprisonment of not more than five (5) years, or both.

WARNING:

Petitioner/applicant is cautioned to avoid submitting personal information in documents filed in a patent application that may contribute to identity theft. Personal information such as social security numbers, bank account numbers, or credit card numbers (other than a check or credit card authorization form PTO-2038 submitted for payment purposes) is never required by the USPTO to support a petition or an application. If this type of personal information is included in documents submitted to the USPTO, petitioners/applicants should consider redacting such personal information from the documents before submitting them to the USPTO. Petitioner/applicant is advised that the record of a patent application is available to the public after publication of the application (unless a non-publication request in compliance with 37 CFR 1.213(a) is made in the application) or issuance of a patent. Furthermore, the record from an abandoned application may also be available to the public if the application is referenced in a published application or an issued patent (see 37 CFR 1.14). Checks and credit card authorization forms PTO-2038 submitted for payment purposes are not retained in the application file and therefore are not publicly available.

LEGAL NAME OF INVENTOR	
Inventor: <u>JÖRG KAUFMANN</u>	Date (Optional): <u>14 Dec, 2012</u>
Signature: <u>[Signature]</u>	

Note: An application data sheet (PTO/SB/14 or equivalent), including naming the entire inventive entity, must accompany this form. Use an additional PTO/SB/AIA01 form for each additional inventor.

This collection of information is required by 35 U.S.C. 115 and 37 CFR 1.63. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.11 and 1.14. This collection is estimated to take 1 minute to complete, including gathering, preparing, and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, VA 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. SEND TO: Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.

If you need assistance in completing the form, call 1-800-PTO-9198 and select option 2.

Under the Paperwork Reduction Act of 1995, no persons are required to respond to a collection of information unless it displays a valid OMB control number.

DECLARATION (37 CFR 1.63) FOR UTILITY OR DESIGN APPLICATION USING AN APPLICATION DATA SHEET (37 CFR 1.76)

Title of
Invention

Interfering RNA Molecules

As the below named inventor, I hereby declare that:

This declaration
is directed to:

☐

The attached application, or

☒

United States application or PCT international application number 13/692,178

filed on December 3, 2012

The above-identified application was made or authorized to be made by me.

I believe that I am the original inventor or an original joint inventor of a claimed invention in the application.

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LEGAL NAME OF INVENTOR

Inventor: ANKE KLIPPEL-GIESE

Date (Optional): Dec 14, 2012

Signature: Anke Klippel-Giese

Note: An application data sheet (PTO/SB/14 or equivalent), including naming the entire inventive entity, must accompany this form. Use an additional PTO/SB/AIA01 form for each additional inventor.

This collection of information is required by 35 U.S.C. 115 and 37 CFR 1.63. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.11 and 1.14. This collection is estimated to take 1 minute to complete, including gathering, preparing, and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, VA 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. SEND TO: Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.

If you need assistance in completing the form, call 1-800-PTO-9199 and select option 2.

I hereby certify that this paper is being transmitted via the Office electronic filing system in accordance with 37 CFR § 1.6(a)(4).

Dated: May 8, 2017

Electronic Signature for Conchita Picazo-Mejia: /Conchita Picazo-Mejia/

Patent
Docket No. 680132000106

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Patent Application of:
Klaus GIESE et al.

Application No.: Not Yet Assigned

Confirmation No.: Not Yet Assigned

Filed: Concurrently Herewith

Art Unit: Not Yet Assigned

For: INTERFERING RNA MOLECULES

Examiner: Not Yet Assigned

**INFORMATION DISCLOSURE
STATEMENT UNDER 37 C.F.R. § 1.97 & § 1.98**

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Dear Sir:

Pursuant to 37 C.F.R. § 1.97 and § 1.98, Applicant submits for consideration in the above-identified application the documents listed on the attached Form PTO/SB/08a/b. Copies of the documents were previously submitted in an Information Disclosure Statement and/or Office Action, directed to the related U.S. Patent Application Number 14/977,710, filed December 22, 2015, and, accordingly, copies are not included herewith. This protocol conforms with 37 C.F.R. § 1.98(d) and M.P.E.P. 609.02(A)(2). The Examiner is requested to make these documents of record in the application.

Applicant would like to draw the Examiner's attention to the fact that document numbered 23. (US-2014/220676-A1), listed on the attached Form PTO/SB/08a/b is the English language counterpart of document numbered 59. (DE-19903713), 60. (DE-19956568-A1), 69. (EP-1550719-A1) and 70. (EP-1798285-A1).

pa-1785674

This Information Disclosure Statement is submitted:

- ☒ With the application; accordingly, no fee or separate requirements are required.
- ☐ With the filing of a Request for Continued Examination under 37 C.F.R. § 1.114; accordingly, no fee or separate requirements are required.
- ☐ Before the mailing of a first Office Action after the filing of a Request for Continued Examination under 37 C.F.R. § 1.114; accordingly, no fee or separate requirements are required. However, if applicable, a certification under 37 C.F.R. § 1.97 (e)(1) has been provided.
- ☐ Within three months of the application filing date or before mailing of a first Office Action on the merits; accordingly, no fee or separate requirements are required. However, if applicable, a certification under 37 C.F.R. § 1.97 (e)(1) has been provided.
- ☐ After receipt of a first Office Action on the merits but before mailing of a final Office Action or Notice of Allowance.
 - ☐ The required fee is submitted herewith.
 - ☐ A Certification under 37 C.F.R. § 1.97(e) is provided above; accordingly, no fee is believed to be due.
- ☐ After mailing of a final Office Action or Notice of Allowance, but before payment of the Issue Fee.
 - ☐ A Certification under 37 C.F.R. § 1.97(e) is provided above and the required fee is submitted herewith.

Applicant would appreciate the Examiner initialing and returning the Form PTO/SB/08a/b, indicating that the information has been considered and made of record herein.

The information contained in this Information Disclosure Statement under 37 C.F.R. § 1.97 and § 1.98 is not to be construed as a representation that: (i) a complete search has been made; (ii) additional information material to the examination of this application does not exist; (iii) the information, protocols, results and the like reported by third parties are accurate or enabling; or (iv) the above information constitutes prior art to the subject invention.

In the unlikely event that the transmittal form is separated from this document and the Patent and Trademark Office determines that an extension and/or other relief (such as payment of a fee under 37 C.F.R. § 1.17 (p)) is required, Applicant petitions for any required relief including extensions of time and authorizes the Commissioner to charge the cost of such petition and/or other fees due in connection with the filing of this document to **Deposit Account No. 03-1952** referencing **Docket No.: 680132000106**. However, the Commissioner is not authorized to charge the cost of the issue fee to the Deposit Account.

Dated: May 8, 2017

Respectfully submitted,

Electronic signature: /Jian Xiao/
Jian Xiao
Registration No.: 55,748
MORRISON & FOERSTER LLP
755 Page Mill Road
Palo Alto, California 94304-1018
(650) 813-5736

Substitute for form 1449/PTO INFORMATION DISCLOSURE STATEMENT BY APPLICANT <i>(Use as many sheets as necessary)</i>				Complete if Known	
				Application Number	Not Yet Assigned
				Filing Date	Concurrently Herewith
				First Named Inventor	Klaus GIESE
				Art Unit	Not Yet Assigned
				Examiner Name	Not Yet Assigned
Sheet	1	of	12	Attorney Docket Number	680132000106

U.S. PATENT DOCUMENTS					
Examiner Initials*	Cite No. ¹	Document Number Number-Kind Code ² (if known)	Publication Date MM-DD-YYYY	Name of Patentee or Applicant of Cited Document	Pages, Columns, Lines, Where Relevant Passages or Relevant Figures Appear
	1.	US-2003/0027783-A1	02-06-2003	Zernicka-Goetz et al.	
	2.	US-2003/0108923-A1	06-12-2003	Tuschl et al.	
	3.	US-2003/0135033-A1	07-17-2003	Klippel-Giese et al.	
	4.	US-2003/0190635-A1	10-09-2003	McSwiggen, James A.	
	5.	US-2004/0053875-A1	03-18-2004	Kreutzer et al.	
	6.	US-2004/0072779-A1	04-15-2004	Kreutzer et al.	
	7.	US-2004/0102408-A1	05-27-2004	Kreutzer et al.	
	8.	US-2004/0106569-A1	06-03-2004	Klippel-Giese et al.	
	9.	US-2004/0175703-A1	09-09-2004	Kreutzer et al.	
	10.	US-2004/0180351-A1	09-16-2004	Giese et al.	
	11.	US-2004/0229266-A1	11-18-2004	Tuschl et al.	
	12.	US-2005/0043263-A1	02-24-2005	Giese et al.	
	13.	US-2005/0100907-A1	05-12-2005	Kreutzer et al.	
	14.	US-2005/0142535-A1	06-30-2005	Damha et al.	
	15.	US-2006/0094678-A1	05-04-2006	Vornlocher et al.	
	16.	US-2006/0142226-A1	06-29-2006	Polisky et al.	
	17.	US-2006/0217329-A1	09-28-2006	Feinstein, Elena	
	18.	US-2006/0240022-A1	10-26-2006	Klippel-Giese et al.	
	19.	US-2007/0027097-A1	02-01-2007	Lewis, D. L.	
	20.	US-2007/0093445-A1	04-26-2007	Tuschl et al.	
	21.	US-2007/0135372-A1	06-14-2007	MacLachlan et al.	
	22.	US-2007/0265220-A1	11-15-2007	Rossi et al.	
	23.	US-2014/220676-A1	08-07-2014	Kreutzer et al.	English Counterpart to DE-19903713; DE-19956568-A1; EP-1550719-A1; and EP-1798285-A1
	24.	US- 4,217,294	08-12-1980	Petty, Herbert E.	
	25.	US- 5,466,786	11-14-1995	Buhr et al.	
	26.	US-5,514,546	05-07-1996	Kool, Eric T.	
	27.	US-5,589,389	12-31-1996	Pages et al.	
	28.	US-5,670,633	09-23-1997	Cook et al.	
	29.	US-5,792,847	08-11-1998	Buhr et al.	
	30.	US-5,872,232	02-16-1999	Cook et al.	
	31.	US-5,898,031	04-27-1999	Crooke, Stanley T.	
	32.	US-5,914,396	06-22-1999	Cook et al.	
	33.	US-5,955,589	09-21-1999	Cook et al.	
	34.	US-6,005,087	12-21-1999	Cook et al.	
	35.	US-6,107,094	08-22-2000	Crooke, Stanley T.	
	36.	US-6,129,912	10-10-2000	Hortin et al.	
	37.	US-6,153,737	11-28-2000	Manoharan et al.	
	38.	US-6,165,772	12-26-2000	Bertling, Wolf	
	39.	US-6,239,272-B1	05-29-2001	Beigelman et al.	
	40.	US-6,284,267-B1	09-04-2001	Aneja, Rajindra	
	41.	US- 60/352,873	02-01-2002	Damha et al.	
	42.	US-60/358,580	02-20-2002	Beigelman et al.	
	43.	US-60/363,124	03-11-2002	Beigelman et al.	
	44.	US-60/386,782	06-06-2002	Beigelman et al.	
	45.	US-6,476,205-B1	11-05-2002	Buhr et al.	
	46.	US-6,506,559-B1	01-14-2003	Fire et al.	
	47.	US-6,531,584-B1	03-11-2003	Cook et al.	
	48.	US-6,617,438-B1	09-09-2003	Beigelman et al.	

Substitute for form 1449/PTO INFORMATION DISCLOSURE STATEMENT BY APPLICANT <i>(Use as many sheets as necessary)</i>				Complete if Known	
				Application Number	Not Yet Assigned
				Filing Date	Concurrently Herewith
				First Named Inventor	Klaus GIESE
				Art Unit	Not Yet Assigned
				Examiner Name	Not Yet Assigned
Sheet	2	of	12	Attorney Docket Number	680132000106

	49.	US-6,812,204-B1	11-02-2004	McHale et al.	
	50.	US-7,056,704-B2	06-06-2006	Tuschl et al.	
	51.	US-7,074,863-B2	07-11-2006	Ekholm et al.	
	52.	US-7,078,196-B2	07-18-2006	Tuschl et al.	
	53.	US-7,138,517-B2	11-21-2006	Cook et al.	
	54.	US-7,148,342-B2	12-12-2006	Tolentino et al.	
	55.	US-7,196,184-B2	03-27-2007	Heidenreich et al.	
	56.	US-7,348,314-B2	03-25-2008	John et al.	

FOREIGN PATENT DOCUMENTS						
Examiner Initials*	Cite No. ¹	Foreign Patent Document	Publication Date MM-DD-YYYY	Name of Patentee or Applicant of Cited Document	Pages, Columns, Lines, Where Relevant Passages Or Relevant Figures Appear	T ⁶
		Country Code ³ -Number ⁴ -Kind Code ⁵ (if known)				
	57.	CA-2476112-A1	08-28-2003	SIRNA Therapeutics, Inc.		
	58.	DE-19618797-A1	11-13-1997	Bertling, Wolf, Prof. Dr.		
	59.	DE-19903713	01-30-1999	KREUTZER ROLAND	English Equivalent US2014220676A1	
	60.	DE-19956568-A1	08-17-2000	KREUTZER ROLAND	English Equivalent US2014220676A1	
	61.	EP-020185724	08-14-2002	Atugen AG		
	62.	EP-0586520-B1	04-19-2000	Isis Pharmaceuticals, Inc. et al.		
	63.	EP-0618925-B1	08-29-2001	Isis Pharmaceuticals, Inc.		
	64.	EP-1144623-B1	08-28-2002	Ribopharma ag		
	65.	EP-1214945-A2	06-19-2002	Ribopharma Ag		
	66.	EP-1230375-B1	07-06-2005	Cancer Research Technology Ltd		
	67.	EP-1407044-B1	09-19-2007	Max-Planck-Gesellschaft zur Forderung et al.		
	68.	EP-1527176-B1	01-03-2007	Atugen AG		
	69.	EP-1550719-A1	07-06-2005	Ainylam Europe AG	English Equivalent US2014/220676A1	
	70.	EP-1798285-A1	06-20-2007	Ainylam Europe AG	English Equivalent US2014/220676A1	
	71.	GB-2417727-B	01-16-2008	Alnylam Europe AG		
	72.	GB-992744-A	05-19-1965	Dennis Harold Ward		
	73.	WO-92/07065-A1	04-30-1992	Max Planck-Gesellschaft Zur Forderung Der Wissenschaften E.V.		
	74.	WO-92/19732-A1	11-12-1992	Genset SA		
	75.	WO-92/20823-A1	11-26-1992	Isis Pharmaceuticals, Inc.		
	76.	WO-93/13121-A1	07-08-1993	Isis Pharmaceuticals, Inc.		
	77.	WO-94/01550-A1	01-20-1994	Hybridon, Inc.		
	78.	WO-95/13834-A1	05-26-1995	Genta Incorporated		
	79.	WO-96/18376-A1	06-20-1996	Henkel KGAA	Google Translation	
	80.	WO-96/18736-A2	06-20-1996	Ribozyme Pharm. Inc.		
	81.	WO-97/41141-A1	11-06-1997	Thomas Jefferson University		
	82.	WO-97/46570-A1	12/11/1997	Isis Pharmaceuticals, Inc.		

Substitute for form 1449/PTO INFORMATION DISCLOSURE STATEMENT BY APPLICANT <i>(Use as many sheets as necessary)</i>				Complete if Known	
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				Filing Date	Concurrently Herewith
				First Named Inventor	Klaus GIESE
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				Examiner Name	Not Yet Assigned
Sheet	3	of	12	Attorney Docket Number	680132000106

				Deutsches Krebsforschungszentrum Stiftung Des Offentlichen Rechts		
83.	WO-98/05770-A2	02-12-1998				
84.	WO-98/53083-A1	11-26-1998		Zeneca LTD		
85.	WO-99/15682-A2	04-01-1999		Plant Bioscience LTD		
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Sheet	4	of	12	Attorney Docket Number	680132000106

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				Application Number	Not Yet Assigned
				Filing Date	Concurrently Herewith
				First Named Inventor	Klaus GIESE
				Art Unit	Not Yet Assigned
				Examiner Name	Not Yet Assigned
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				First Named Inventor	Klaus GIESE
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				First Named Inventor	Klaus GIESE
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				First Named Inventor	Klaus GIESE
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Docket No.: 680132000106
(PATENT)

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Patent Application of:
Klaus GIESE et al.

Application No.: Not Yet Assigned

Confirmation No.: Not Yet Assigned

Filed: Concurrently Herewith

Art Unit: Not Yet Assigned

For: INTERFERING RNA MOLECULES

Examiner: Not Yet Assigned

STATEMENT REGARDING SEQUENCE LISTING

Commissioner for Patents
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Dear Sir:

The undersigned hereby states that the content of the computer readable copy of the Sequence Listing (provided on an ASCII text file), included as part of the application filed herewith, does not include new matter.

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Dated: May 8, 2017

Respectfully submitted,

Electronic signature: /Jian Xiao/
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Electronic Patent Application Fee Transmittal				
Application Number:				
Filing Date:				
Title of Invention:		INTERFERING RNA MOLECULES		
First Named Inventor/Applicant Name:		Klaus GIESE		
Filer:		Jian Xiao/Conchita Picazo-Mejia		
Attorney Docket Number:		680132000106		
Filed as Large Entity				
Filing Fees for Track I Prioritized Examination - Nonprovisional Application under 35 USC 111(a)				
Description	Fee Code	Quantity	Amount	Sub-Total in USD(\$)
Basic Filing:				
UTILITY APPLICATION FILING	1011	1	280	280
UTILITY SEARCH FEE	1111	1	600	600
UTILITY EXAMINATION FEE	1311	1	720	720
REQUEST FOR PRIORITIZED EXAMINATION	1817	1	4000	4000
Pages:				
Claims:				
CLAIMS IN EXCESS OF 20	1202	10	80	800
Miscellaneous-Filing:				

Description	Fee Code	Quantity	Amount	Sub-Total in USD(\$)
PUBL. FEE- EARLY, VOLUNTARY, OR NORMAL	1504	1	0	0
PROCESSING FEE, EXCEPT PROV. APPLS.	1830	1	140	140
Petition:				
Patent-Appeals-and-Interference:				
Post-Allowance-and-Post-Issuance:				
Extension-of-Time:				
Miscellaneous:				
Total in USD (\$)				6540

Electronic Acknowledgement Receipt

EFS ID:	29150490
Application Number:	15589968
International Application Number:	
Confirmation Number:	8531
Title of Invention:	INTERFERING RNA MOLECULES
First Named Inventor/Applicant Name:	Klaus GIESE
Customer Number:	25226
Filer:	Jian Xiao/Conchita Picazo-Mejia
Filer Authorized By:	Jian Xiao
Attorney Docket Number:	680132000106
Receipt Date:	08-MAY-2017
Filing Date:	
Time Stamp:	22:20:56
Application Type:	Utility under 35 USC 111(a)

Payment information:

Submitted with Payment	yes
Payment Type	DA
Payment was successfully received in RAM	\$6540
RAM confirmation Number	050917INTEFSW00008058031952
Deposit Account	031952
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Information:					
2	Application Data Sheet	Application_Data_Sheet_Fillable_PDF_68013_2000106.pdf	1823364	no	10
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3	TrackOne Request	Certification_and_Request_for_Prioritized_Examination_68013_2000106.pdf	33856	no	1
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	Preliminary Amendment		1	1	
	Specification		2	2	
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National Stage of an International Application under 35 U.S.C. 371

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Application Data Sheet 37 CFR 1.76		Attorney Docket Number	680132000106
		Application Number	
Title of Invention	INTERFERING RNA MOLECULES		
<p>The application data sheet is part of the provisional or nonprovisional application for which it is being submitted. The following form contains the bibliographic data arranged in a format specified by the United States Patent and Trademark Office as outlined in 37 CFR 1.76.</p> <p>This document may be completed electronically and submitted to the Office in electronic format using the Electronic Filing System (EFS) or the document may be printed and included in a paper filed application.</p>			

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Inventor	1				Remove
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	Klaus		GIESE		
Residence Information (Select One) US Residency <input checked="" type="radio"/> Non US Residency Active US Military Service					
City	Berlin	Country of Residence ⁱ		DE	
Mailing Address of Inventor:					
Address 1		KROWELSTR. 27			
Address 2					
City	Berlin	State/Province			
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Inventor	2				Remove
Legal Name					
Prefix	Given Name	Middle Name	Family Name	Suffix	
	Jörg		KAUFMANN		
Residence Information (Select One) US Residency <input checked="" type="radio"/> Non US Residency Active US Military Service					
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Mailing Address of Inventor:					
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Inventor	3				Remove
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Application Data Sheet 37 CFR 1.76		Attorney Docket Number	680132000106
		Application Number	
Title of Invention	INTERFERING RNA MOLECULES		

Prefix	Given Name	Middle Name	Family Name	Suffix
	Anke		KLIPPEL-GIESE	
Residence Information (Select One) US Residency <input checked="" type="radio"/> Non US Residency Active US Military Service				
City	Berchtesgaden	Country of Residence ⁱ	DE	

Mailing Address of Inventor:				
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Application Information:

Title of the Invention	INTERFERING RNA MOLECULES		
Attorney Docket Number	680132000106	Small Entity Status Claimed	<input type="checkbox"/>
Application Type	Nonprovisional		
Subject Matter	Utility		
Total Number of Drawing Sheets (if any)	29	Suggested Figure for Publication (if any)	

Filing By Reference:

Only complete this section when filing an application by reference under 35 U.S.C. 111(c) and 37 CFR 1.57(a). Do not complete this section if application papers including a specification and any drawings are being filed. Any domestic benefit or foreign priority information must be provided in the appropriate section(s) below (i.e., "Domestic Benefit/National Stage Information" and "Foreign Priority Information").

For the purposes of a filing date under 37 CFR 1.53(b), the description and any drawings of the present application are replaced by this reference to the previously filed application, subject to conditions and requirements of 37 CFR 1.57(a).

Application number of the previously filed application	Filing date (YYYY-MM-DD)	Intellectual Property Authority or Country ⁱ

Application Data Sheet 37 CFR 1.76		Attorney Docket Number	680132000106
		Application Number	
Title of Invention	INTERFERING RNA MOLECULES		

Publication Information:

<input type="checkbox"/>	Request Early Publication (Fee required at time of Request 37 CFR 1.219)
<input type="checkbox"/>	Request Not to Publish. I hereby request that the attached application not be published under 35 U.S.C. 122(b) and certify that the invention disclosed in the attached application has not and will not be the subject of an application filed in another country, or under a multilateral international agreement, that requires publication at eighteen months after filing.

Representative Information:

Representative information should be provided for all practitioners having a power of attorney in the application. Providing this information in the Application Data Sheet does not constitute a power of attorney in the application (see 37 CFR 1.32). Either enter Customer Number or complete the Representative Name section below. If both sections are completed the customer Number will be used for the Representative Information during processing.			
Please Select One:	<input checked="" type="radio"/> Customer Number	US Patent Practitioner	<input type="radio"/> Limited Recognition (37 CFR 11.9)
Customer Number	25226		

Domestic Benefit/National Stage Information:

This section allows for the applicant to either claim benefit under 35 U.S.C. 119(e), 120, 121, 365(c), or 386(c) or indicate National Stage entry from a PCT application. Providing benefit claim information in the Application Data Sheet constitutes the specific reference required by 35 U.S.C. 119(e) or 120, and 37 CFR 1.78.

When referring to the current application, please leave the "Application Number" field blank.

Prior Application Status	Pending	Remove			
Application Number	Continuity Type	Prior Application Number	Filing or 371(c) Date (YYYY-MM-DD)		
	Continuation of	14977710	2015-12-22		
Prior Application Status	Patented	Remove			
Application Number	Continuity Type	Prior Application Number	Filing Date (YYYY-MM-DD)	Patent Number	Issue Date (YYYY-MM-DD)
14977710	Continuation of	14578636	2014-12-22	9222092	2015-12-29
Prior Application Status	Patented	Remove			
Application Number	Continuity Type	Prior Application Number	Filing Date (YYYY-MM-DD)	Patent Number	Issue Date (YYYY-MM-DD)
14578636	Continuation of	13692178	2012-12-03	8933215	2015-01-13
Prior Application Status	Patented	Remove			
Application Number	Continuity Type	Prior Application Number	Filing Date (YYYY-MM-DD)	Patent Number	Issue Date (YYYY-MM-DD)
13692178	Continuation of	12986389	2011-01-07	8324370	2012-12-04

Application Data Sheet 37 CFR 1.76		Attorney Docket Number		680132000106	
		Application Number			
Title of Invention		INTERFERING RNA MOLECULES			

Prior Application Status		Patented		Remove	
Application Number	Continuity Type	Prior Application Number	Filing Date (YYYY-MM-DD)	Patent Number	Issue Date (YYYY-MM-DD)
12986389	Continuation of	12200296	2008-08-28	7893245	2011-02-22
Prior Application Status		Patented		Remove	
Application Number	Continuity Type	Prior Application Number	Filing Date (YYYY-MM-DD)	Patent Number	Issue Date (YYYY-MM-DD)
12200296	Continuation of	10633630	2003-08-05	7452987	2008-11-18
Prior Application Status		Expired		Remove	
Application Number	Continuity Type	Prior Application Number	Filing or 371(c) Date (YYYY-MM-DD)		
10633630	Claims benefit of provisional	60402541	2002-08-12		
Additional Domestic Benefit/National Stage Data may be generated within this form by selecting the Add button.					Add

Foreign Priority Information:

This section allows for the applicant to claim priority to a foreign application. Providing this information in the application data sheet constitutes the claim for priority as required by 35 U.S.C. 119(b) and 37 CFR 1.55. When priority is claimed to a foreign application that is eligible for retrieval under the priority document exchange program (PDX)ⁱ the information will be used by the Office to automatically attempt retrieval pursuant to 37 CFR 1.55(i)(1) and (2). Under the PDX program, applicant bears the ultimate responsibility for ensuring that a copy of the foreign application is received by the Office from the participating foreign intellectual property office, or a certified copy of the foreign priority application is filed, within the time period specified in 37 CFR 1.55(g)(1).

Remove			
Application Number	Country ⁱ	Filing Date (YYYY-MM-DD)	Access Code ⁱ (if applicable)
02017601.2	EP	2002-08-05	
Remove			
Application Number	Country ⁱ	Filing Date (YYYY-MM-DD)	Access Code ⁱ (if applicable)
03008383.6	EP	2003-04-10	
Additional Foreign Priority Data may be generated within this form by selecting the Add button.			
Add			

Statement under 37 CFR 1.55 or 1.78 for AIA (First Inventor to File) Transition Applications

Application Data Sheet 37 CFR 1.76		Attorney Docket Number	680132000106
		Application Number	
Title of Invention	INTERFERING RNA MOLECULES		

☐ This application (1) claims priority to or the benefit of an application filed before March 16, 2013 and (2) also contains, or contained at any time, a claim to a claimed invention that has an effective filing date on or after March 16, 2013.

NOTE: By providing this statement under 37 CFR 1.55 or 1.78, this application, with a filing date on or after March 16, 2013, will be examined under the first inventor to file provisions of the AIA.

Application Data Sheet 37 CFR 1.76		Attorney Docket Number	680132000106
		Application Number	
Title of Invention	INTERFERING RNA MOLECULES		

Authorization or Opt-Out of Authorization to Permit Access:

When this Application Data Sheet is properly signed and filed with the application, applicant has provided written authority to permit a participating foreign intellectual property (IP) office access to the instant application-as-filed (see paragraph A in subsection 1 below) and the European Patent Office (EPO) access to any search results from the instant application (see paragraph B in subsection 1 below).

Should applicant choose not to provide an authorization identified in subsection 1 below, applicant **must opt-out** of the authorization by checking the corresponding box A or B or both in subsection 2 below.

NOTE: This section of the Application Data Sheet is **ONLY** reviewed and processed with the **INITIAL** filing of an application. After the initial filing of an application, an Application Data Sheet cannot be used to provide or rescind authorization for access by a foreign IP office(s). Instead, Form PTO/SB/39 or PTO/SB/69 must be used as appropriate.

1. Authorization to Permit Access by a Foreign Intellectual Property Office(s)

A. Priority Document Exchange (PDX) - Unless box A in subsection 2 (opt-out of authorization) is checked, the undersigned hereby **grants the USPTO authority** to provide the European Patent Office (EPO), the Japan Patent Office (JPO), the Korean Intellectual Property Office (KIPO), the State Intellectual Property Office of the People's Republic of China (SIPO), the World Intellectual Property Organization (WIPO), and any other foreign intellectual property office participating with the USPTO in a bilateral or multilateral priority document exchange agreement in which a foreign application claiming priority to the instant patent application is filed, access to: (1) the instant patent application-as-filed and its related bibliographic data, (2) any foreign or domestic application to which priority or benefit is claimed by the instant application and its related bibliographic data, and (3) the date of filing of this Authorization. See 37 CFR 1.14(h)(1).

B. Search Results from U.S. Application to EPO - Unless box B in subsection 2 (opt-out of authorization) is checked, the undersigned hereby **grants the USPTO authority** to provide the EPO access to the bibliographic data and search results from the instant patent application when a European patent application claiming priority to the instant patent application is filed. See 37 CFR 1.14(h)(2).

The applicant is reminded that the EPO's Rule 141(1) EPC (European Patent Convention) requires applicants to submit a copy of search results from the instant application without delay in a European patent application that claims priority to the instant application.

2. Opt-Out of Authorizations to Permit Access by a Foreign Intellectual Property Office(s)

☐ A. Applicant **DOES NOT** authorize the USPTO to permit a participating foreign IP office access to the instant application-as-filed. If this box is checked, the USPTO will not be providing a participating foreign IP office with any documents and information identified in subsection 1A above.

☐ B. Applicant **DOES NOT** authorize the USPTO to transmit to the EPO any search results from the instant patent application. If this box is checked, the USPTO will not be providing the EPO with search results from the instant application.

NOTE: Once the application has published or is otherwise publicly available, the USPTO may provide access to the application in accordance with 37 CFR 1.14.

Application Data Sheet 37 CFR 1.76		Attorney Docket Number	680132000106
		Application Number	
Title of Invention	INTERFERING RNA MOLECULES		

Applicant Information:

Providing assignment information in this section does not substitute for compliance with any requirement of part 3 of Title 37 of CFR to have an assignment recorded by the Office.

Applicant	1	Remove
<p>If the applicant is the inventor (or the remaining joint inventor or inventors under 37 CFR 1.45), this section should not be completed. The information to be provided in this section is the name and address of the legal representative who is the applicant under 37 CFR 1.43; or the name and address of the assignee, person to whom the inventor is under an obligation to assign the invention, or person who otherwise shows sufficient proprietary interest in the matter who is the applicant under 37 CFR 1.46. If the applicant is an applicant under 37 CFR 1.46 (assignee, person to whom the inventor is obligated to assign, or person who otherwise shows sufficient proprietary interest) together with one or more joint inventors, then the joint inventor or inventors who are also the applicant should be identified in this section.</p> <p style="text-align: right;">Clear</p>		
<input checked="" type="radio"/> Assignee	Legal Representative under 35 U.S.C. 117	Joint Inventor
Person to whom the inventor is obligated to assign.		Person who shows sufficient proprietary interest
If applicant is the legal representative, indicate the authority to file the patent application, the inventor is:		
<div style="border: 1px solid black; height: 20px; width: 100%;"></div>		
Name of the Deceased or Legally Incapacitated Inventor: <div style="border: 1px solid black; height: 20px; width: 100%;"></div>		
If the Applicant is an Organization check here. <input checked="" type="checkbox"/>		
Organization Name	Silence Therapeutics GMBH	
Mailing Address Information For Applicant:		
Address 1	ROBERT-ROSSLE-STR. 10	
Address 2		
City	BERLIN	State/Province
Country	DE	Postal Code
Phone Number		Fax Number
Email Address		
Additional Applicant Data may be generated within this form by selecting the Add button. Add		

Assignee Information including Non-Applicant Assignee Information:

Providing assignment information in this section does not substitute for compliance with any requirement of part 3 of Title 37 of CFR to have an assignment recorded by the Office.

Application Data Sheet 37 CFR 1.76		Attorney Docket Number	680132000106
		Application Number	
Title of Invention	INTERFERING RNA MOLECULES		

Assignee	1		
Complete this section if assignee information, including non-applicant assignee information, is desired to be included on the patent application publication. An assignee-applicant identified in the "Applicant Information" section will appear on the patent application publication as an applicant. For an assignee-applicant, complete this section only if identification as an assignee is also desired on the patent application publication.			
Remove			
If the Assignee or Non-Applicant Assignee is an Organization check here. <input checked="" type="checkbox"/>			
Organization Name	Silence Therapeutics GMBH		
Mailing Address Information For Assignee including Non-Applicant Assignee:			
Address 1	ROBERT-ROSSLE-STR. 10		
Address 2			
City	BERLIN	State/Province	
Country ⁱ	DE	Postal Code	D-13125
Phone Number		Fax Number	
Email Address			
Additional Assignee or Non-Applicant Assignee Data may be generated within this form by selecting the Add button. Add			

Signature:[Remove](#)

NOTE: This Application Data Sheet must be signed in accordance with 37 CFR 1.33(b). However, if this Application Data Sheet is submitted with the **INITIAL** filing of the application and either box A or B is **not** checked in subsection 2 of the "Authorization or Opt-Out of Authorization to Permit Access" section, then this form must also be signed in accordance with 37 CFR 1.14(c).

This Application Data Sheet **must** be signed by a patent practitioner if one or more of the applicants is a **juristic entity** (e.g., corporation or association). If the applicant is two or more joint inventors, this form must be signed by a patent practitioner, **all** joint inventors who are the applicant, or one or more joint inventor-applicants who have been given power of attorney (e.g., see USPTO Form PTO/AIA/81) on behalf of **all** joint inventor-applicants.

See 37 CFR 1.4(d) for the manner of making signatures and certifications.

Signature	/Jian Xiao/		Date (YYYY-MM-DD)	2017-05-08
First Name	Jian	Last Name	Xiao	Registration Number
				55748
Additional Signature may be generated within this form by selecting the Add button. Add				

Application Data Sheet 37 CFR 1.76		Attorney Docket Number	680132000106
		Application Number	
Title of Invention	INTERFERING RNA MOLECULES		

This collection of information is required by 37 CFR 1.76. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.14. This collection is estimated to take 23 minutes to complete, including gathering, preparing, and submitting the completed application data sheet form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, VA 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. **SEND TO: Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.**

Privacy Act Statement

The Privacy Act of 1974 (P.L. 93-579) requires that you be given certain information in connection with your submission of the attached form related to a patent application or patent. Accordingly, pursuant to the requirements of the Act, please be advised that: (1) the general authority for the collection of this information is 35 U.S.C. 2(b)(2); (2) furnishing of the information solicited is voluntary; and (3) the principal purpose for which the information is used by the U.S. Patent and Trademark Office is to process and/or examine your submission related to a patent application or patent. If you do not furnish the requested information, the U.S. Patent and Trademark Office may not be able to process and/or examine your submission, which may result in termination of proceedings or abandonment of the application or expiration of the patent.

The information provided by you in this form will be subject to the following routine uses:

- 1 The information on this form will be treated confidentially to the extent allowed under the Freedom of Information Act (5 U.S.C. 552) and the Privacy Act (5 U.S.C. 552a). Records from this system of records may be disclosed to the Department of Justice to determine whether the Freedom of Information Act requires disclosure of these records.
- 2 A record from this system of records may be disclosed, as a routine use, in the course of presenting evidence to a court, magistrate, or administrative tribunal, including disclosures to opposing counsel in the course of settlement negotiations.
- 3 A record in this system of records may be disclosed, as a routine use, to a Member of Congress submitting a request involving an individual, to whom the record pertains, when the individual has requested assistance from the Member with respect to the subject matter of the record.
- 4 A record in this system of records may be disclosed, as a routine use, to a contractor of the Agency having need for the information in order to perform a contract. Recipients of information shall be required to comply with the requirements of the Privacy Act of 1974, as amended, pursuant to 5 U.S.C. 552a(m).
- 5 A record related to an International Application filed under the Patent Cooperation Treaty in this system of records may be disclosed, as a routine use, to the International Bureau of the World Intellectual Property Organization, pursuant to the Patent Cooperation Treaty.
- 6 A record in this system of records may be disclosed, as a routine use, to another federal agency for purposes of National Security review (35 U.S.C. 181) and for review pursuant to the Atomic Energy Act (42 U.S.C. 218(c)).
- 7 A record from this system of records may be disclosed, as a routine use, to the Administrator, General Services, or his/her designee, during an inspection of records conducted by GSA as part of that agency's responsibility to recommend improvements in records management practices and programs, under authority of 44 U.S.C. 2904 and 2906. Such disclosure shall be made in accordance with the GSA regulations governing inspection of records for this purpose, and any other relevant (i.e., GSA or Commerce) directive. Such disclosure shall not be used to make determinations about individuals.
- 8 A record from this system of records may be disclosed, as a routine use, to the public after either publication of the application pursuant to 35 U.S.C. 122(b) or issuance of a patent pursuant to 35 U.S.C. 151. Further, a record may be disclosed, subject to the limitations of 37 CFR 1.14, as a routine use, to the public if the record was filed in an application which became abandoned or in which the proceedings were terminated and which application is referenced by either a published application, an application open to public inspections or an issued patent.
- 9 A record from this system of records may be disclosed, as a routine use, to a Federal, State, or local law enforcement agency, if the USPTO becomes aware of a violation or potential violation of law or regulation.

CERTIFICATION AND REQUEST FOR PRIORITIZED EXAMINATION UNDER 37 CFR 1.102(e) (Page 1 of 1)			
First Named Inventor:	Klaus GIESE	Nonprovisional Application Number (if known):	Not Yet Assigned
Title of Invention:	INTERFERING RNA MOLECULES		
APPLICANT HEREBY CERTIFIES THE FOLLOWING AND REQUESTS PRIORITIZED EXAMINATION FOR THE ABOVE-IDENTIFIED APPLICATION.			
<p>1. The processing fee set forth in 37 CFR 1.17(i)(1) and the prioritized examination fee set forth in 37 CFR 1.17(c) have been filed with the request. The publication fee requirement is met because that fee, set forth in 37 CFR 1.18(d), is currently \$0. The basic filing fee, search fee, and examination fee are filed with the request or have been already been paid. I understand that any required excess claims fees or application size fee must be paid for the application.</p> <p>2. I understand that the application may not contain, or be amended to contain, more than four independent claims, more than thirty total claims, or any multiple dependent claims, and that any request for an extension of time will cause an outstanding Track I request to be dismissed.</p> <p>3. The applicable box is checked below:</p> <p>I. <input checked="checked" type="checkbox"/> Original Application (Track One) - Prioritized Examination under § 1.102(e)(1)</p> <p>i. (a) The application is an original nonprovisional utility application filed under 35 U.S.C. 111(a). This certification and request is being filed with the utility application via EFS-Web. ---OR---</p> <p>(b) The application is an original nonprovisional plant application filed under 35 U.S.C. 111(a). This certification and request is being filed with the plant application in paper.</p> <p>ii. An executed inventor's oath or declaration under 37 CFR 1.63 or 37 CFR 1.64 for each inventor, or the application data sheet meeting the conditions specified in 37 CFR 1.53(f)(3)(i) is filed with the application.</p> <p>II. <input type="checkbox"/> Request for Continued Examination - Prioritized Examination under § 1.102(e)(2)</p> <p>i. A request for continued examination has been filed with, or prior to, this form.</p> <p>ii. If the application is a utility application, this certification and request is being filed via EFS-Web.</p> <p>iii. The application is an original nonprovisional utility application filed under 35 U.S.C. 111(a), or is a national stage entry under 35 U.S.C. 371.</p> <p>iv. This certification and request is being filed prior to the mailing of a first Office action responsive to the request for continued examination.</p> <p>v. No prior request for continued examination has been granted prioritized examination status under 37 CFR 1.102(e)(2).</p>			

Signature /Jian Xiao/	Date May 8, 2017
Name (Print/Typed) Jian Xiao	Practitioner Registration Number 55,748
<small>Note: This form must be signed in accordance with 37 CFR 1.33. See 37 CFR 1.4(d) for signature requirements and certifications. Submit multiple forms if more than one signature is required.*</small>	
<div style="border: 1px solid black; display: inline-block; padding: 2px 5px;"><input checked="checked" type="checkbox"/> X</div> *Total of <u>1</u> forms are submitted.	

Attorney Docket No.: 680132000106

I hereby certify that this paper is being transmitted via the Office electronic filing system in accordance with 37 CFR § 1.6(a)(4).	
Dated: May 8, 2017	Electronic Signature for Conchita Picazo-Mejia: /Conchita Picazo-Mejia/

PATENT APPLICATION FEE DETERMINATION RECORD Substitute for Form PTO-875	Application or Docket Number 15/589,968	Filing Date 05/08/2017	<input type="checkbox"/> To be Mailed
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ENTITY: ☒ LARGE ☐ SMALL ☐ MICRO

APPLICATION AS FILED – PART I

(Column 1)

(Column 2)

FOR	NUMBER FILED	NUMBER EXTRA	RATE (\$)	FEE (\$)
<input type="checkbox"/> BASIC FEE (37 CFR 1.16(a), (b), or (c))	N/A	N/A	N/A	
<input type="checkbox"/> SEARCH FEE (37 CFR 1.16(k), (l), or (m))	N/A	N/A	N/A	
<input type="checkbox"/> EXAMINATION FEE (37 CFR 1.16(o), (p), or (q))	N/A	N/A	N/A	
TOTAL CLAIMS (37 CFR 1.16(i))	minus 20 =	*	X \$ =	
INDEPENDENT CLAIMS (37 CFR 1.16(h))	minus 3 =	*	X \$ =	
<input type="checkbox"/> APPLICATION SIZE FEE (37 CFR 1.16(s))	If the specification and drawings exceed 100 sheets of paper, the application size fee due is \$310 (\$155 for small entity) for each additional 50 sheets or fraction thereof. See 35 U.S.C. 41(a)(1)(G) and 37 CFR 1.16(s).			
<input type="checkbox"/> MULTIPLE DEPENDENT CLAIM PRESENT (37 CFR 1.16(j))				
* If the difference in column 1 is less than zero, enter "0" in column 2.			TOTAL	

APPLICATION AS AMENDED – PART II

(Column 1)

(Column 2)

(Column 3)

AMENDMENT	05/08/2017	CLAIMS REMAINING AFTER AMENDMENT	MINUS	HIGHEST NUMBER PREVIOUSLY PAID FOR	PRESENT EXTRA	RATE (\$)	ADDITIONAL FEE (\$)
	Total (37 CFR 1.16(i))	* 30	Minus	** 30	= 0	X \$80 =	0
	Independent (37 CFR 1.16(h))	* 1	Minus	***3	= 0	X \$420 =	0
	<input type="checkbox"/> Application Size Fee (37 CFR 1.16(s))						
	<input type="checkbox"/> FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM (37 CFR 1.16(j))						
TOTAL ADD'L FEE						0	

(Column 1)

(Column 2)

(Column 3)

AMENDMENT	CLAIMS REMAINING AFTER AMENDMENT	MINUS	HIGHEST NUMBER PREVIOUSLY PAID FOR	PRESENT EXTRA	RATE (\$)	ADDITIONAL FEE (\$)
	Total (37 CFR 1.16(i))	*	Minus	**	=	X \$ =
	Independent (37 CFR 1.16(h))	*	Minus	***	=	X \$ =
	<input type="checkbox"/> Application Size Fee (37 CFR 1.16(s))					
	<input type="checkbox"/> FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM (37 CFR 1.16(j))					
TOTAL ADD'L FEE						

* If the entry in column 1 is less than the entry in column 2, write "0" in column 3.

** If the "Highest Number Previously Paid For" IN THIS SPACE is less than 20, enter "20".

*** If the "Highest Number Previously Paid For" IN THIS SPACE is less than 3, enter "3".

The "Highest Number Previously Paid For" (Total or Independent) is the highest number found in the appropriate box in column 1.

LIE
CORALIA BETANCOURT

This collection of information is required by 37 CFR 1.16. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.14. This collection is estimated to take 12 minutes to complete, including gathering, preparing, and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, VA 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. **SEND TO: Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.**

If you need assistance in completing the form, call 1-800-PTO-9199 and select option 2.

INTERFERING RNA MOLECULES

CROSS-REFERENCE TO RELATED APPLICATIONS

This application is a continuation of U.S. Patent Application 14/578,636, filed on
5 December 22, 2014, now U.S. Patent No. 9,222,092, which is a continuation of U.S. Patent
Application 13/692,178, filed on December 3, 2012, now U.S. Patent No. 8,933,215, which is
a continuation of U.S. Patent Application 12/986,389, filed on January 7, 2011, now U.S.
Patent No. 8,324,370, which is a continuation of U.S. Patent Application 12/200,296, filed on
10 August 28, 2008, now U.S. Patent 7,893,245, which is a continuation of U.S. Patent
Application No. 10/633,630, filed on August 5, 2003, now U.S. Patent No. 7,452,987, which
claims the benefit of U.S. Provisional Application No. 60/402,541, filed August 12, 2002.
Each of these applications are incorporated herein by reference in their entirety, including all
figures, tables and amino acid or nucleic acid sequences.

The Sequence Listing for this application is labeled "Seq-List.txt" which was created
15 on August 28, 2008 and is 84 KB. The entire contents of the sequence listing is incorporated
herein by reference in its entirety.

FIELD OF THE INVENTION

The invention provides novel forms of interfering ribonucleic acid molecules having a
20 double-stranded structure. The first strand comprises a first stretch of contiguous nucleotides
that is at least partially complementary to a target nucleic acid, and the second strand
comprises a second stretch of contiguous nucleotides that is at least partially identical to a
target nucleic acid. Methods for using these molecules, for example for inhibiting expression
of a target gene, and pharmaceutical compositions, cells and organisms containing these
25 molecules also are provided.

BACKGROUND OF THE INVENTION

RNA-mediated interference (RNAi) is a post-transcriptional gene silencing
mechanism initiated by double stranded RNA (dsRNA) homologous in sequence to the
30 silenced gene (Fire (1999), Trends Genet 15, 358-63, Tuschl, *et al.* (1999), Genes Dev 13,
3191-7, Waterhouse, *et al.* (2001), Nature 411, 834-42, Elbashir, *et al.* (2001), Nature 411,
494-8, for review see Sharp (2001), Genes Dev 15, 485-90, Barstead (2001), Curr Opin
Chem Biol 5, 63-6). RNAi has been used extensively to determine gene function in a number

of organisms, including plants (Baulcombe (1999), Curr Opin Plant Biol 2, 109-13),
nematodes (Montgomery, *et al.* (1998), Proc Natl Acad Sci U S A 95, 15502-7), *Drosophila*
(Kennerdell, *et al.* (1998), Cell 95, 1017-26, Kennerdell, *et al.* (2000), Nat Biotechnol 18,
896-8). In the nematode *C. elegans* about one third of the genome has already been subjected
5 to functional analysis by RNAi (Kim (2001), Curr Biol 11, R85-7, Maeda, *et al.* (2001), Curr
Biol 11, 171-6).

Until recently RNAi in mammalian cells was not generally applicable, with the
exception of early mouse development (Wianny, *et al.* (2000), Nat Cell Biol 2, 70-5). The
discovery that transfection of duplexes of 21-nt into mammalian cells interfered with gene
10 expression and did not induce a sequence independent interferon-driven anti-viral response
usually obtained with long dsRNA led to new potential application in differentiated
mammalian cells (Elbashir *et al.* (2001), Nature 411, 494-8). Interestingly these small
interfering RNAs (siRNAs) resemble the processing products from long dsRNAs suggesting
a potential bypassing mechanism in differentiated mammalian cells. The Dicer complex, a
15 member of the RNase III family, necessary for the initial dsRNA processing has been
identified (Bernstein, *et al.* (2001), Nature 409, 363-6, Billy, *et al.* (2001), Proc Natl Acad Sci
U S A 98, 14428-33). One of the problems previously encountered when using unmodified
ribooligonucleotides was the rapid degradation in cells or even in the serum-containing
medium (Wickstrom (1986), J Biochem Biophys Methods 13, 97-102, Cazenave, *et al.*
20 (1987), Nucleic Acids Res 15, 10507-21). It will depend on the particular gene function and
assay systems used whether the respective knock down induced by transfected siRNA will be
maintained long enough to achieve a phenotypic change.

It is apparent, therefore, that synthetic interfering RNA molecules that are both stable
and active in a biochemical environment such as a living cell are greatly to be desired.

SUMMARY OF THE INVENTION

It is therefore an object of the present invention to provide compositions and methods
using interfering RNA molecules having enhanced stability.

In accomplishing this object, there has been provided, in accordance with a first
30 aspect of the present invention, a ribonucleic acid comprising a double stranded structure
whereby the double- stranded structure comprises a first strand and a second strand, whereby
the first strand comprises a first stretch of contiguous nucleotides and whereby said first
stretch is at least partially complementary to a target nucleic acid, and the second strand

comprises a second stretch of contiguous nucleotides whereby said second stretch is at least partially identical to a target nucleic acid, and whereby the double stranded structure is blunt ended.

5 In accordance with a second aspect of the present invention there has been provided a ribonucleic acid comprising a double stranded structure whereby the double-stranded structure comprises a first strand and a second strand, whereby the first strand comprises a first stretch of contiguous nucleotides and whereby said first stretch is at least partially complementary to a target nucleic acid, and the second strand comprises a second stretch of contiguous nucleotides, whereby said second stretch is at least partially identical to a target
10 nucleic acid, whereby the first stretch and/or the second stretch have a length of 18 or 19 nucleotides.

In an embodiment of the ribonucleic acid according to the first aspect of the invention the first stretch and/or the second stretch have a length of 18 or 19 nucleotides.

15 In a further embodiment of the ribonucleic acid according to the first aspect of the invention the double stranded structure is blunt ended on both sides of the double strand.

In an alternative embodiment of the ribonucleic acid according to the first aspect of the invention the double stranded structure is blunt ended on the double stranded structure which is defined by the 5'-end of the first strand and the 3'-end of the second strand.

20 In a further alternative embodiment of the ribonucleic acid according to the first and the second aspect of the invention the double stranded structure is blunt ended on the double stranded structure which is defined by the 3'-end of the first strand and the 5'-end of the second strand.

25 In accordance with a third aspect of the present invention there has been provided a ribonucleic acid comprising a double stranded structure whereby the double-stranded structure comprises a first strand and a second strand, whereby the first strand comprises a first stretch of contiguous nucleotides and whereby said first stretch is at least partially complementary to a target nucleic acid, and the second strand comprises a second stretch of contiguous nucleotides and whereby said second stretch is at least partially identical to a target nucleic acid, and whereby at least one of the two strands has an overhang of at least
30 one nucleotide at the 5'-end.

In an embodiment of the ribonucleic acid according to the third aspect of the present invention the overhang consists of at least one nucleotide which is selected from the group comprising ribonucleotides and deoxyribonucleotides

In a more preferred embodiment of the ribonucleic acid according to the third aspect of the present invention the nucleotide has a modification whereby said modification is preferably selected from the group comprising nucleotides being an inverted abasic and nucleotides having an NH₂-modification at the 2'-position.

5 In a preferred embodiment of the ribonucleic acid according to the third aspect of the present invention at least one of the strands has an overhang of at least one nucleotide at the 3'-end consisting of ribonucleotide or deoxyribonucleotide.

10 In another preferred embodiment of the ribonucleic acid according to the third aspect of the present invention the first stretch and/or the second stretch have a length of 18 or 19 nucleotides.

In an embodiment of the ribonucleic acid according to any aspect of the present invention the double-stranded structure has a length of 17 to 21 nucleotides, preferably 18 to 19 nucleotides.

15 In an embodiment of the ribonucleic acid according to the third aspect of the present invention the overhang at the 5'-end is on the second strand.

In a preferred embodiment of the ribonucleic acid according to the third aspect of the present invention the first strand comprises also an overhang, preferably at the 5'-end.

In an embodiment of the ribonucleic acid according to the third aspect of the present invention the 3'-end of the first strand comprises an overhang.

20 In an alternative embodiment of the ribonucleic acid according to the third aspect of the present invention the overhang at the 5'-end is on the first strand.

In a preferred embodiment thereof the second strand also comprises an overhang, preferably at the 5'-end.

25 In an embodiment of the ribonucleic acid according to the third aspect of the present invention the 3'-end of the first strand comprises an overhang.

In an embodiment of the ribonucleic acid according to any aspect of the present invention at least one nucleotide of the ribonucleic acid has a modification at the 2'-position and the modification is preferably selected from the group comprising amino, fluoro, methoxy, alkoxy and alkyl.

30 In accordance with a fourth aspect of the present invention there has been provided a ribonucleic acid comprising a double stranded structure, whereby the double-stranded structure comprises a first strand and a second strand, whereby the first strand comprises a first stretch of contiguous nucleotides and whereby said first stretch is at least partially

complementary to a target nucleic acid, and the second strand comprises a second stretch of contiguous nucleotides and whereby said second stretch is at least partially identical to a target nucleic acid, whereby said first strand and/or said second strand comprises a plurality of groups of modified nucleotides having a modification at the 2'-position whereby within
5 the strand each group of modified nucleotides is flanked on one or both sides by a flanking group of nucleotides whereby the flanking nucleotides forming the flanking group of nucleotides is either an unmodified nucleotide or a nucleotide having a modification different from the modification of the modified nucleotides.

10 In an embodiment of the ribonucleic acid according to the fourth aspect of the present invention the ribonucleic acid is the ribonucleic acid according to the first, second or third aspect of the present invention.

In a further embodiment of the ribonucleic acid according to the fourth aspect of the present invention said first strand and/or said second strand comprise said plurality of modified nucleotides.

15 In another embodiment of the ribonucleic acid according to the fourth aspect of the present invention said first strand comprises said plurality of groups of modified nucleotides.

In yet another embodiment of the ribonucleic acid according to the fourth aspect of the present invention said second strand comprises said plurality of groups of modified nucleotides.

20 In a preferred embodiment of the ribonucleic acid according to the fourth aspect of the present invention the group of modified nucleotides and/or the group of flanking nucleotides comprises a number of nucleotides whereby the number is selected from the group comprising one nucleotide to 10 nucleotides.

25 In another embodiment of the ribonucleic acid according to the fourth aspect of the present invention the pattern of modified nucleotides of said first strand is the same as the pattern of modified nucleotides of said second strand.

In a preferred embodiment of the ribonucleic acid according to the fourth aspect of the present invention the pattern of said first strand aligns with the pattern of said second strand.

30 In an alternative embodiment of the ribonucleic acid according to the fourth aspect of the present invention the pattern of said first strand is shifted by one or more nucleotides relative to the pattern of the second strand.

In an embodiment of the ribonucleic acid according to the fourth aspect of the present invention the modification is selected from the group comprising amino, fluoro, methoxy, alkoxy and alkyl.

In another embodiment of the ribonucleic acid according to the fourth aspect of the present invention the double stranded structure is blunt ended.

In a preferred embodiment of the ribonucleic acid according to the fourth aspect of the present invention the double stranded structure is blunt ended on both sides.

In another embodiment of the ribonucleic acid according to the fourth aspect of the present invention the double stranded structure is blunt ended on the double stranded structure's side which is defined by the 5'-end of the first strand and the 3'-end of the second strand.

In still another embodiment of the ribonucleic acid according to the fourth aspect of the present invention the double stranded structure is blunt ended on the double stranded structure's side which is defined at the 3'-end of the first strand and the 5'-end of the second strand.

In another embodiment of the ribonucleic acid according to the fourth aspect of the present invention at least one of the two strands has an overhang of at least one nucleotide at the 5'-end.

In a preferred embodiment of the ribonucleic acid according to the fourth aspect of the present invention the overhang consists of at least one deoxyribonucleotides.

In a further embodiment of the ribonucleic acid according to the fourth aspect of the present invention at least one of the strands has an overhang of at least one nucleotide at the 3'-end.

In an embodiment of the ribonucleic acid according to any of the aspects of the present invention the length of the double-stranded structure has a length from about 17 to 21 and more preferably 18 or 19 bases.

In another embodiment of the ribonucleic acid according to any of the aspects of the present invention the length of said first strand and/or the length of said second strand is independently from each other selected from the group comprising the ranges of from about 15 to about 23 bases, 17 to 21 bases and 18 or 19 bases.

In a preferred embodiment of the ribonucleic acid according to any of the aspects of the present invention the complementarity between said first strand and the target nucleic acid is perfect.

In an embodiment of the ribonucleic acid according to any of the aspects of the present invention the duplex formed between the first strand and the target nucleic acid comprises at least 15 nucleotides wherein there is one mismatch or two mismatches between said first strand and the target nucleic acid forming said double-stranded structure.

5 In an embodiment of the ribonucleic acid according to any of the aspects of the present invention, wherein both the first strand and the second strand each comprise at least one group of modified nucleotides and at least one flanking group of nucleotides, whereby each group of modified nucleotides comprises at least one nucleotide and whereby each flanking group of nucleotides comprising at least one nucleotide; with each group of
10 modified nucleotides of the first strand being aligned with a flanking group of nucleotides on the second strand, whereby the most terminal 5' nucleotide of the first strand is a nucleotide of the group of modified nucleotides, and the most terminal 3' nucleotide of the second strand is a nucleotide of the flanking group of nucleotides.

15 In a preferred embodiment of the ribonucleic acid according to the fourth aspect, wherein each group of modified nucleotides consists of a single nucleotide and/or each flanking group of nucleotides consists of a single nucleotide.

20 In a further embodiment of the ribonucleic acid according to the fourth aspect, wherein on the first strand the nucleotide forming the flanking group of nucleotides is an unmodified nucleotide which is arranged in a 3' direction relative to the nucleotide forming the group of modified nucleotides, and wherein on the second strand the nucleotide forming the group of modified nucleotides is a modified nucleotide which is arranged in 5' direction relative to the nucleotide forming the flanking group of nucleotides.

25 In another embodiment of the ribonucleic acid according to the fourth aspect, wherein the first strand comprises eight to twelve, preferably nine to eleven, groups of modified nucleotides, and wherein the second strand comprises seven to eleven, preferably eight to ten, groups of modified nucleotides.

30 In a preferred embodiment of the ribonucleic acid according to any of the aspects of the present invention the target gene is selected from the group comprising structural genes, housekeeping genes, transcription factors, motility factors, cell cycle factors, cell cycle inhibitors, enzymes, growth factors, cytokines and tumor suppressors.

In a further embodiment of the ribonucleic acid according to any of the aspects of the present invention the first strand and the second strand are linked by a loop structure.

In a preferred embodiment of the ribonucleic acid according to any of the aspects of the present invention the loop structure is comprised of a non-nucleic acid polymer.

In a preferred embodiment thereof the non-nucleic acid polymer is polyethylene glycol.

5 In an alternative embodiment thereof the loop structure is comprised of a nucleic acid.

In an embodiment of the ribonucleic acid according to any of the aspects of the present invention the 5'-terminus of the first strand is linked to the 3'-terminus of the second strand.

10 In a further embodiment of the ribonucleic acid according to any of the aspects of the present invention the 3'-end of the first strand is linked to the 5'-terminus of the second strand.

In accordance with a fifth aspect of the present invention there have been provided methods of using a ribonucleic acid according to any of the aspects of the present invention for target validation.

15 In accordance with a sixth aspect of the present invention there have been provided medicaments and pharmaceutical compositions containing a ribonucleic acid according to any of the aspects of the present invention, and methods of making such medicaments and compositions.

20 In a preferred embodiment of the use according to the sixth aspect of the present invention methods are provided for the treatment of a disease or of a condition which is selected from the group comprising glioblastoma, prostate cancer, breast cancer, lung cancer, liver cancer, colon cancer, pancreatic cancer and leukaemia, diabetes, obesity, cardiovascular diseases, and metabolic diseases.

25 In accordance with a seventh aspect of the present invention there has been provided a cell, for example a knockdown cell, containing a ribonucleic acid according to any of the aspects of the present invention.

In accordance with an eighth aspect of the present invention there has been provided an organism, for example a knockdown organism, containing a ribonucleic acid according to any of the aspects of the present invention.

30 In accordance with a ninth aspect of the present invention there has been provided a composition containing a ribonucleic acid according to any of the aspects of the present invention.

In accordance with a tenth aspect of the present invention there has been provided a pharmaceutical composition containing a ribonucleic acid according to any of the aspects of the present invention, and a pharmaceutically acceptable carrier.

In accordance with an eleventh aspect of the present invention there has been provided a method for inhibiting the expression of a target gene in a cell or derivative thereof comprising introducing a ribonucleic acid according to any of the aspects of the present invention into a cell in an amount sufficient to inhibit expression of the target gene, wherein the target gene is the target gene of a ribonucleic acid according to any of the aspects of the present invention.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 shows a schematic illustration defining the terminology as used herein. The upper of the two strands is the first strand and the antisense strand of the targeted nucleic acid such as mRNA. The second strand is the one which essentially corresponds in its sequence to the targeted nucleic acid and thus forms the sense strand. Both, the first strand and second strand form a double-stranded structure, typically through Watson Crick base pairing.

Fig. 2 illustrates some embodiments of the ribonucleic acid molecules of the present invention with patterns of modified and unmodified groups of nucleotides which are also referred to herein as a pattern of modification. The modified groups of nucleotides are also referred to herein as a group of modified nucleotides. The unmodified nucleotides or unmodified groups of nucleotides referred to as flanking group(s) of nucleotides herein, as used herein may also have one or several of the modification(s) as disclosed herein which, however, is/are different from the modification of the nucleotides forming the group(s) of modified nucleotides. In Fig. 2A the modified and unmodified groups of nucleotides, *i.e.* the groups of modified nucleotides and the flanking groups of nucleotides on both the first stretch and the second stretch are located on corresponding parts of the stretches and are thus aligned to each other (groups of modified nucleotides on the first strand aligned with groups of modified nucleotides on the second strand and flanking groups of nucleotides on the first strand aligned with flanking group of nucleotides on the second strand), whereas in Fig. 2B the pattern realised on the first strand is also realised on the second strand, however, with a phase shift such that the modified group of nucleotides of the first stretch is base pairing with an unmodified group of nucleotides of the second stretch and vice versa so that a group of modified nucleotides on the first strand aligns with a flanking group of nucleotides on the

second strand. In Fig. 2C a further possibility of arranging the modified and unmodified groups of nucleotides is realised. It is also within the present invention that the pattern of the first stretch is independent from the pattern of the second stretch and that both patterns partially overlap in terms of relative position to each other in the double-stranded structure defined by base pairing. In a further embodiment the extent of this overlapping can vary over the length of the stretch(es) and strand(s), respectively.

Figs. 3A-3C show the result of a knockdown experiment using RNAi molecules with different end protection groups. More particularly Fig. 3A shows that the various forms of end protected RNAi molecules are functional on the knockdown of PTEN mRNA.

Fig. 3B (SEQ ID NOs: 1-8, respectively in order of appearance) shows the sequence of the different RNAi molecules used in the experiment the result of which is depicted in Fig. 3A. Fig. 3C shows the result of an immunoblot analysis of PTEN protein after treatment with modified RNAi molecules in comparison to PTEN specific antisense constructs.

Figs. 4A-4B show that the 3' overhang of RNAi molecules is not important for RNA interference. More particularly, Fig. 4A shows a dose response curve of different RNAi molecules and Fig. 4B (SEQ ID NOs: 9-20, respectively in order of appearance) shows the sequence of the RNAi molecules used in the experiment the result of which is shown in Fig. 4A.

Figs. 5A-5B show that duplex length of the RNAi molecules has to be at least 18-19 nucleotides. More particularly, Fig. 5B (SEQ ID NOs: 21-28, respectively in order of appearance) shows the sequence of the PTEN specific RNAi molecules used in the experiment the result of which is depicted in Fig. 5A as dose response curve.

Figs. 6A-6B show that four terminal mismatched nucleotides in RNAi molecules with a length of 19 nucleotides are still functional in mediating Akt1 knockdown. More particularly, Fig. 6B (SEQ ID NOs: 29-36, respectively in order of appearance) shows the sequence of the RNAi molecules used in the experiment the result of which is depicted in Fig. 6A.

Figs. 7A-7C show further results on duplex length requirements and tolerance for mutation in siRNAs. More particularly, Fig. 7A (SEQ ID NOs: 37-52, respectively in order of appearance) shows the various constructs used (left panel) and the respective impact on inhibition of Akt1 mRNA expression in HeLa cells relative to the expression of p110 α used in the indicated amounts of siRNA molecules (right panel). The nucleotide changes in the

mismatch siRNA molecules are indicated by arrows; the 3' deoxyribonucleotides, if any, are indicated in capital letters. Fig. 7B (SEQ ID NOs: 53-62, respectively in order of appearance) shows the various PTEN specific siRNAs (left panel), the inhibition of PTEN mRNA expression in HeLA cells expressed as ratio PTEN/p110 α , at various amounts of siRNA (middle panel) and Fig. 7C a Western Blot analysis depicting the inhibition of PTEN protein expression using PTEN specific siRNA (30nM) and respective mismatch siRNA after 48 and 96 hours, respectively, with p100 α being used as loading control.

Figs. 8A-8B show the result of studies on the stability in serum conferred to RNAi molecules by 2'-O-methylation and that end modifications have no beneficial effects on RNAi stability. More particularly, Fig. 8A shows the result of a gel electrophoresis of the various RNAi molecules depicted in Fig. 8B (SEQ ID NOs: 9, 10, 63-68, 13, 14, 17 and 69-71, respectively in order of appearance) being subject to incubation with fetal calf serum.

Figs. 9A-9C show that an amino end modification results in loss of activity. Fig. 9B (SEQ ID NOs: 72, 77, 73, 77, 74, 77, 75, 77, 78, 77, 76, 77, 80, 77, 78, and 79, respectively in order of appearance) shows the particular RNAi molecules used in the experiments the result of which is shown in Fig. 9A expressed as PTEN/p110 α expression level ratio. Fig. 9C shows the design principles which may be deduced from the results depicted in Fig. 9A. As used in Fig. 9C the term functional means functionally active in the particular assay system as described in the example and "not functional" means not functionally active in said system.

Figs. 10A-10C show that 2'-O-Alkyl (methyl) modifications stabilize RNAi molecules but also result in reduction of their activity. More particularly, Fig. 10C shows the sequence of the RNAi molecules used in the experiment the result of which is depicted as a dose response curve in Fig. 10A. Fig. 10B shows the result of a gel electrophoresis of the various RNAi molecules depicted in Fig. 10C (SEQ ID NOs: 13, 14, 81-90, 80, 90, 89, 14, 87 and 90, respectively in order of appearance) being subject to a two hour incubation in fetal calf serum.

Figs. 11A-11C show the result of an experiment on the efficacy of RNAi molecules with blocks of 2'-O-methyl modifications with Fig. 11A graphically depicting the results of said experiments as a dose response curve and with Fig. 11C showing the sequences of the particular RNAi molecules used in said experiments. Fig. 11B shows the result of a gel electrophoresis of the various RNAi molecules depicted in Fig. 11C (SEQ ID NOs: 91-97,

90, 98-100 and 90, respectively in order of appearance) being subject to a two hour incubation in fetal calf serum.

Figs. 12A-12D show that alternating 2'-O-methyl modification result in activity of the modified RNAi molecules compared to unmodified forms. More particularly, Fig. 12B (SEQ ID NOs: 101-118, respectively in order of appearance) shows the sequence of the RNAi molecules used in this experiment the result of which is depicted in Fig. 12A. Fig. 12C shows the stability of said RNAi molecules following incubation in serum for two hours, whereas Fig. 12D shows an immunoblot for PTEN protein upon application of different RNAi molecules to HeLa cells. As may be taken therefrom RNAi molecules with alternating modifications are stabilized against endonuclease degradation and active in mediating a PTEN protein knock down.

Fig. 13 shows the result of a Western Blot analysis to determine the time course of PTEN protein knock down. Cells were continuously transfected with 2'-O-Methyl modified versus unmodified RNAi molecules using cationic lipids for 72 h. Protein lysates were prepared and analysed by immunoblot after 48 and 120 h. For the 96 h and 120 h timepoints the cells were split, replated and incubated in the absence of RNAi molecules for an additional 24 and 48 h.

Fig. 14 shows a Western Blot depicting the protein knock down of PTEN being persistent using alternating modified RNAi molecules versus unmodified RNAi molecules. Transfections were performed for only 5 h and new medium without transfection reagents was added. Lysates were analysed by immunoblot 72h and 96h post transfection with the indicated RNAi molecules.

Figs. 15A-15D show that siRNA molecules with distinct 2'-O-methyl ribonucleotides modifications show increased stability in serum and mediate protein knock-down in HeLa cells. More particularly, Fig. 15A (SEQ ID NOs: 119-146, respectively in order of appearance) indicates the various siRNA molecule constructs used (left panel), whereby 2'-O-methyl ribonucleotides modifications are underlined and indicated by bold letters in the sequence. Inhibition of PTEN mRNA expression in HeLa cells transfected with the indicated amounts of modified siRNA molecules is expressed as ratio PTEN/p110 α and indicated on the right panel. Fig. 15B (SEQ ID NOs: 119, 120, 123, 124, 135-138, 141, 142, 147-152, 145 and 146, respectively in order of appearance) shows on the left panel the various siRNA constructs used and on the right panel a PAA gel electrophoresis of modified and unmodified

siRNA molecules after incubation in serum; the various constructs with 2'-O-methyl ribonucleotides are indicated by underlining and bold printing. Fig. 15C shows an SDS-PAGE based immunoblot illustrating the inhibition of PTEN protein expression using various of the siRNA constructs (30 nM) as depicted in Fig. 15A and 15B, respectively. Again, p110 α is used as loading control. Finally, Fig. 15D is an immunoblot indicating a prolonged protein knock-down, i.e. the inhibition of PTEN protein expression, upon administration of siRNA molecules (30 nM) with distinct 2'-O-methylribonucleotides modifications after 48 and 128 hours. As in Fig. 15C, p 110 α is used as loading control.

Figs. 16A-16C show that siRNA molecules with distinct 2'-O-methylribonucleotides modifications which are specific for Akt1 and p110 β mRNA show increased stability in serum and mediate protein knock-down in HeLa cells. More particularly, Fig. 16A (SEQ ID NOs: 153-164, respectively in order of appearance) indicates on the left panel the various constructs used whereby again 2'-O-methylribonucleotides are underlined and printed in bold. The integrity of the indicated siRNA molecules after incubation in serum is shown in the right panel. Fig. 16B shows an immunoblot of Akt1, Akt2 and Akt phosphorylation and p110 being used as a loading control upon transfection of the cells with the indicated siRNAs (30 mM). Fig. 16C (SEQ ID NOs: 165-174, respectively in order of appearance) shows various p110 β specific siRNA constructs (left panel) with the 2'-O-methyl modifications being underlined and printed in bold, and the result of an immunoblot analysis (right panel) of the inhibition of the phosphorylation of the downstream kinase Akt1 by said siRNA constructs. p110 α has been used as a loading control.

Figs. 17A-17B show the efficacy of various RNAi molecules with hairpin structures as dose response curve while Fig. 17B shows the structure of the RNAi molecules the result of which is depicted in Fig. 17A. Synthetic siRNAs with different loops are functional in reducing the p110 β , Akt1 and Akt 2 expression. (14A) Inhibition of p110 β mRNA expression in siRNA transfected HeLa cells. Samples were analyzed in parallel for the level of p110 β mRNA expression 24h after transfection of the indicated siRNAs. The transfected bimolecular siRNAs (21mer with 3' TT overhangs, molecule 1AB) or the monomolecular siRNAs with loop structures are schematically shown. Note that the position of the loops (HIV derived pA-loop; (A)₁₂-loop) (SEQ ID NO: 175) relative to the antisense sequence is reversed in 3A, 4A relative to 3B, 4B. The 2AB siRNA molecule contains 6 mismatches in the 21mer duplex and serves as a negative control together with the untreated sample. RNA

was prepared and subjected to real time RT-PCR (Taqman) analysis. p110 β mRNA levels are shown relative to the mRNA levels of p110 α , which serve as an internal reference. Each bar represents triplicate transfections (\pm standard deviation). HeLa cells were transfected at 50% confluency (2500 cells per 96 well) with siRNAs at the indicated concentrations in growth medium.

Figs. 18A-18C show the efficacy of various RNAi molecules with intermolecular and intramolecular loop structures as dose response curves. (18A) (SEQ ID NO: 175) Inhibition of Akt1 mRNA expression in siRNA transfected HeLa cells. Samples were analysed in parallel for the level of Akt1 and Akt2 mRNA expression 24h after transfection of the indicated siRNAs. The different loops (A-loops; GAGA-loop and a polyethyleneglycol (PEG)-linker) and their putative secondary structure are shown schematically. The siRNA molecule 9A is specific for Akt2 and serves as a negative control. Note that 10A and 10B do not contain self-complementary sequences and are transfected in combination. Akt1 mRNA levels are shown relative to the mRNA levels of p110 β , which served as internal control. (18B) (SEQ ID NO: 175) Inhibition of Akt2 mRNA expression in HeLa cells transfected with the indicated siRNA molecules. Akt2 mRNA levels are shown relative to the mRNA levels of p110 β . The Akt1 specific molecule 7A serves here as a negative control.

Fig. 18C shows a Western Blot analysis on Akt protein depicting the functionality of synthetic siRNAs with different loops in specifically reducing the Akt1 and Akt 2 expression. Inhibition of Akt1 and Akt2 protein expression were analysed by immunoblot. The cells were harvested 48h after transfection of the indicated hairpin siRNAs (20nM). Cell extracts were separated by SDS-PAGE and analysed by immunoblotting using anti-p110 antibody, anti Akt 1/2. Similar results were obtained with an antibody specific for the phosphorylated form of Akt1. The positions of p110 α , another catalytic subunit of PI 3-kinase, which was used as a loading control, and of Akt1, Akt2 and phosphorylated Akt (P*-Akt) are indicated on the left.

Figs. 19A-19B show an NH₂ modification, also referred to herein as amino modification, which may be present at either the 3'-OH terminal nucleotide or the 5' terminal nucleotide. The amino group is attached to the phosphate which in turn is attached to the OH group of the sugar moiety, through an alkyl group comprising an alkyl chain of 1 to 8, preferably 6 C atoms, whereby the second C atom close to the phosphate group has a CH₂OH group attached thereto. As an alternative the linker may be formed by an ether whereby the ether is comprised of two alcohols whereby one alcohol is an amino alcohol and the other is a

dialcohol with one alcohol group involved in the formation of the ether group and the other one being an OH group located at either of the C atoms, preferably at the second C atom relative to the phosphate group.

5

DETAILED DESCRIPTION

The present inventors have surprisingly found that small interfering RNAs can be designed that are both highly specific and active as well as stable under the reaction conditions typically encountered in biological systems such as biochemical assays or cellular environments. The various interfering RNAs previously described by Tuschl *et al.* (see, for example, WO01/75164) provide for a length of 21 to 23 nucleotides and a modification at the 3' end of the double-stranded RNA. The present inventors found that stability problems of interfering RNA, including small interfering RNA (siRNA) which is generally referred to herein in the following as RNAi, actually result from attack by endonucleases rather than exonucleases as previously thought. This surprising observation permitted the present inventors to develop the methods and compositions that are the subject of the present invention.

15

Structure of RNAi molecules

The present invention provides new forms of interfering RNA. RNAi consists of a ribonucleic acid comprising a double-stranded structure, formed by a first strand and a second strand. The first strand comprises a stretch of contiguous nucleotides (the first stretch of contiguous nucleotides) that is at least partially complementary to a target nucleic acid. The second strand also comprises a stretch of contiguous nucleotides where the second stretch is at least partially identical to a target nucleic acid. The very basic structure of this ribonucleic acid is schematically shown in Fig. 1. The first strand and said second strand may be hybridized to each other to form a double-stranded structure. The hybridization typically occurs by Watson Crick base pairing.

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The ribonucleic acids of the invention are, however, not necessarily limited in length to such a double-stranded structure. For example, further nucleotides may be added to each strand and/or to each end of any of the strands forming the RNAi. Depending on the particular sequence of the first stretch and the second stretch, the hybridization or base pairing is not necessarily complete or perfect, which means that the first stretch and the second stretch are not 100 % base paired due to mismatches. One or more mismatches may also be present within the duplex. Such mismatches have no effect on the RNAi activity if

placed outside a stretch of preferably 15, 16 or 17 matching nucleotides. If mismatches are placed to yield only 15 or less contiguous matching nucleotides, the RNAi molecule typically shows a reduced activity in down regulating mRNA for a given target compared to a 17 matching nucleotide duplex.

5 The first stretch of contiguous nucleotides of the first strand is essentially complementary to a target nucleic acid, more advantageously to a part of the target nucleic acid. The term "complementary" as used herein preferably means that the nucleotide sequence of the first strand hybridizes to a nucleic acid sequence of a target nucleic acid sequence or a part thereof. Typically, the target nucleic acid sequence or target nucleic acid
10 is, in accordance with the mode of action of interfering ribonucleic acids, a single stranded RNA, more preferably an mRNA. Such hybridization occurs most likely through Watson Crick base pairing but, however, is not necessarily limited thereto. The extent to which the first strand and more particularly the first stretch of contiguous nucleotides of the first strand is complementary to a target nucleic acid sequence can be as high as 100% and be as little as
15 80%, advantageously 80-100%, more advantageously 85-100%, most advantageously 90-100%. Optimum complementarity seems to be 95-100%. Complementarity in this sense means that the aforementioned range of nucleotides, such as, *e.g.*, 80%-100%, depending on the particular range, of the nucleotides are perfect by Watson Crick base pairing.

Complementarity of the strands

20 It is shown in one aspect of the present invention that the complementarity between the first stretch of nucleotides and the target RNA has to be 18-19 nucleotides; stretches of as little as 17 nucleotides even with two sequence specific overhangs are not functional in mediating RNAi. Accordingly, in a duplex having a length of 19 nucleotides or base pairs a minimum complementarity of 17 nucleotides or nucleotide base pairs is acceptable, allowing
25 for a mismatch of two nucleotides. In the case of a duplex consisting of 20 nucleotides or base pairs a complementarity of 17 nucleotides or nucleotide base pairs is allowable and functionally active. The same principle applies to a duplex of 21 nucleotides or base pairs with a total of 17 complementary nucleotides or base pairs. The extent of complementarity required for a length of a duplex, *i.e.* of a double stranded structure, can also be based on the
30 melting temperature of the complex formed by either the double stranded structure as described herein or by the complex of the first stretch of the first strand and the target nucleic acid.

The skilled artisan will understand that all of the ribonucleic acids of the present invention are suitable to cause or being involved in methods of RNA mediated interference such as those described, for example, in WO 99/32619, WO 00/44895 and WO 01/75164.

Length of the RNAi molecules

5 The first strategy by which an interfering ribonucleic acid molecule may be designed according to the present invention is to have an optimum length of 18 or 19 nucleotides of the stretch that is complementary to the target nucleic acid. It is also within the scope of the present invention that this optimum length of 18 or 19 nucleotides is the length of the double
10 stranded structure in the RNAi used. This length requirement clearly differs from that described in, for example, WO 01/75164. It is within the scope of the present invention that any further design, both according to the present invention and as previously described by others, can be realised in connection with an interfering ribonucleic acid having such length characteristics, i.e. a length of 18 or 19 nucleotides.

End modification of the RNAi molecules

15 The second strategy for the design of an interfering ribonucleic acid molecule is to have a free 5' hydroxyl group, (also referred to herein as a free 5' OH-group) at the terminus of the first strand. A free 5' OH-group means that the most terminal nucleotide forming the first strand is present and is thus not modified, particularly not by an end modification. Typically, the terminal 5'-hydroxy group of the second strand, respectively, is also present in
20 an unmodified manner. In a more preferred embodiment, the 3'-end of the first strand and first stretch, respectively, also is unmodified so as to present a free OH-group (also referred to herein as a free 3'OH-group), whereby the design of the 5' terminal nucleotide is the one of any of the embodiments described above. Preferably such a free OH-group also is present at the 3'-end of the second strand and second stretch, respectively. In other embodiments of
25 the ribonucleic acid molecules as described previously according to the present invention the 3'-end of the first strand and first stretch, respectively, and/or the 3'-end of the second strand and second stretch, respectively, may have an end modification at the 3' end.

30 As used herein the terms free 5'OH-group and 3'OH-group also indicate that the respective 5' and 3' terminal nucleotides of the polynucleotide present an OH-group. Such OH-group may stem from either the sugar moiety of the nucleotide, more preferably from the 5' position in the case of the 5'OH-group and from the 3' position in the case of the 3'OH-group, or from a phosphate group attached to the sugar moiety of the respective terminal nucleotide. The phosphate group may in principle be attached to any OH-group of the sugar

moiety of the nucleotide. Preferably, the phosphate group is attached to the 5'OH-group of the sugar moiety in the case of the free 5'OH-group and/or to the 3'OH-group of the sugar moiety in the case of the free 3'OH-group still providing what is referred to herein as free 5' or 3' OH-group.

5 The term "end modification" as used herein in connection with any strategy for the design of RNAi or any embodiment of RNAi disclosed herein, means a chemical entity added to the most 5' or 3' nucleotide of the first and/or second strand. Examples of such end modifications include, but are not limited to, inverted (deoxy) abasics, amino, fluoro, chloro, bromo, CN, CF, methoxy, imidazole, carboxylate, thioate, C₁ to C₁₀ lower alkyl, substituted
10 lower alkyl, alkaryl or aralkyl, OCF₃, OCN, O-, S-, or N-alkyl; O-, S-, or N-alkenyl; SOCH₃; SO₂CH₃; ONO₂; NO₂; N₃; heterocycloalkyl; heterocycloalkaryl; aminoalkylamino; polyalkylamino or substituted silyl, as, among others, described in European patents EP 0 586 520 B1 or EP 0 618 925 B1.

15 As used herein, alkyl or any term comprising "alkyl" means any carbon atom chain comprising 1 to 12, preferably 1 to 6 and more, preferably 1 to 2 C atoms.

20 A further end modification is a biotin group, which may preferably be attached to either the most 5' or the most 3' nucleotide of the first and/or second strand, or to both ends. In a more preferred embodiment the biotin group is coupled to a polypeptide or a protein. It is also within the scope of the present invention that the polypeptide or protein is attached
25 through any of the end modifications described herein. The polypeptide or protein may confer further characteristics to the nucleic acid molecules of the invention. For example, the polypeptide or protein may act as a ligand to another molecule. If the other molecule is a receptor the receptor's function and activity may be activated by the binding ligand. The receptor may show an internalization activity which provides for effective transfection of the
30 ligand bound inventive nucleic acid molecules. An example of a ligand that may be coupled to the nucleic acid molecules of the invention is VEGF and the corresponding receptor is the VEGF receptor.

 Various possible embodiments of the RNAi of the present invention having different kinds of end modification(s) are presented in the Table 1 below:

Table 1: Various embodiments of the interfering ribonucleic acid according to the present invention

		1 st strand/1 st stretch	2 nd strand/ 2 nd stretch
1.)	5'-end 3'-end	free OH free OH	free OH free OH
2.)	5'-end 3'-end	free OH end modification	free OH end modification
3.)	5'-end 3'-end	free OH free OH	free OH end modification
4.)	5'-end 3'-end	free OH end modification	free OH free OH
5.)	5'-end 3'-end	free OH free OH	end modification free OH
6.)	5'-end 3'-end	free OH end modification	end modification free OH
7.)	5'-end 3'-end	free OH free OH	end modification end modification
8.)	5'-end 3'-end	free OH end modification	end modification end modification

The various end modifications as disclosed herein are preferably located at the ribose moiety of a nucleotide of the ribonucleic acid. More particularly, the end modification may be attached to or replace any of the OH-groups of the ribose moiety, including but not limited to the 2'OH, 3'OH and 5'OH position, provided that the nucleotide thus modified is a terminal nucleotide. Inverted abasics are nucleotides, either deoxyribonucleotides or ribonucleotides which do not have a nucleobase moiety. This kind of compound is, among others, described in Sternberger et al.(2002), Antisense. Nucl. Ac. Drug Dev. 12:143.

Any of the aforementioned end modifications may be used in connection with the various embodiments of RNAi depicted in Table 1. It is particularly advantageous to inactivate the sense strand of any of the RNAi forms or embodiments disclosed herein, preferably via an end modification, and more preferably a 5' end modification. The advantage of this strategy arises from the inactivation of the sense strand which corresponds

to the second strand of the ribonucleic acids described herein, which might otherwise interfere with an unrelated single-stranded RNA in the cell. Thus the expression and more particularly the translation pattern of the transcriptome of a cell is more specifically influenced. This effect is also referred to as an off-target effect. Referring to Table 1 those
5 embodiments depicted as embodiments 7 and 8 are particularly advantageous in the above sense as the modification results in an inactivation of the – target unspecific – part of the RNAi (which is the second strand) thus reducing any unspecific interaction of the second strand with single-stranded RNA in a cellular or similar system where the RNAi according to the present invention is going to be used to knock down specific ribonucleic acids and
10 proteins, respectively.

Blunt-ended RNAi molecules

A third strategy provided by the present invention involves a ribonucleic acid comprising a double-stranded structure having a first strand and a second strand, where the first strand comprises a first stretch of contiguous nucleotides and where the first stretch is at
15 least partially complementary to a target nucleic acid, and the second strand comprises a second stretch of contiguous nucleotides where the second stretch is at least partially identical to a target nucleic acid, where the double-stranded structure is blunt-ended. As used herein the term double-stranded structure also is referred to as duplex. This design of RNAi clearly differs from, *e.g.*, that described by Tuschl *et al.* in WO 01/75164, which contains a 3'-
20 overhang. As used herein the term overhang refers to a double-stranded structure where at least one end of one strand is longer than the corresponding end of the other strand ("the counter strand") forming the double-stranded structure. Preferably, the first stretch is identical to the first strand and the second stretch is identical to the second strand.

It also is advantageous to employ a combination of the design principles described
25 above, *i.e.* to prepare RNAi molecules that are blunt-ended and that have an end modification of either the first or the second strand or both. In other words, it is within the scope of the present invention to have blunt-ended RNAi carrying any end modification scheme as depicted in Table 1.

RNAi molecules having a 5' overhang

30 The fourth strategy of the present invention is to have an overhang at the 5'-end of the ribonucleic acid. More particularly, such overhang may in principle be present at either or both the first strand and second strand of the ribonucleic acid according to the present invention. The length of the overhang may be as little as one nucleotide and as long as 2 to 8

nucleotides, preferably 2, 4, 6 or 8 nucleotides. It is within the present invention that the 5' overhang may be located on the first strand and/or the second strand of the ribonucleic acid. The nucleotide(s) forming the overhang may be (a) deoxyribonucleotide(s), (a) ribonucleotide(s) or a continuation thereof.

5 The overhang preferably comprises at least one deoxyribonucleotide, whereby said one deoxyribonucleotide is preferably the most 5'-terminal one. It is within the present invention that the 3'-end of the respective counter-strand of the inventive ribonucleic acid does not have an overhang, more preferably not a deoxyribonucleotide overhang. Here again, any of the inventive ribonucleic acids may comprise an end modification scheme as
10 outlined in connection with Table 1 and/or an end modification as outlined herein.

RNAi molecules having modified nucleotides

A fifth strategy for the design of interfering ribonucleic acids subject to the present application resides in the formation of a certain pattern of modified nucleotides on at least one of the strands and more particularly on one of the stretches of contiguous nucleotides of
15 the ribonucleic acid(s) according to the present invention. The type of modification of the nucleotides may be the same as that discussed in connection with the other strategies for designing interfering RNA disclosed herein. Advantageously, the modification is an end modification as described herein, such as, e.g., inverted abasics, methoxy, or amino and the like at the ribose moiety of at least one nucleotide forming the ribonucleotide acids according
20 to the present application. The modification of the nucleotides may be any form of modification described herein, more particularly the kind of modification as described herein as end modification, except that the so-called end modification is not necessarily located at terminal nucleotides. Rather the modification may occur at a non-terminal nucleotide. Under such conditions the modification is preferably attached to the ribose moiety of the - to be -
25 modified nucleotide and even more preferably to the 2' position of the ribose moiety.

Combinations of design elements

It is also within the present invention that any ribonucleic acid designed according to this strategy may also have the features conferred to a ribonucleic acid according to the present application by any of the other design strategies disclosed herein. Accordingly, the
30 interfering ribonucleic acid having a pattern of modified nucleotides may have an end modification, an end modification scheme, may be blunt ended or may have a 5' overhang or any combination of two or more of these elements or characteristics.

Apart from the aforementioned modifications which may be presented either as end modifications or as modification pattern, the ribonucleic acid backbone as such may be further modified by forming different links between the nucleotides. Examples of such different links are described, among others, in EP 0 586 520 B1 and EP 0 618 925 B1. Of particular interest here are internal modification(s) of the ribonucleic acid backbone which have been shown to confer higher nuclease resistance of ribooligonucleotides. In a preferred embodiment the modification of the modified nucleotide is a methylation of the 2'-OH-group of the ribose moiety of the nucleotide to form a methoxy group.

In a preferred embodiment, both strands, and more particularly both the first stretch and the second stretch show this kind of modification of the nucleotides forming said strands and stretches, respectively. However, it is also within the present invention that either the first strand and first stretch, respectively, or the second strand and second stretch, respectively, show this particular pattern of modification of the nucleotides. As used herein, the term group of modified nucleotides or flanking group of nucleotides may comprise or represent one or more nucleotides.

A pattern of modification of the nucleotides in a contiguous stretch of nucleotides may be realised such that the modification is contained within a single nucleotide or group of nucleotides that are covalently linked to each other via standard phosphodiester bonds or, at least partially, through phosphorothioate bonds. In the event that such a modified nucleotide or group of modified nucleotides does not form the 5'-end or 3'-end of the stretch, a flanking nucleotide or group of nucleotides is arrayed on both sides of the modified nucleotide or group, where the flanking nucleotide or group either is unmodified or does not have the same modification of the preceding nucleotide or group of nucleotides. The flanking nucleotide or group of nucleotides may, however, have a different modification. This sequence of modified nucleotide or group of modified nucleotides, respectively, and unmodified or differently modified nucleotide or group of unmodified or differently modified nucleotides may be repeated one or more times. Preferably, the sequence is repeated more than one time. The pattern of modification is discussed in more detail below, generally referring to a group of modified nucleotides or a group of unmodified nucleotides whereby each of said group may actually comprise as little as a single nucleotide. The term "unmodified nucleotide" as used herein means either not having any of the aforementioned modifications at the nucleotide forming the respective nucleotide or group of nucleotides, or having a modification which is different from the one of the modified nucleotide and group of nucleotides, respectively.

It is also within the present invention that the modification of the unmodified nucleotide(s) wherein such unmodified nucleotide(s) is/are actually modified in a way different from the modification of the modified nucleotide(s), can be the same or even different for the various nucleotides forming said unmodified nucleotides or for the various flanking groups of nucleotides. That is, a group of modified nucleotides may contain two or more different modified nucleotides within a single group.

The pattern of modified and unmodified nucleotides may be such that the 5'-terminal nucleotide of the strand or of the stretch starts with a modified group of nucleotides or starts with an unmodified group of nucleotides. However, in an alternative embodiment it is also possible that the 5'-terminal nucleotide is formed by an unmodified group of nucleotides.

This kind of pattern may be realised either on the first stretch or the second stretch of the interfering RNA or on both. A 5' phosphate on the target-complementary strand of the siRNA duplex is required for siRNA function, suggesting that cells check the authenticity of siRNAs through a free 5' OH (which can be phosphorylated) and allow only such bona fide siRNAs to direct target RNA destruction (Nykanen, *et al.* (2001), *Cell* 107, 309-21).

Preferably, the first stretch shows a pattern of modified and unmodified groups of nucleotides, i.e. of group(s) of modified nucleotides and flanking group(s) of nucleotides, whereas the second stretch does not show this kind of pattern. This may be useful insofar as the first stretch is actually the more important one for the target-specific degradation process underlying the interference phenomenon of RNA so that for specificity reasons the second stretch can be chemically modified so it is not functional in mediating RNA interference.

However, it is also within the present invention that both the first stretch and the second stretch have this kind of pattern. Preferably, the pattern of modification and non-modification is the same for both the first stretch and the second stretch.

In a particular embodiment, the modified nucleotides or groups of modified nucleotides of one strand of the molecule are complementary in position to the modified nucleotides or groups of nucleotides of the other strand. This possibility is schematically depicted in Fig. 2A. In an alternative embodiment, there is a phase shift between the patterns of modification of the first stretch and first strand, respectively, relative to the pattern of modification of the second stretch and second strand, respectively. Preferably, the shift is such that the modified group of nucleotides of the first strand corresponds to the unmodified group of nucleotides of the second strand and vice versa. This possibility is illustrated schematically in Fig. 2B. In another embodiment, there is a partial shift of the pattern of

modification so that the modified groups overlap as illustrated in Fig. 2C. The groups of modified nucleotides in any given strand may optionally be the same length, but may be of different lengths. Similarly, the groups of unmodified nucleotides in any given strand may optionally be the same length, or of different lengths.

5 In a preferred embodiment the second (penultimate) nucleotide at the terminus of the strand and stretch, respectively, is an unmodified nucleotide or the beginning group of unmodified nucleotides. Preferably, this unmodified nucleotide or unmodified group of nucleotides is located at the 5'-end of the first and second strand, respectively, and even more preferably at the terminus of the first strand. In a further preferred embodiment the
10 unmodified nucleotide or unmodified group of nucleotides is located at the 5'-end of the first strand and first stretch, respectively. In a preferred embodiment the pattern consists of alternating single modified and unmodified nucleotides.

In a particular embodiment of this aspect of the present invention the interfering ribonucleic acid subject comprises two strands, whereby a 2'-O-methyl modified nucleotide
15 and a non-modified nucleotide, preferably a nucleotide which is not 2'-O-methyl modified, are incorporated on both strands in an alternating fashion, resulting in a strand having a pattern MOMOMOMOM etc. where M is a 2'-O-methyl nucleotide and O is a non-modified nucleotide. The same sequence of 2'-O-methyl modification and non-modification exists on the second strand, and preferably there is a phase shift between the two strands such that the
20 2'-O-methyl modified nucleotide on the first strand base pairs with a non-modified nucleotide(s) on the second strand and vice versa. This particular arrangement, *i.e.* base pairing of 2'-O-methyl modified and non-modified nucleotide(s) on both strands is particularly preferred in case of short interfering ribonucleic acids, *i.e.* short base paired double-stranded ribonucleic acids because it is assumed, although the present inventors do
25 not wish to be bound by that theory, that a certain repulsion exists between two base-pairing 2'-O-methyl modified nucleotides which would destabilise a duplex, and particularly short duplexes, containing such pairings.

In a "phase shifted" arrangement of this nature, it is preferred if the antisense strand starts with a 2'-O-methyl modified nucleotide at the 5' end whereby consequently the second
30 nucleotide is non-modified, the third, fifth, seventh and so on nucleotides are thus again 2'-O-methyl modified whereas the second, fourth, sixth, eighth and the like nucleotides are non-modified nucleotides. Again, not wishing to be bound by any theory, it seems that a particular importance may be ascribed to the second, and optionally fourth, sixth, eighth and/or similar

position(s) at the 5' terminal end of the antisense strand which should not comprise any modification, whereas the most 5' terminal nucleotide, *i.e.* the first 5' terminal nucleotide of the antisense strand may exhibit such modification with any uneven positions such as first, optionally third, fifth and similar position(s) at the antisense strand may be modified. In further embodiments the modification and non-modification, respectively, of the modified and non-modified nucleotide(s), respectively, may be any modification as described herein.

Although not limited thereto, the double-stranded structure of the inventive ribonucleic acid, which is also referred to as duplex, is formed by the first strand and second strand, respectively, or the first and second stretch of contiguous nucleotides. The length of the first stretch and second stretch, respectively, is typically about 15 to about 23, preferably 17 to 21, and more preferably 18 or 19 bases. A length of less than 30 nucleotides, preferably less than 21 nucleotides does not induce an interferon response in any biological system which is capable of showing RNA interference and an interferon response. This apparently is because a given cell experiences profound physiological changes when double-stranded RNA longer than 30 base pairs binds and activates the protein kinase PKR and 2',5'-oligoadenylate synthetase. Activated PKR stalls translation via phosphorylation of eIF2a, activated 2',5'-AS causes mRNA degradation. These effects are not desired in target validation and animal models because they override the effect of the target specific knockdown on the phenotype.

RNAi oligonucleotides with loop structures

In a sixth strategy for designing interfering ribonucleic acids of the present invention, the ribonucleic acid comprises a double-stranded structure where the double-stranded structure has a first strand comprising a first stretch of contiguous nucleotides that is at least partially complementary to a target nucleic acid, and a second strand comprising a second stretch of contiguous nucleotides that is at least partially identical to a target nucleic acid, and where one terminus of the first strand and one terminus of the second strand are linked by a loop structure.

In one embodiment the loop structure is comprised of a non-nucleic acid polymer. Suitable non-nucleic acid polymers include polyethylene glycol or similar polymers. The polymer may be chosen in such a manner that the two linked strands may hybridize to each other. This requires that the polymer has to have a certain molecular "hinge" structure or molecular flexibility to allow the bending of the molecule so as to allow that both stretches get in close contact and in a three-dimensional orientation which permits hybridization. In principle any molecule which complies with this requirement may be used in connection with

the present invention. For example, amino acid based molecules may also be used. Such amino acid based molecules may be either homopolymers or heteropolymers. A useful example is a homopolymer consisting of seven glycine residues which allows the generation of a hinge as required to bring the two stretches to hybridize in the close proximity as needed.

5 A suitable glycine based hinge is described, *e.g.*, in Guan K. L. and Dixon J. E. (1991), *Anal. Biochem.* 192, 262. In another embodiment the hinge may be formed by crown ethers of a type that is known in the art.

In an alternative embodiment the loop is comprised of a nucleic acid moiety. In the context of these loops, LNA as described in Elayadi and Corey (2001) *Curr. Opin. Investig. Drugs*, 2(4):558-61 and Orum and Wengel (2001) *Curr. Opin. Mol. Ther.* 3(3):239-43; and PNA are regarded as nucleic acids and may also be used as loop forming polymers. Basically, the 5'-terminus of the first strand may be linked to the 3'-terminus of the second strand. As an alternative, the 3'-end of the first strand may be linked to the 5'-terminus of the second strand. The nucleotide sequence forming the loop structure is, in general, not critical.

15 However, the length of the nucleotide sequence forming such loop seems to be critical for steric reasons. Accordingly, a minimum length of four nucleotides appears to be appropriate to form the required loop structure. In principle, the maximum number of nucleotides forming the hinge or the link between both stretches to be hybridized is not limited. However, the longer a polynucleotide is, the more likely secondary and tertiary structures are formed and this can affect the required orientation of the stretches that must form the hybridised structure. Preferably, a maximum number of nucleotides forming the hinge is about 12 nucleotides. It is within the scope of the present invention that any of the designs described above may be combined with this sixth strategy, *i.e.* by linking the two strands covalently in a manner that back folding (loop) can occur through a loop structure or similar structure.

20

25 The present inventors surprisingly have found that if the loop is placed 3' of the antisense strand, *i.e.* the first strand of the ribonucleic acid(s) according to the present invention, the resulting RNAi molecules have higher activity than molecules where the loop is placed 5' of the antisense strand. This result is contrary to the conventional wisdom that the orientation of the loop is irrelevant, based on the assumption that any RNAi, is subject to a processing during which non-loop linked RNAi is generated. If this was the case, however,

30 the clearly observed increased activity of those structures having the loop placed 3' of the

antisense could not be explained. Accordingly, a preferred arrangement in 5' → 3' direction of this kind of small interfering RNAi is second strand - loop - first strand.

Expression of RNAi from a vector

The respective constructs may be incorporated into suitable vector systems. Preferably the vector comprises a promoter for the expression of RNAi. Suitable promoters include pol III and the U6, H1, 7SK promoters as described in Good et al. (1997) *Gene Ther*, 4, 45-54.

Use of RNAi for gene knockdown and target validation

The ribonucleic acid molecules according to the present invention have general applicability and permit knockdown or knockout of any desired coding nucleotide such as an mRNA, *i.e.* the expression of any gene producing an RNA may be modified. A particular application is the use of the inventive ribonucleic acid for target validation. As used herein, target validation means a process that involves determining whether a DNA, RNA, or protein molecule is directly involved in a biological process. Preferably the biological process is causally involved in a disease or non-standard condition and the gene under study is therefore a suitable target for development of a new therapeutic compound.

Sequence homology studies have successfully classified genes into target families. The enormous task of deciphering which of these targets are key players in diseases and which should be used for subsequent drug development needs to be addressed in an efficient manner. Therefore, the knockdown of gene expression should be reduced by 50-100%, preferably by 90% to see significant effects on the phenotype. In other cases depending on the gene, a knockdown of as little as 20% might be sufficient to yield a phenotype. A phenotype will be defined by comparison of cells containing functional RNAi molecules with cells containing non functional RNAi molecules. This will ensure a significant readout even under conditions where the protein function is inhibited only partially. Generally there is no linear correlation between the degree of mRNA reduction and the extent of the change in phenotype. For some proteins a reduction of about 20 % of the protein is sufficient to create a change in the phenotype whereas in case of other genes and mRNA, respectively, as little as 5 to 10 % remaining protein is sufficient to maintain an observed phenotype.

A further use of the ribonucleic acid molecules according to the present invention is its use for the manufacture of a medicament or its use as a medicament. Such a medicament may either be used for the treatment and/or prevention of diseases or conditions such as any

type of cancer where a gene and its product have been linked to the onset, cause or progression of this disease. Additionally, such a medicament may be used to treat diseases where the presence or overexpression of a gene product causes a pathological phenotype. In a preferred embodiment the disease is characterised by a gain of function and may be remedied through application or administration of the corresponding, biologically active RNAi. Diseases or conditions which may be treated by the medicament comprising a ribonucleic acid as disclosed herein, may be selected from the group comprising cancer, heart diseases, metabolic diseases, dermatological diseases, inflammatory diseases, immune system disorders and autoimmune disorders. The various forms of cancer include, but are not limited to, solid tumors and tumors of the hematopoietic system, such as glioblastoma, prostate cancer, breast cancer, lung cancer, liver cancer, pancreatic cancer and leukaemia. Metabolic diseases include, but are not limited to, obesity and diabetes. Dermatological diseases include, but are not limited to, psoriasis.

In another aspect the ribonucleic acid molecules according to the present invention may be used as diagnostics, preferably for those diseases as specified in connection with the above-mentioned diseases and conditions. Such diagnosis could be based on the observation that upon applying the ribonucleic acid molecules according to the present invention to a sample which preferably contains cells, a change in the expression pattern of the sample occurs. Preferably such sample comprises cells from a subject from whom it is assumed that it may exhibit said disease or condition to be treated or a predisposition therefor.

A further application of the nucleic acids according to the present invention resides in their use in the screening of pharmaceutically active compounds and/or lead optimization. The latter is done such as to monitor or determine the effect of candidate drugs such as small molecules and compare the effect created by said candidate drugs with the effect observed upon administering specific RNAi designed on the basis of the principles disclosed herein. In doing so candidate drugs having off-target effects may be eliminated from the screening process whereas those candidate drugs which create a similar or identical phenotype are deemed as highly relevant lead compound or may even be a pharmaceutically active compound themselves. In this approach, the highly specific RNAi molecules act as a gold standard against which candidate drugs are measured.

In a further aspect the invention is related to a cell, preferably a knockdown cell, which contains a ribonucleic acid as disclosed herein. Such a cell preferably is either isolated or contained in a tissue or even organ which again preferably is not contained in an organism.

However, the cell may also be contained in an organism. The cell is preferably a cell which is involved in the disease or condition which is to be treated by the ribonucleic acids of the invention. This kind of knock-down cell may be used to generate an expression profile based on, *e.g.*, mRNA or protein, in order to elucidate functional relationship and to determine downstream targets.

In a further aspect the invention is related to an organism containing a ribonucleic acid as disclosed herein. Preferably such organism is a vertebrate organism and more preferably the vertebrate organism is a mammal. A mammal as used herein is, among others and not limited thereto, an ape, a dog, a cat, a goat, a sheep, a pig, a guinea pig, a rabbit, a mouse, a rat and a human being.

In a still further aspect the present invention provides compositions containing one or more ribonucleic acids according to the present invention. Preferably such composition comprises negative and positive controls either in combination with the effective ribonucleic acid or separated therefrom. Such composition may further comprise a solvent, preferably a buffer.

In a further aspect the present invention is related to a pharmaceutical composition containing a ribonucleic acid according to the present invention and a pharmaceutically acceptable carrier. Pharmaceutically acceptable carriers are known in the art and comprise, among others, diluent, buffers and the like. Suitable carriers are described, for example, in Remington's Pharmaceutical Sciences (18th edition, 1990, Mack Publishing Co.). The pharmaceutical composition may comprise additional pharmaceutically active compounds. In cases where the disease or condition to be treated using the ribonucleic acid molecules of the present invention is the same or related disease or condition as that treated by the additional pharmaceutically active compound(s) then the different mode of action of the ribonucleic acid molecules and the additional compounds will produce synergistic effects.

The invention is now further illustrated by reference to the figures and examples from which further features, embodiments and advantages of the present invention may be taken.

EXAMPLE 1: DOSE RESPONSE OF SYNTHETIC DUPLEX RNAI MOLECULES

In this example the impact of NH₂ end protection groups on the activity of duplex RNAi molecules was investigated. Synthetic siRNAs were purchased from Biospring (Frankfurt, Germany). The ribooligonucleotides were resuspended in RNase free TE to a final concentration of 50 μ M. In the case of bimolecular siRNA molecules equal aliquots

(100 μ M) were combined to a final concentration of 50 μ M. For the formation of intramolecular duplexes the siRNAs were incubated at 50°C for 2 min in annealing buffer (25mM NaCl; 5mM MgCl₂) and were cooled down to RT. Transfections were carried out in 96 well or 10-cm plates (at 30% to 50% confluency) by using various cationic lipids such as Oligofectamine, Lipofectamine (Life Technologies), NC388 (Ribozyme Pharmaceuticals, Inc., Boulder, CO), or FuGene 6 (Roche) according to the manufacturer's instructions. RNAi molecules were transfected by adding pre-formed 5x concentrated complex of annealed RNAi and lipid in serum-free medium to cells in complete medium. Prior to transfection 2500 HeLa cells were plated per well 15 – 18 hours before transfection for the 96 well format.

The total transfection volume was 100 μ l for cells plated in 96-wells and 10 ml for cells in 10 cm plates. The final lipid concentration was 0.8 to 1.2 μ g/ml depending on cell density; the RNAi concentration is indicated in each experiment.

Complex formation was allowed to take place for 30 min at 37°C. Complexes were added to cells to yield a final 1x concentration of both lipid and RNAi. Depending on the analysis performed following transfection, cells were lysed using a standard cell lysis buffer for protein extraction (Klippel, A., Escobedo, J. A., Hirano, M. and Williams, L. T. (1994). Mol Cell Biol 14, 2675-2685) or a denaturing buffer for RNA isolation according to the RNA isolation kit (Invitex, Berlin (Germany) 24 to 48 hours post transfection for RNA analysis and 48 to 72 hours post transfection for protein analysis by Western Blot.

Determination of the relative amounts of RNA levels by Taqman analysis:

24h post transfection the RNA of cells transfected in 96-wells was isolated and purified using the Invisorb RNA HTS 96 kit (Invitex GmbH, Berlin). Inhibition of PTEN mRNA expression was detected by real time RT-PCR (Taqman) analysis using 300 nM PTEN 5' primer CACCGCCAAATTTAACTGCAGA (SEQ ID NO: 176), 300 nM PTEN 3' primer AAGGGTTTGATAAGTTCTAGCTGT (SEQ ID NO: 177) and 100 nM of the PTEN Taqman probe Fam-TGCACAGTATCCTTTTGAAGACCATAACCCA-Tamra (SEQ ID NO: 178) in combination with 40 nM β -actin 5' primer GTTTGAGACCTTCAACACCCCA (SEQ ID NO: 179), 40 nM β -actin 3' primer GACCAGAGGCATACAGGGACA (SEQ ID NO: 180) and 100 nM of the β -actin Taqman probe Vic-CCATGTACGTAGCCATCCAGGCTGTG-Tamra (SEQ ID NO: 181). The Akt primers and

probes are determined in Sternberger et al. (Sternberger, supra) and are used according to the manufacturer's instructions (Applied Biosystem; use of Amplicon Set). Also said primers and probes may be designed using the software program Primer Express (Applied Biosystem). The reaction was carried out in 50 µl and assayed on the ABI PRISM 7700 Sequence detector (Applied Biosystems) according to the manufacturer's instructions under the following conditions: 48°C for 30 min, 95°C for 10 min, followed by 40 cycles of 15 sec at 95°C and 1 min at 60°C.

RNA knockdown was shown by real time RT-PCR analysis of HeLa cells transfected with 21 nt long siRNA duplex molecules unmodified and modified with either NH₂ or inverted Abasics groups at the 5'-end at a lipid carrier concentration of 1.0 µg/ml. Cell density was 2000 cells/well. Modifications on the 3'-end were either RNA overhangs, RNA overhangs with amino groups or DNA overhangs.

Preparation of cell extracts and immunoblotting. Cells were washed twice with cold phosphate-buffered saline and lysed at 4°C in lysis buffer containing 20 mM Tris (pH 7.5), 137 mM NaCl, 15% (vol/vol) glycerol, 1% (vol/vol) Nonidet P-40 (NP-40), 2 mM phenylmethylsulfonyl fluoride, 10 mg aprotinin per ml, 20 mM leupeptin, 2 mM benzamidine, 1 mM sodium vanadate, 25 mM β-glycerol phosphate, 50 mM NaF and 10 mM NaPPi. Lysates were cleared by centrifugation at 14,000 x g for 5 minutes and aliquots of the cell extracts containing equal amounts of protein were analyzed for protein expression by Western-blotting. Samples were separated by SDS-PAGE and transferred to nitrocellulose-filters (Schleicher & Schuell). Filters were blocked in TBST buffer (10 mM Tris-HCl (pH 7.5), 150 mM NaCl, 0.05% (vol/vol) Tween 20, 0.5% (wt/vol) sodium azide) containing 5% (wt/vol) dried milk. The respective antibodies were added in TBST at appropriate dilutions. Bound antibody was detected using anti-mouse- or anti-rabbit-conjugated horseradish peroxidase (Transduction Laboratories) in TBST, washed, and developed using the SuperSignal West Dura (Pierce) or ECL (Amersham) chemoluminescence substrates (c.f. Sternberger et al. (2002). *Antisense. Nucl. Ac. Drug Dev. in press*).

Antibodies. The murine monoclonal anti-p110 antibody U3A and the murine monoclonal anti-p85 antibody N7B have been described (Klippel et al., 1994, supra). Rabbit polyclonal anti-Akt and anti-phospho Akt (S473) antibodies were obtained from Cell Signaling Technology. The murine monoclonal anti-PTEN antibody was from Santa Cruz Biotechnology. The PTEN 53 specific antisense molecule, *i.e.* geneBloc, is described in

Sternberger *et al.* [Sternberger, *supra*] having the following sequence (ucuccuuTTGTTTCTGcuaacga) (SEQ ID NO: 182), whereby the nucleotide depicted in lower case are ribonucleotides whereas the nucleotide in capital letters are deoxyribonucleotide. This antisense molecule is also identical to RNAi 1A without TT.

5 The results are shown in Fig. 3A and the respective RNAi molecules in Fig. 3B which are directed to the mRNA of PTEN. The nucleotides written in lower case letters represent ribonucleotides whereas capital letters represent deoxyribonucleotide. The term NH₂ indicates that the 3'-position of the ribonucleotide was modified by an amino group. The RNAi molecules used in this and other examples disclosed herein are also referred to as small
10 interfering RNA molecules siRNA. It is to be noted that in any of the figures contained herein the upper strand is the antisense or first strand, whereas the lower strand is the sense or second strand of the interfering RNA molecule.

As can be taken from Fig. 3A amino end modifications such as amino modification and inverted abasics modification of the terminal OH group of the nucleic acid are as potent
15 as unmodified ends when the modification is located at the 3' end of the antisense strand (see also Fig. 8A; 8B). Therefore chemical modification to stabilize or with other beneficial properties (delivery) will be tolerated without activity loss when located at the 3' OH; especially when the 3'OH is located on an overhanging nucleotide.

For the experiment shown in Fig. 3C similar conditions as outlined above were used.
20 The first strand and the second strand of the RNAi were either modified by a NH₂ group at the 3'-position of the ribose moiety or by an inverted abasic at said positions. The first construct is designated as siRNA-NH₂ (3A3B) the second as siRNA-iB (4A4B). The sequence of both molecules is depicted in Fig. 3B. The term 3A3B indicates that the interfering ribonucleic acid consists of strand 3A as the antisense strand and strand 3B as the
25 sense strand. For reason of comparison an antisense oligonucleotide designated GB53 (Sternberger *et al.*, *supra*) was generated which was directed against the PTEN mRNA as well. The particularities of this latter experiment were as follows.

As may be taken from Fig. 3C end protected RNAi molecules depicted in Fig. 3B are functional in yielding a PTEN protein knockdown.

30 From this example it can be taken that both end protection groups render RNAi molecules active in knocking down PTEN protein. This inhibition is as efficient as inhibition with antisense constructs but at lower concentrations used which is a clear advantage over the already very powerful antisense technology.

EXAMPLE 2: OVERHANG REQUIREMENTS FOR RNAI DUPLEX ACTIVITY IN VIVO

The experimental procedures were the same as depicted in connection with Example 1 except that the PTEN mRNA targeting interfering RNAi molecules were differently designed. The results are shown in Fig. 4A as dose response curves with Fig. 4B showing the particular sequence and modifications of the interfering RNAi molecules used to generate the data depicted in Fig. 4A. The nomenclature is such that, *e.g.*, RNAi 18 is composed of strand 18A as antisense strand and strand 18B as sense strand.

Blunt ended molecules were compared to molecules with 3'-overhangs (RNAi 18) and 5'-overhangs (RNAi 30 and RNAi 31) in their activity to knockdown PTEN mRNA in HeLa cells. The activity of blunt ended molecules (RNAi 28) and molecules with 5'-overhangs was comparable to the activity of molecules with 3'-overhangs. This shows that 3'-overhangs are not required for RNAi activity.

EXAMPLE 3: DUPLEX LENGTH REQUIREMENTS OF INTERFERING RNA MOLECULES FOR RNAI ACTIVITY IN VIVO

The experimental approach was similar to the one outlined in connection with Example 1 except that the interfering RNA molecules were directed against the mRNA of Akt1. The negative control to show the specificity of the RNAi molecules was again p110 mRNA. The experimental results are shown in Fig. 5A with the particularities of the interfering RNAi molecules used being represented in Fig. 5B. Similar experiments were carried out with further siRNA constructs which are depicted in Fig. 7A, left panel, whereby the arrows indicate mismatches and deoxyribonucleotide are expressed in capital letters. The inhibition of Akt1 mRNA expression in HeLa cells transfected with the indicated amounts of siRNA molecules is depicted on the right panel of Fig. 7A.

Taqman analysis on Akt RNA from HeLa cells transfected with different RNAi molecules shows that the double strand duplex of the siRNA molecules has to be longer than 17 base pairs to show activity whereas molecules with 17 base pair long duplexes or shorter are not functional even if sequence-specific overhangs are added. The shortest RNAi molecules successfully tested were 18 to 19 nucleotides or base pairs in length. It is to be noted that the design of the interfering RNA molecule 51A/51B referred to as RNAi 51 corresponds to the one as described in international patent application WO 01/75164. The

RNAi molecule 55A/55B comprises a stretch of 17 nucleotides and has a clearly decreased activity in terms of degradation of Akt1 mRNA.

As may be taken from Fig. 7A 19 nt long duplexes are highly efficient in reducing Akt1 mRNA levels independent of the nature (deoxy- or ribonucleotides) of the 3' overhang (compare molecules 1AB, 2AB, 3AB, 4AB). The 17 nucleotide long siRNA (molecule 5AB) showed a dramatically reduced silencing activity confirming the above expressed understanding that active siRNA duplexes should be at least 18 nt or longer. Without wishing to be bound by any theory this result may be explained mechanistically by two different requirements. First, a minimum base pairing of 18 nt between the antisense of the siRNA and the target mRNA may be obligatory, or second, incorporation into the RNA-induced silencing complex (RISC) requires a minimum length of the siRNA duplex. To address this question a 19 nt long siRNA duplex molecule with one and two terminal mutations (CG and UA inversion) relative to the wild type sequence was synthesised (molecules 6AB and 7AB). Both molecules, even the molecule with a stretch of only 15 nt base pairing to the target mRNA were functional in inducing the Akt1 mRNA level. Therefore, it can be concluded that the duplex length itself, but not the base pairing of the antisense siRNA with the target mRNA seems to determine the minimum length of functional siRNAs. This suggests that the length of the double-stranded helix is an important determinant for the incorporation into the RISC complex. The introduced mismatches at the terminal ends of the siRNA duplexes had little effect on RNA interference.

Given the experimental results, the minimum requirement for optimum RNAi mediated interference is thus a duplex length of 18 or 19 nucleotides, independent of the further design of the RNAi molecules such as blunt end or 5'-overhang or any other form as disclosed herein, but generally applicable to RNAi molecules. However, it has to be acknowledged that the particular design of the RNAi molecules may confer further advantages to said molecules, such as, *e.g.*, increased efficiency and increased stability, respectively.

EXAMPLE 4: TARGET-ANTISENSE HOMOLOGY REQUIREMENTS FOR RNAI IN VIVO

The experimental set-up was similar to the one described in example 1, whereby the RNAi is specific for Akt1. In addition, a PTEN specific interfering RNA molecule was designed and used as negative control. The results are shown in Fig. 6A and Fig. 6B.

Basically the same experiment was carried out using further siRNA molecules as depicted in Fig. 7B with the results being indicated in Fig. 7B (right panel) and Fig. 7C, respectively.

Having established the minimal duplex length of 18 or more than 18 nucleotides for functional siRNA molecules we asked the question how many matching nucleotides between target mRNA and siRNA are necessary for silencing activity. As shown by Taqman analysis on Akt1 RNA a stretch of 19 to 15 nucleotides perfectly matching to the target RNA, in the case of Akt 1, was sufficient to mediate RNAi activity. A PTEN specific RNAi molecule did not reduce RNA amounts of Akt1 thus confirming the specificity of this approach. Molecules containing mismatches of one or two nucleotides at any or both ends of a strand were functional suggesting that a homolog stretch of 15 nt between a target mRNA and RNAi is sufficient for gene silencing.

It can be concluded from these data that unspecific gene silencing can occur by chance through unspecific binding to unrelated targets. This is based on the understanding that a stretch of 15 to 17 matching base pairs is not specific for a single gene and will occur by chance considering the complexity and size of the genome or transcriptome of vertebrates. Apart from the above mentioned experiments the location of the mismatch also was subsequently analysed. For this purpose a 19 nt long blunt siRNA directed against PTEN mRNA was used. The sequence changes in one siRNA strand were compensated by complementary changes in the other strand to avoid disrupting duplex formation. As may be seen from both Figs. 7B and C, respectively, a siRNA with only one point mutation in the center of the molecule was severely compromised in its ability to use mRNA and protein expression levels. This result indicates that the RNA machinery is highly discriminative between perfect and imperfect base pairing between target mRNA and siRNA in the center of the duplex. This extreme dependence on a perfect complementarity between target and siRNA has already been described for RNAi interference in the Drosophila system, however, not yet in connection with mammalian systems such as HeLa.

Based on this observation the present invention reduces this off-target problem of siRNA by two approaches: first, by reducing the molecule length of the siRNA molecules to the minimal requirements (18-19 nt) and thereby reducing the chance of homology to off-targets; second, by inactivation of the sense strand to prevent an unwanted RNA silencing caused by accidental complementarity of the sense strand to an unrelated target RNA (see also Example 6).

EXAMPLE 5: STABILITY OF MODIFIED RNAI MOLECULES IN SERUM

Oligonucleotides were incubated in human serum for 15 min and two hours and loaded on 10% polyacrylamide gel with untreated controls. The results are shown in Fig. 8A. The various RNAi molecules used are shown and described in more detail in Fig. 8B.

From this example it can be taken that the RNAi duplex of RNA molecules with all nucleotides modified with 2'-O-methyl groups (RNAi molecules 79A79B and 28A28B) have higher stability in serum. It is also shown that a blunt duplex is more stable than the duplex molecule with overhangs. From this the conclusion may be drawn that end protection (*e.g.* iB or amino) does not increase the stability in serum. In addition, it can also be concluded that, in contrast to the understanding in the art before the filing of the present application, endonucleases are more important than exonucleases in the protection of RNAi molecules.

In view of this, in addition to the various modifications or designs of the inventive RNAi molecules as disclosed above, further or additional modification of the nucleotides may include the use of a phosphorothioate backbone of the RNAi molecules which may be either complete or partial in order to inhibit endonuclease function. A complete phosphorothioate backbone means that any of the nucleotides exhibits a phosphorothioate group whereas a partial phosphorothioate backbone means that not all of the nucleotides forming the RNAi molecule have a phosphorothioate modification. This modification is suitable to increase the lifetime of RNAi molecules irrespective of the further design of RNAi molecules. In this regard, the present invention encompasses a partially or completely phosphorothioate modified RNAi which may be realized in connection with the different strategies for the design of interfering RNA molecules as disclosed herein or with any of the designs known in the art.

EXAMPLE 6: INACTIVATION OF THE SENSE STRAND BY NH₂ END PROTECTION GROUPS ON THE 5' AND 3' ENDS

The experimental set-up was similar to the one described in connection with Example 1 with the target nucleic acid sequence being PTEN mRNA. The concentration of HeLa cells was 2,000 cells per well. RNA of PTEN was analysed in Taqman assays after transfection of differently modified RNAi molecules. The different interfering RNA molecules used are depicted in Fig. 9B and the experimental results are shown in Fig. 9A.

The dose response curves of various RNAi molecules depicted in Fig. 8A show that RNAi molecules are functional when the sense strand, *i.e.* the second stand, is modified on

both ends with amino groups. Particularly effective are RNAi molecules 20A26B, 18A26B, and 28A26B. The lowest activity is shown by RNAi molecule 26A26B which corresponds to end modification on all 4 ends of the duplex (the molecules described by Tuschl are 18AB).

However, RNAi activity is also achieved when the antisense strand, *i.e.* the first strand, is modified only at the 3' end leaving a free OH group at the 5' end (RNAi constructs 22A26B; 20A26B). There is no activity when the antisense strand is modified with amino groups on both the 5' and the 3' end (26A26B). This leads to the conclusion that any end of the antisense strand and more particularly the 5' end of the antisense should be kept without modifications. Additionally, it is worth stating that the NH₂ end modification can be used to inactivate the sense strand on the 5' and 3' end and therefore reduce off-target effects mediated by an otherwise functional sense strand which results in a significantly increased specificity of the RNAi molecule which is advantageous for target validation as well as for any medical use of the RNAi molecule.

The further generalisation of the results from this experiment is depicted in Fig. 9C. Functionally active RNAi are accordingly those not having an amino modification at the antisense strand or having an amino modification only at the 3' end of the antisense strand whereas an amino modification at both ends of the antisense strand is not functional, *i.e.* does not result in a knockdown of the target mRNA.

EXAMPLE 7: IMPACT OF 2'-O-METHYL MODIFICATION OF RNAI MOLECULES FOR ENDONUCLEASE PROTECTION.

RNA knockdown was again shown using real time RT-PCR analysis on HeLa cells transfected with RNAi duplex molecules directed against the PTEN mRNA as represented in Fig. 10A. Experimental procedures were basically the same as specified in Example 1. The structure of the RNAi molecules investigated and their dose responses, which are depicted in Fig. 10A, are shown in Fig. 10C. The nucleotides printed in bold are those having a 2'-O-methyl modification.

It is illustrated by the dose response curves shown for various RNAi molecules in Fig. 10A that internal 2'-O-alkyl groups reduce RNAi activity. Preferably such 2'-O-alkyl groups are 2'-O-methyl or 2'-O-ethyl groups. However, molecules with unmodified nucleotides in combination with 2'-O-alkyl modification show significant activity. As is also depicted in Fig. 10A no activity was obtained when the antisense strand is all modified with 2'-O-methyl groups and the sense strand is not modified (*c.f.*, *e.g.*, RNAi molecule 79A28B). Taken the

results of a stability test such as incubation of the various RNAi molecules in serum, as depicted in Fig. 10B, shows that 2'-O-alkyl modifications stabilize RNAi molecules against degradation. This clearly beneficial effect, however, is at least to a certain degree counterbalanced by the effect that 2'-O-alkyl modifications generally result in a reduced knockdown activity. Accordingly, the design of RNAi molecules has to balance stability against activity which makes it important to be aware of the various design principles as disclosed in the present application.

EXAMPLE 8: IMPACT OF BLOCKS OF INTERNAL 2'-O-METHYL MODIFICATIONS ON THE STABILITY OF RNAI MOLECULES IN SERUM.

The experimental approach in connection with this study was the same as depicted in Example 1. Again, PTEN RNA was analysed by real time RT-PCR on HeLa cells at a density of 2000 cells/well which were transfected with different doses of RNAi molecules. RNAi molecules were incubated in serum for two hours and analysed on a 10% polyacrylamide gel. The results of this study are illustrated in Figs. 11A to 11C, whereby Fig. 11A shows the dose response of the various RNAi molecules depicted in Fig. 11C, and Fig. 11B shows the result of a stability test using some of the RNAi molecules depicted in Fig. 11C. The nucleotides written in bold in Fig. 11C are the ones carrying a modification, in this case a 2'-O-methyl modification of the ribose moiety of the nucleotides.

A dose dependent inhibition by the unmodified RNAi molecules was observed. It was also shown that the 2'-O-methyl modification of the core 9 nucleotides made the RNAi stable in serum and allowed activity of the duplex in mediating the interference phenomenon leading to a degradation of the PTEN mRNA. Total modification of the sense strand makes the RNAi molecule stable in serum and allows certain activity.

Alternating blocks of 5 nucleotides with 2'-O-methyl modification renders the RNAi molecule stable in serum and allows activity on PTEN RNA as shown by incubating the RNAi duplex in serum for two hours and loading the samples on a 10% polyacrylamide gel. As may be taken from Fig. 11B the duplex comprising strands 80A and 80B was strongly degraded after incubation in serum for two hours. The duplex consisting of strands 82A and 82B confirmed the result that the 5'-end of the first strand which comprises the antisense strand should not be modified at the 5'-terminal nucleotides (compare 82A82B with the reverse orientated 81A81B). This was also confirmed by the results obtained having the duplex consisting of the strands 86A and 86B which is both active and stabilised in serum. It

is noteworthy that molecules with unmodified blocks at the terminal 5' of the antisense strand are more active whereby the 5' terminal OH group is preferably not derivatized.

Further experiments were carried out using different modification patterns of 2'-O-methyl modification of the nucleotides. The results thereof are shown in Fig. 12A to 12C and further discussed herein in example 9.

EXAMPLE 9: THE IMPACT OF ALTERNATING INTERNAL 2'-O-ALKYL MODIFICATION ON SERUM STABILITY OF RNAI MOLECULES.

The experimental set-up for performing this kind of study was the same as used in connection with the studies reported in Example 1 and Example 8, respectively, with the targeted nucleic acid being again PTEN mRNA. HeLa cells were transfected with the different RNAi molecules depicted in Fig. 12B and RNA knockdown was demonstrated using real time RT-PCR on PTEN RNA in a dose-dependent manner (Fig. 12A). The stability of the various RNAi molecules after 15 min and two hours in serum at 37°C is depicted in Fig. 12C and a Western Blot for p110 and PTEN as the target-protein of the various RNAi molecules is depicted in Fig. 12D with the RNAi molecules tested being the same in both the experiments underlying Fig. 12C and Fig. 12D.

As illustrated in Fig. 12A and Fig. 12C nucleotides modified with 2'-O-methyl groups alternating with unmodified nucleotides rendered RNAi molecules stable in serum while still allowing them to be active in the sense of interfering with the target mRNA. It was shown that incubation of RNAi duplex molecules for 15 min and two hours in serum degraded the unmodified duplex and the duplex where the 10 most 5'-positioned nucleotides were unmodified.

In the RNAi molecules represented in Fig. 12B various patterns of modified and unmodified nucleotides were prepared. The RNAi molecule 94A1/94B1 comprises a structure wherein a modified nucleotide is flanked by an unmodified nucleotide with the unmodified nucleotide being located at the 5' end of the first strand. The RNAi molecule comprised of strands 94A2 and 94B2 was another example where the modified nucleotides and the unmodified nucleotides of the first and the second strand were located at opposing sites. In contrast to this the RNAi molecule comprised of strands 94A1 and 94B2 had the same pattern of modified and unmodified nucleotides. However, there was a phase shift such that the modified nucleotide base paired with an unmodified nucleotide. The two RNAi molecules comprised of strands 94A1 and 94B1 and strands 94A2 and 94B2 differed from

lasting PTEN protein knock down as unmodified molecules. The lack of protein knock down also seen in Fig. 12 with molecules of the 94A1B1 and 94A1B2 version was confirmed in this experiment. Unmodified molecules (80AB) were not as potent in supporting long lasting protein knock down when the cells were not continuously transfected.

EXAMPLE 11: PERSISTENT PTEN PROTEIN KNOCK DOWN WITH ALTERNATING 2'-O-METHYL MODIFICATIONS OF RNAI MOLECULES

The experimental approach was similar to the one outlined in connection with example 10 with the exception that the transfection was terminated after 5 h by replacing the transfection medium with new medium. The protocol was slightly modified such that for each of the RNAi molecules a 40 nM concentration was realized using a stock solution of 1 µg RNAi / ml cationic lipid as described in connection with example 1. 5 hours after transfection the medium was withdrawn and fresh EMEM added. The cells were split after 72 h, with half of the cells being lysed and the other half newly plated and lysed 24 h later (96h post transfection). The result of a Western Blot analysis using 3 different RNAi molecules (80AB, 94A1/B2, 94A2/B1 are depicted in Fig. 14. As a positive control untreated cells were used. Fig. 14 shows the expression of PTEN after 72 h and 96 h, respectively. Taken the structural particularities of the various RNAi molecules it can be taken from Fig. 14 that protein knockdown is persistent with alternating molecules of the 94A2B1 kind even over 96 h after splitting and replating cells compared to unmodified RNAi molecules (such as 80AB) and RNAi molecule 94A1B2.

A further experiment was carried out using the siRNA constructs as depicted in Fig. 15A (left panel). From the results as depicted as ratio of PTEN/p110α mRNA degradation at the various concentrations of siRNA constructs administered to the test system, it can be taken that siRNA molecules with either one or both strands consisting of 2'-O-methyl residues were not able to induce RNA interference in the mammalian system (Fig. 15A, molecules V2, V5, V6). However, the decrease in activity was less pronounced when only parts of the strands were modified. Interestingly, a molecule having an unmodified antisense strand (which is the upper strand in the representation throughout this specification if not indicated otherwise) and a completely modified sense strand was significantly more active when compared to the reversed version (Fig. 5A, molecules V5 and V6). This result suggests that the antisense strand of the siRNA seems to be more critical and sensitive to modification.

The most efficient molecules inducing PTEN mRNA had only stretches of modifications leaving the 5' end unmodified or were modified on alternating positions on both strands (Fig. 15A, molecules V10, V12).

To test the nuclease resistance the different siRNA versions were incubated in serum followed by PAA gel electrophoresis. The result is shown in Fig. 15B (right panel with the various sequences indicated on the left panel of Fig. 15B). As shown earlier, blunt ended siRNA molecules with unmodified ribonucleotides were very rapidly degraded whereas a complete substitution with 2'-O-methyl nucleotides mediated resistance against serum-derived nucleases (Fig. 15B, compare molecule AB with V1). siRNA molecules with partial 2'-O-methyl modification also showed an increased stability when compared to unmodified siRNAs. In particular, molecules with alternating modifications on both strands showed a significant improvement in instability (Fig. 15B, molecules V13, V14, V15 and V12). More importantly, transfection of three of these molecules into HeLa cells resulted in a significant down regulation of PTEN protein expression as depicted in Fig. 15C, length 6, 9 and 10). In this RNA interference activity assay an unexpected preference for molecules was observed which were modified at every second nucleotide beginning with the most 5' terminal nucleotide of the antisense strand (molecules V15 and V12). Molecules which contained modifications beginning with the second nucleotide at the 5' end of the antisense strand were more stable but had a strongly reduced activity in gene silencing (molecules V13, V14). This result points towards highly specific interactions between the involved enzymes and precise nucleotides in the siRNA duplex. Taken together the data shown herein demonstrate that 2'-O-methyl modifications at particularly selected positions in the siRNA duplex can increase nuclease resistance and do not necessarily abolish RNAi completely.

Although an increased stability of synthetic siRNA has primarily implication for *in vivo* application, it was also analysed whether the particular modification can also lead to an extended protein knock-down in cell culture systems. Accordingly, HeLa cells were transiently transfected for six hours using different versions of PTEN specific siRNAs. The lipid siRNA complex was then washed away and the PTEN protein knock-down was analysed 48 hours and 120 hours later. Although knock-down experiments without continued transfection of siRNAs were complicated due to rapid growth of untransfected cells in this time period resulting in a very transient knock-down, the present inventors were able to demonstrate a prolonged PTEN protein knock-down with siRNA molecules stabilised by the described 2'-O-methyl modification. At 48 hours post transfection the unmodified siRNA

(AB) showed the biggest reduction in PTEN protein levels, however, at 120 hours post transfection the reduction in PTEN protein expression was superior with the siRNA stabilised by alternating 2'-O-methyl modifications (Fig. 15D, compare lane 2 with lanes 4, 6 and 7).

From the results it can also be surmised that the starting nucleotide position of the alternating modification can affect the activity of the duplex. To test this preference in more detail two additional series of siRNAs were synthesized, one specific for the kinase Akt1 and the other one specific for p110 β which is one of the two catalytic subunits of PI(3-) kinase. The particular constructs are shown in Fig. 16A. Only 19 nt long siRNA duplexes either without any or with 2'-O-methyl modification on every second other nucleotide were used. Using Akt1 as a target an efficient protein knock-down as well as a dramatic reduction in phospho-Akt levels were observed with blunt, unmodified siRNAs (Fig. 16A, right panel). From the different versions of molecules with modifications on every second other nucleotide only one was efficiently mediating RNAi (Fig. 16A, molecule V5). This siRNA molecule contained an antisense strand which was modified at the most terminal 5' and 3' nucleotides. The sense strand started with the unmodified nucleotides at the most terminal position, resulting in a structure in which the modified and unmodified ribonucleotides of both strands face each other. As expected, this molecule was also protected against serum-derived nucleases as depicted in Fig. 16B (molecule V5).

Interestingly, a very similar 19 nt long siRNA molecule (V4) with modifications beginning at the second nucleotide of the antisense strand showed no RNA interference activation in the particular assay used. The version V6 in which the modified nucleotides of the antisense strand face modified nucleotides on the sense strand, was also inactive in this experiment. An identical series of 19 nt long siRNA molecules specific for p110 β confirmed these observations as depicted in Fig. 16C. Again the similarly modified siRNA molecule (V5) was the most active, as indicated by reducing Akt phosphorylation, which is indicative for a reduced P (I)-3 kinase activity due to reduced p110 β levels. The reduced activity of the molecules V6 might be explained by reduced duplex stability since the same structure was active in the PTEN knock-down experiment with 21mer siRNAs. Although it is known that 2'-O-methyl modification on both strands facing each other reduces the stability of nucleic acid duplexes, the difference between the activity of siRNA molecules V4 and V5 (Figs. 16B and C) probably is not due to differences in duplex stability since the number of base pairing of modified and unmodified nucleotides is identical. This difference in activity might be due

to specific requirements in the interacting proteins involved in the degradation of the target mRNA. Also it can be taken from these experiments that the most terminal nucleotides of the antisense strand can be modified at the 2'-OH-group with significant loss of silencing activity.

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EXAMPLE 12: DIFFERENT LOOP STRUCTURES ARE FUNCTIONAL IN MEDIATING RNA INTERFERENCE

To test whether RNAi molecules, preferably synthetic RNAi molecules with self complementary structures, can inhibit gene expression as efficiently as standard double stranded siRNA molecules, HeLa cells were transfected with p110 β specific synthetic siRNAs. Transfections were carried out in 96 well or 10-cm plates (at 30% to 50% confluency) by using various cationic lipids such as Oligofectamine, Lipofectamine (Life Technologies), GeneBlocs were transfected by adding pre-formed 5x concentrated complex of GB and lipid in serum-free medium to cells in complete medium. The total transfection volume was 100 μ l for cells plated in 96-wells and 10 ml for cells in 10 cm plates. The final lipid concentration was 0.8 to 1.2 μ g/ml depending on cell density; the RNAi molecule concentration is indicated in each experiment.

A dose dependent titration showed no significant difference in efficiency of mRNA knock-down achieved by the standard bimolecular double strand 21mer and the corresponding monomolecular molecules as analysed by realtime PCR (Taqman) (Fig. 17A). Two different loop structures a (A)₁₂ loop (SEQ ID NO: 175) and a HIV derived pA-loop were tested in parallel with similar results. A comparison of the relative position of the antisense sequence and the loop structure revealed an improved knock-down efficiency with the antisense sequence being located 3' to the loop (Fig. 17B; compare construct 3A, 3B and 4A, 4B).

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EXAMPLE 13: STUDIES ON THE EFFICACY OF DIFFERENT INTRAMOLECULAR HAIRPIN LOOPS AND INTERMOLECULAR "BUBBLES"

The influence of different loop structures on inhibition of mRNA and protein expression was tested. For these experiments Akt1 and Akt2 were chosen as targets. The experimental approach was similar to the one described in example 12.

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Significantly, the reduction in Akt1 mRNA as depicted in Fig. 18A and Fig. 18B as well as Akt1 protein levels as depicted in Fig. 18C was completely independent of the loop structure tested (compare molecules 5A, 6A, 7A, 8A) (the structure of the RNAi molecule tested is always depicted underneath the bar diagram). Even a molecule containing a rather unphysiological structure such as a polyethyleneglycol linker (PEG) as a loop efficiently reduced Akt1 expression indicating that size and nucleotide sequence of the loop is not crucial (Fig. 18A; molecule 8A). A synthetic siRNA molecule specific for Akt2 (9A) was used to control for specificity, and had no effect on Akt1 levels as shown in Fig. 15A. This molecule, however, efficiently silenced Akt2 expression (Fig. 18B; Fig. 18C). Self-complementary RNA molecules with loop structures have the possibility to anneal as double strands in monomolecular or bimolecular structures under physiological hybridization conditions (Fig. 18B, loop or bubble structure). To address the question of whether the siRNA molecules exert their function via adapting an intra-molecular loop or an inter-molecular “bubble” (schematically shown in Fig. 18B) two molecules not capable of folding back on themselves were transfected. These constructs contained Akt1- and Akt2-specific sequences within the same molecule (Fig. 18B, constructs 10A, 10B) and were designed so as to prevent formation of an intramolecular duplex (*i.e.* a bimolecular duplex or “bubble” was formed). Surprisingly, this molecule efficiently mediated both Akt1 and Akt2 mRNA knock-down as well as protein knock-down when transfected after annealing of both strands.

Whether loop and bubble structures are indeed substrates for RNA processing enzymes, *e.g.* *Dicer*, is not clear at this point. A recent study by Paddison and coworkers suggests that hairpin containing siRNAs are more dependent on *Dicer* activity than double stranded siRNAs. However, these data demonstrating RNA interference activity using a PEG linker molecule indicate that the linker sequence is likely to be irrelevant.

The features of the present invention disclosed in the specification, the claims and/or the drawings may both separately and in any combination thereof be material for realizing the invention in various forms thereof.

CLAIMS

1. A double-stranded siRNA molecule against a target nucleic acid, wherein the double-stranded siRNA molecule comprises a first strand and a second strand, wherein the first strand comprises a first stretch that is complementary to the target nucleic acid, wherein the second strand comprises a second stretch that is the same length as the first stretch and is complementary to the first stretch, wherein the first strand and the second strand form a double-stranded structure comprising the first stretch and the second stretch, wherein the first stretch and the second stretch each comprises contiguous alternating 2'-O-alkyl ribonucleotides, wherein the 2'-O-alkyl ribonucleotides alternate with unmodified or differently modified ribonucleotides, wherein the 2'-O-alkyl ribonucleotides of the first strand are shifted by one ribonucleotide relative to the 2'-O-alkyl ribonucleotides of the second strand, and wherein each of the first stretch and the second stretch consists of at least 17 and fewer than 30 ribonucleotides.
2. The double-stranded siRNA molecule of claim 1, wherein the double-stranded siRNA molecule is blunt ended on both ends.
3. The double-stranded siRNA molecule of claim 1, wherein at least one end of the double-stranded siRNA molecule has an overhang.
4. The double-stranded siRNA molecule of claim 3, wherein the overhang has one to eight nucleotides.
5. The double-stranded siRNA molecule of claim 3, wherein the 3' end of the first strand has an overhang.
6. The double-stranded siRNA molecule of claim 3, wherein the 5' end of the first strand has an overhang.

7. The double-stranded siRNA molecule of claim 3, wherein the 3' end of the second strand has an overhang.
8. The double-stranded siRNA molecule of claim 3, wherein the 5' end of the second strand has an overhang.
9. The double-stranded siRNA molecule of claim 1, wherein each of the first strand and the second strand consists of at least 18 nucleotides.
10. The double-stranded siRNA molecule of claim 1, wherein the 2'-O-alkyl ribonucleotides are 2'-O-methyl ribonucleotides.
11. The double-stranded siRNA molecule of claim 10, wherein the 2'-O-methyl ribonucleotides alternate with the unmodified ribonucleotides.
12. The double-stranded siRNA molecule of claim 1, wherein the differently modified ribonucleotides have an amino, fluoro, different alkoxy, or alkyl modification at the 2' position.
13. The double-stranded siRNA molecule of claim 10, wherein the 2'-O-methyl ribonucleotides alternate with 2'-fluoro ribonucleotides.
14. The double-stranded siRNA molecule of claim 1, wherein the first stretch and the second stretch each comprises contiguous alternating single 2'-O-alkyl ribonucleotides, and wherein the single 2'-O-alkyl ribonucleotides alternate with the unmodified ribonucleotides.
15. The double-stranded siRNA molecule of claim 14, wherein the single 2'-O-alkyl ribonucleotides are 2'-O-methyl ribonucleotides.

16. The double-stranded siRNA molecule of claim 1, wherein the first stretch and the second stretch each comprises contiguous alternating single 2'-O-alkyl ribonucleotides, and wherein the single 2'-O-alkyl ribonucleotides alternate with the differently modified ribonucleotides.
17. The double-stranded siRNA molecule of claim 16, wherein the single 2'-O-alkyl ribonucleotides are 2'-O-methyl ribonucleotides.
18. The double-stranded siRNA molecule of claim 16, wherein the differently modified ribonucleotides have an amino, fluoro, different alkoxy, or alkyl modification at the 2'-position.
19. The double-stranded siRNA molecule of claim 16, wherein the single 2'-O-alkyl ribonucleotides are 2'-O-methyl ribonucleotides, and the differently modified ribonucleotides are 2'-fluoro ribonucleotides.
20. The double-stranded siRNA molecule of claim 1, wherein the 5' ribonucleotide of the first stretch is a 2'-O-alkyl modified ribonucleotide.
21. The double-stranded siRNA molecule of claim 11, wherein the 5' ribonucleotide of the first stretch is a 2'-O-methyl ribonucleotide.
22. The double-stranded siRNA molecule of claim 13, wherein the 5' ribonucleotide of the first stretch is a 2'-O-methyl ribonucleotide.
23. The double-stranded siRNA molecule of claim 14, wherein the 5' ribonucleotide of the first stretch is a 2'-O-alkyl modified ribonucleotide that alternates with an unmodified ribonucleotide.
24. The double-stranded siRNA molecule of claim 16, wherein the 5' ribonucleotide of the first stretch is a 2'-O-alkyl modified ribonucleotide that alternates with a differently modified ribonucleotide.

25. The double-stranded siRNA molecule of claim 19, wherein the double-stranded siRNA molecule is blunt on at least one end.
26. The double-stranded siRNA molecule of claim 19, wherein at least one end of the double-stranded siRNA molecule has an overhang of one to eight nucleotides.
27. The double-stranded siRNA molecule of claim 26, wherein the 3' end of the first strand has an overhang.
28. The double-stranded siRNA molecule of claim 26, wherein the 5' end of the first strand has an overhang.
29. The double-stranded siRNA molecule of claim 26, wherein the 3' end of the second strand has an overhang.
30. The double-stranded siRNA molecule of claim 26, wherein the 5' end of the second strand has an overhang.

ABSTRACT

The present invention is related to a ribonucleic acid comprising a double stranded structure whereby the double- stranded structure comprises a first strand and a second strand, whereby the first strand comprises a first stretch of contiguous nucleotides and whereby said first stretch is at least partially complementary to a target nucleic acid, and the second strand comprises a second stretch of contiguous nucleotides whereby said second stretch is at least partially identical to a target nucleic acid, and whereby the double stranded structure is blunt ended.

5

Fig. 1

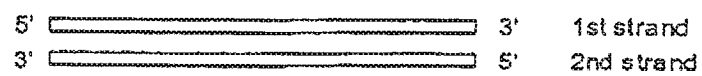


Fig. 2

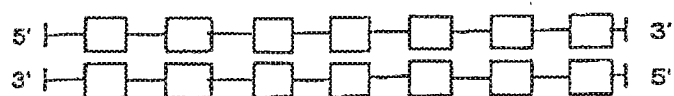


Fig. 2B

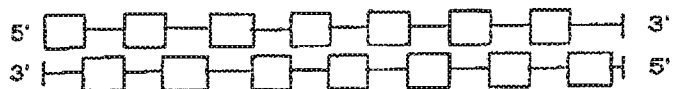
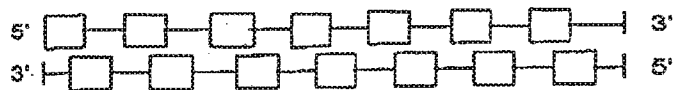


Fig. 2C



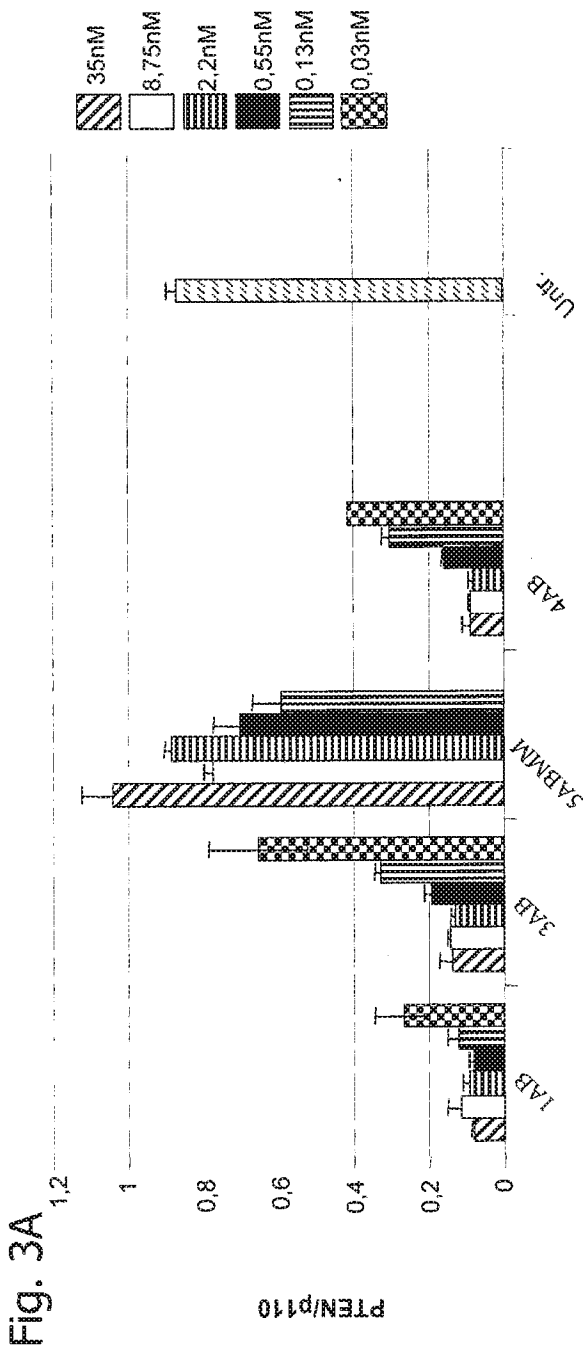


Fig. 3B

ONLY RNA WITH 2 DEOXY AT EACH END

1A 5'- cuccuuuuuuuugcuuacg-TT (SEQ ID NO:1)
1B 3'-TT-gaggaaaaacaaagacgaugc (SEQ ID NO:2)

ONLY RNA WITH NH2 GROUPS AT EACH 3' END AND 2 DEOXY

3A 5'- cuccuuuuuuuugcuuacg-TT-NH2 (SEQ ID NO:3)
3B 3'-NH2-TT-gaggaaaaacaaagacgaugc (SEQ ID NO:4)

ONLY RNA WITH INVERTED ABASIC AND 2 TT

4. 16153-iB (iB at the 3' ends)
4A 5'- cuccuuuuuuuugcuuacg-TT-iB (SEQ ID NO:5)
4B 3'-iB-TT-gaggaaaaacaaagacgaugc (SEQ ID NO:6)

ONLY RNA WITH 2 DEOXY AT EACH END

5A 5'- cucauuuuuuuugcuuacg-TT (SEQ ID NO:7)
5B 3'-TT-gaguaaaagaaacacagugc (SEQ ID NO:8)

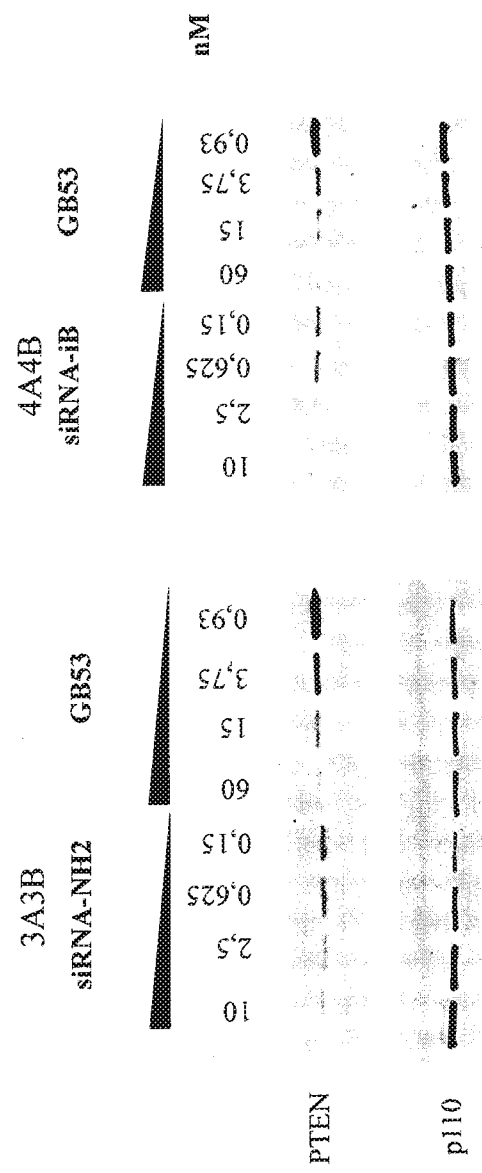


Fig. 3C

Fig. 4A

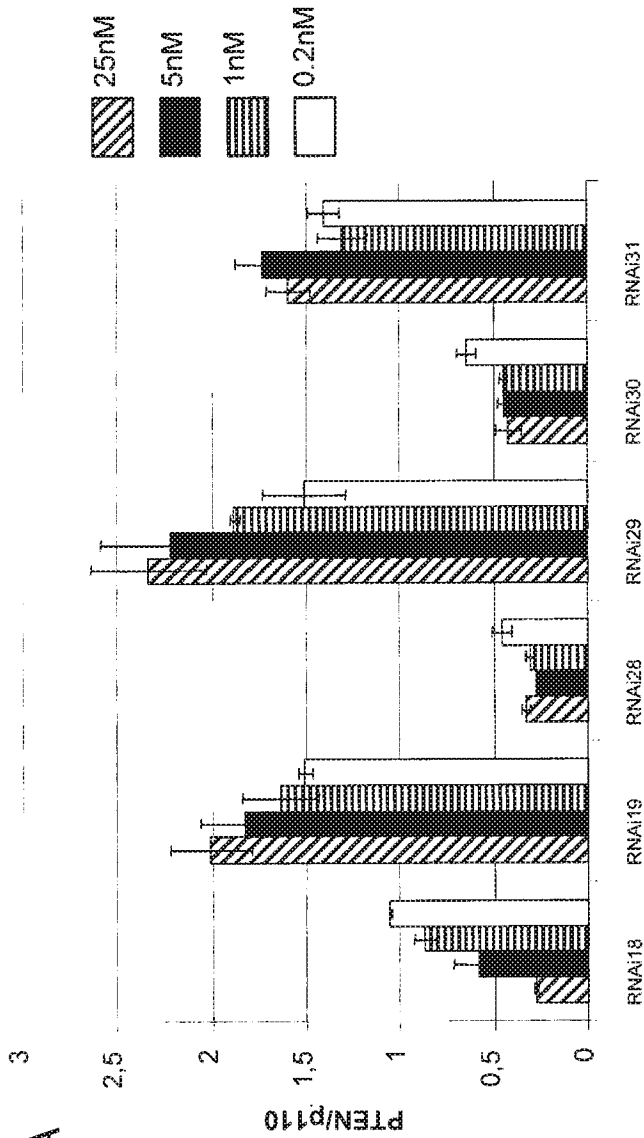


Fig. 4B

18A	5'-	cuccuuuuuuuucugcuaacg-TT (SEQ ID NO:9)
18B	3'-TT-	gaggaaaacaaagacgaugc (SEQ ID NO:10)
19A (MM)	5'-	cucauuuuuuuugucucacg-TT (SEQ ID NO:11)
19B (MM)	3'-TT-	gaguaaaaaagaaacacgagugc (SEQ ID NO:12)
28A	5'-	cuccuuuuuuuucugcuaacg- (SEQ ID NO:13)
28B	3'-	gaggaaaacaaagacgaugc- (SEQ ID NO:14)
29A (MM)	5'-	cucauuuuuuuugucucacg- (SEQ ID NO:15)
29B (MM)	3'-	gaguaaaaaagaaacacgagugc (SEQ ID NO:16)
30A	5'-TT-	cuccuuuuuuuucugcuaacg- (SEQ ID NO:17)
30B	3'-	-gaggaaaacaaagacgaugc-TT (SEQ ID NO:18)
31A (MM)	5'-TT-	cucauuuuuuuugucucacg- (SEQ ID NO:19)
31B (MM)	3'-	-gaguaaaaaagaaacacgagugc-TT (SEQ ID NO:20)

Fig. 5A

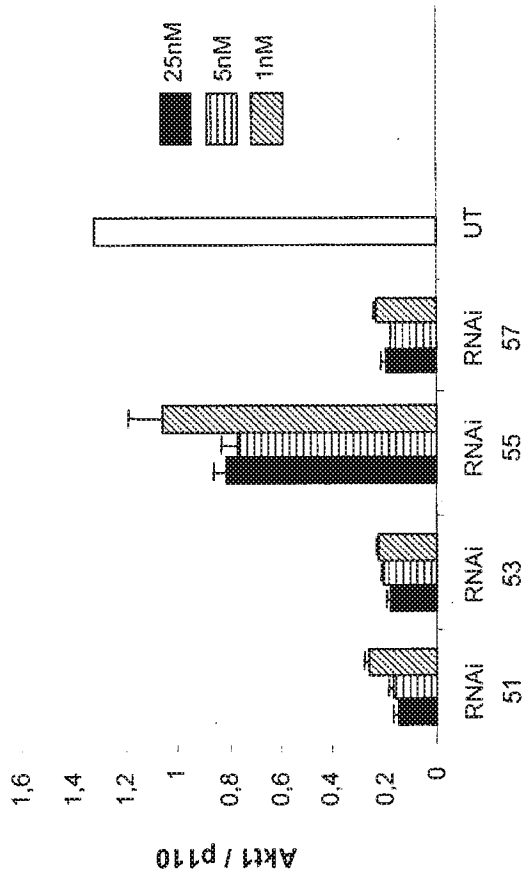
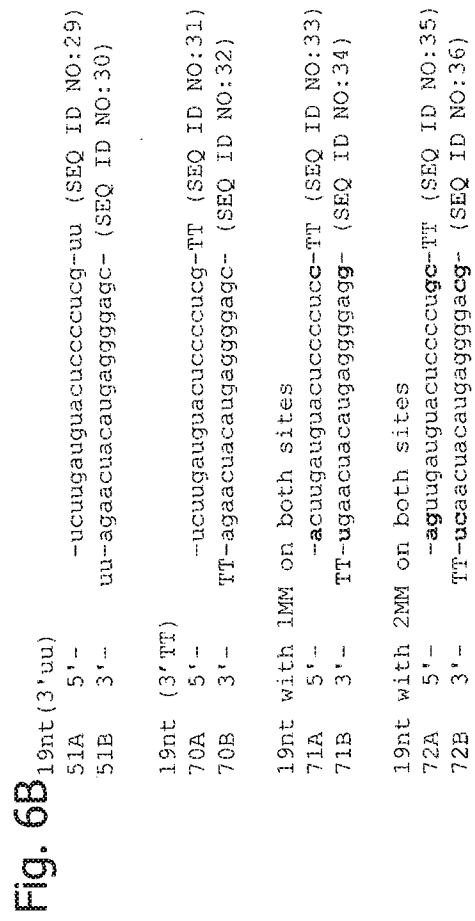
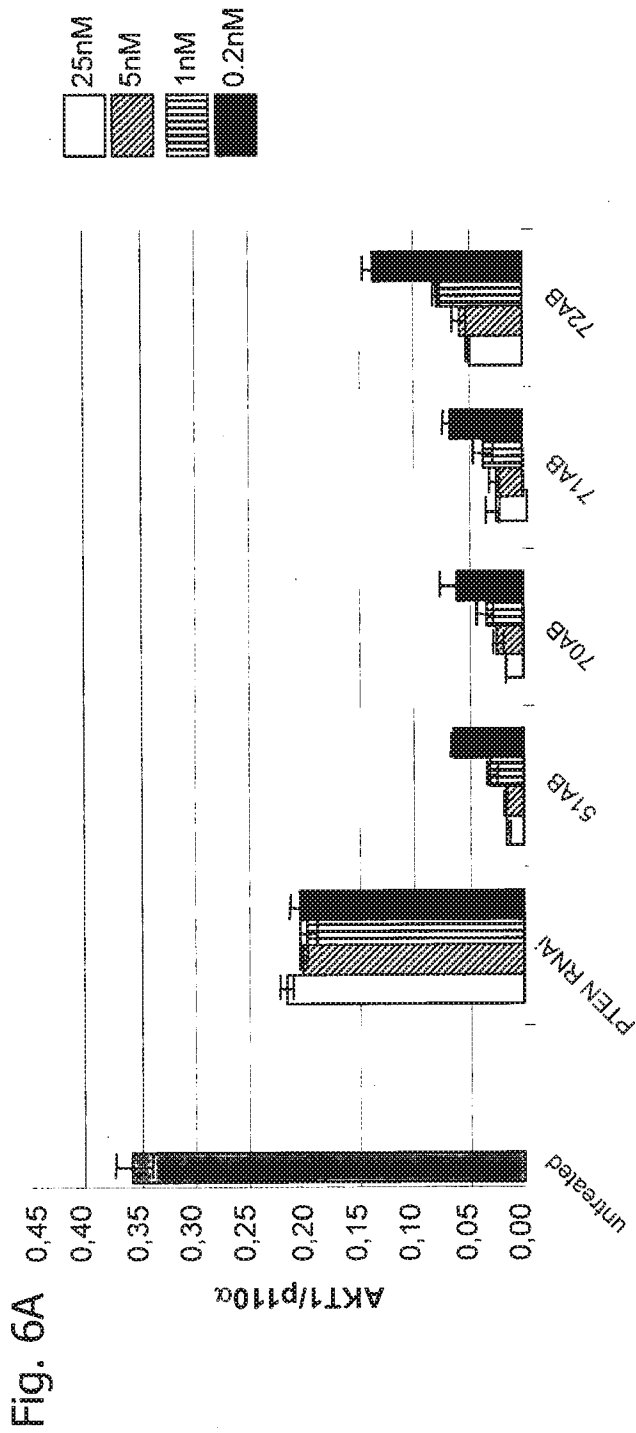


Fig. 5B

51A	5'-	-ucuugauguacuccccucg-uu	(SEQ ID NO:21)	19nt 3'uu
51B	3'-	uu-agaacuacaugaggagc-	(SEQ ID NO:22)	
53A	5'-	-ucuugauguacuccccucg-uu	(SEQ ID NO:23)	19nt 3'seq.specific RNA
53B	3'-	cc-agaacuacaugaggagc-	(SEQ ID NO:24)	
55A	5'-	-cuugauguacuccccuc-gu	(SEQ ID NO:25)	17nt 3'seq.specific RNA
55B	3'-	ca-gaacuacaugaggag-	(SEQ ID NO:26)	
57A	5'-	-ucuugauguacuccccucg-TT	(SEQ ID NO:27)	19nt 3'seq.specific DNA
57B	3'-	CC-agaacuacaugaggagc-	(SEQ ID NO:28)	



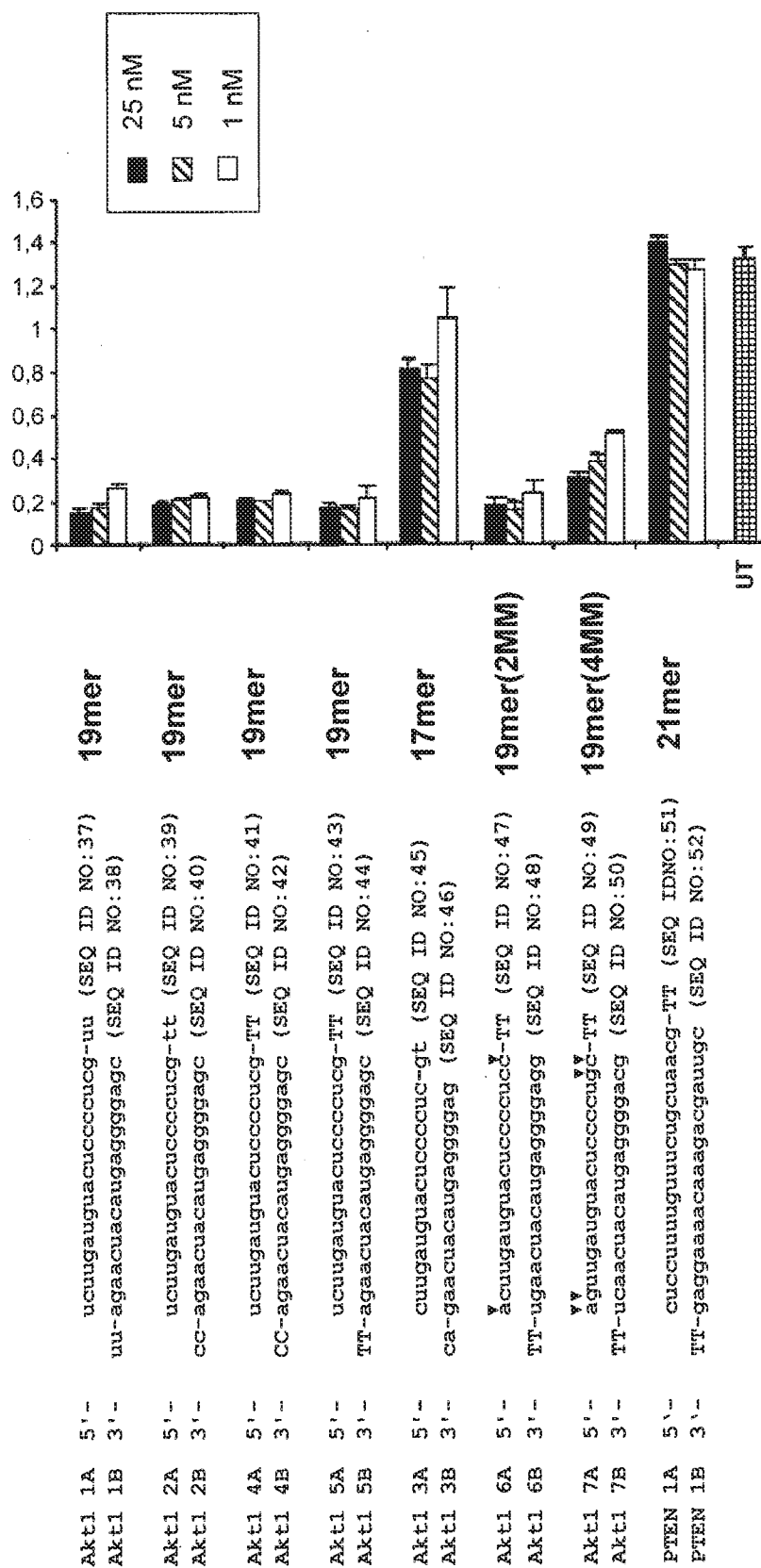


Fig. 7A

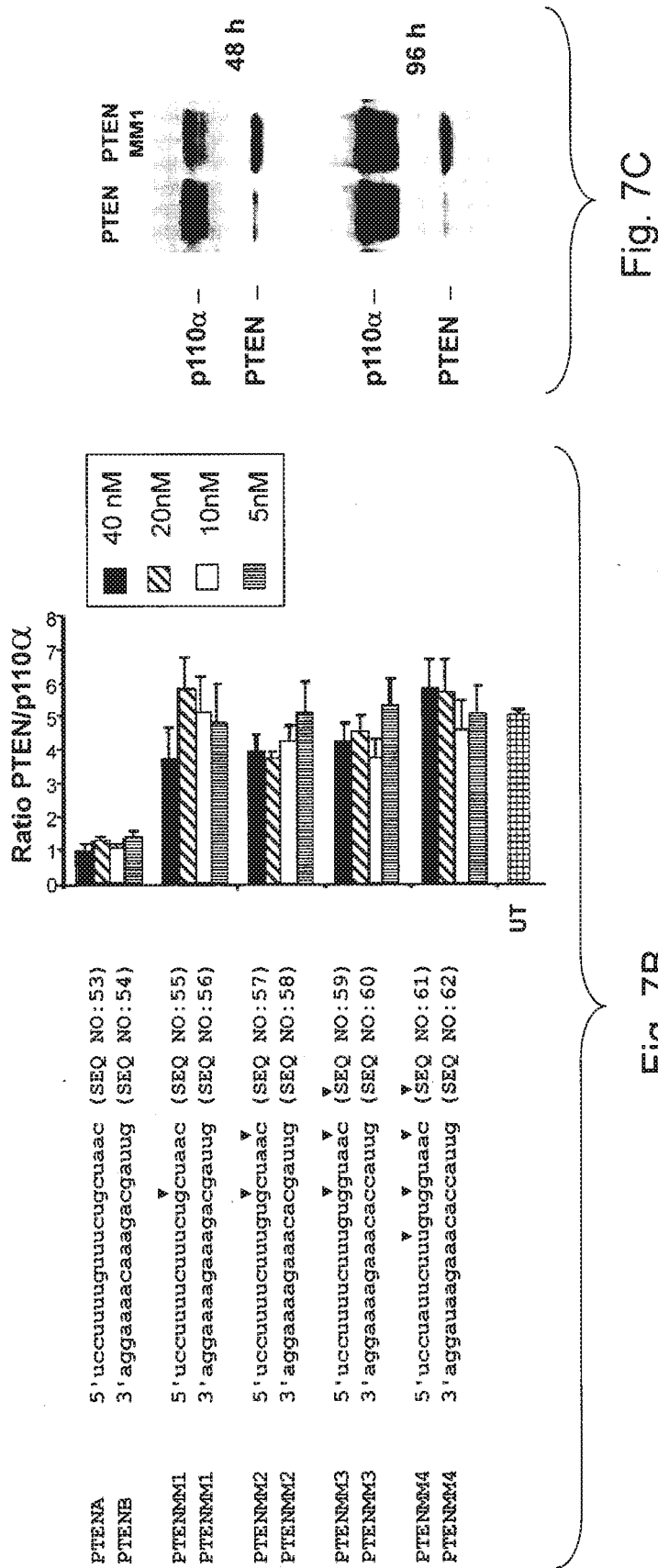


Fig. 8A



Fig. 8B

Tuschl	
18A	5'- cuccuuuuuuucugcuaacg-TT (SEQ ID NO:9)
18B	3'-TT-gaggaaaaaagacgaugc (SEQ ID NO:10)
inverted abasic on both ends and 2TT	
24A	5'- iB-cuccuuuuuuucugcuaacg-TT-iB (SEQ ID NO:63)
24B	3'-iB-TT-gaggaaaaaagacgaugc-iB (SEQ ID NO:64)
NH2groups at both ends AND 2-deoxy	
26A	5'- NH2-cuccuuuuuuucugcuaacg-TT-NH2 (SEQ ID NO:65)
26B	3'-NH2-TT-gaggaaaaaagacgaugc-NH2 (SEQ ID NO:66)
2'-O-Methyl modified with 2-deoxy at each end	
79A	5'- cuccuuuuuuucugcuaacg-TT (SEQ ID NO:67)
79B	3'- TT-gaggaaaaaagacgaugc (SEQ ID NO:68)
Only RNA	
28A	5'- cuccuuuuuuucugcuaacg- (SEQ ID NO:13)
28B	3'- gaggaaaaaagacgaugc- (SEQ ID NO:14)
Only RNA with 2-deoxy at each 5' end	
30A	5'-TT-cuccuuuuuuucugcuaacg- (SEQ ID NO:17)
30B	3'- gaggaaaaaagacgaugc-TT (SEQ ID NO:69)
NH2 groups at each 3'end and 2-deoxy	
3A	5'- cuccuuuuuuucugcuaacg-TT-NH2 (SEQ ID NO:70)
3B	3'-NH2-TT-gaggaaaaaagacgaugc- (SEQ ID NO:71)

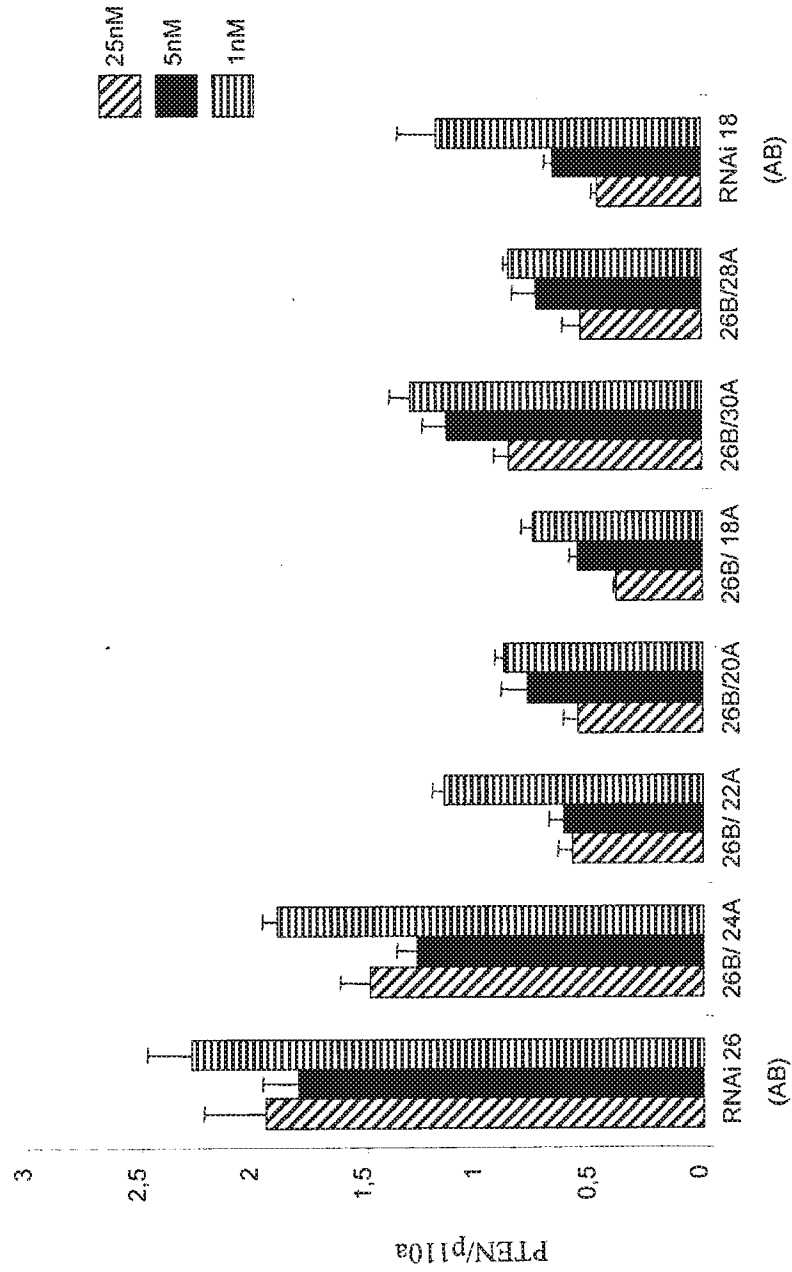
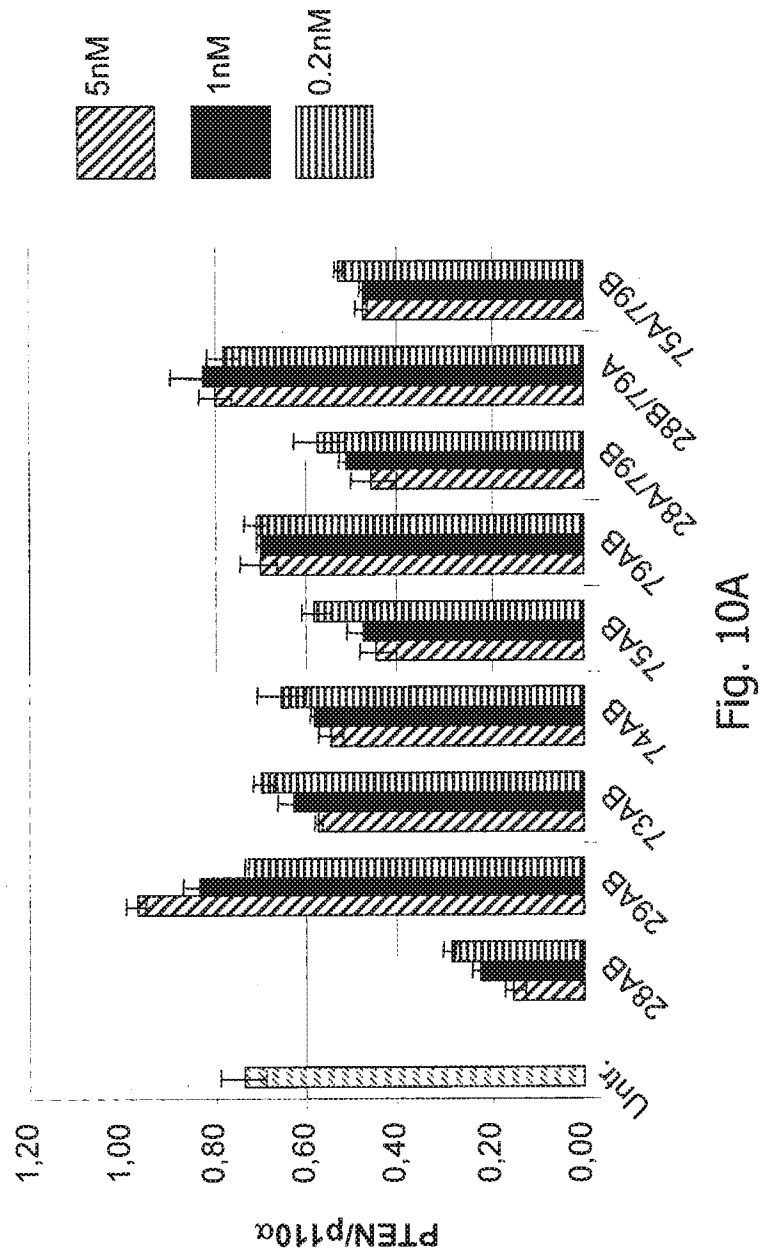


Fig. 9A

১৯৬৬

-tttttttttttttttttttttt	functional
NH2-ttttttttttttttttttttt-NH2	functional
-tttttttttttttttttttttt-NH2	functional
NH2-ttttttttttttttttttttt-NH2	not functional



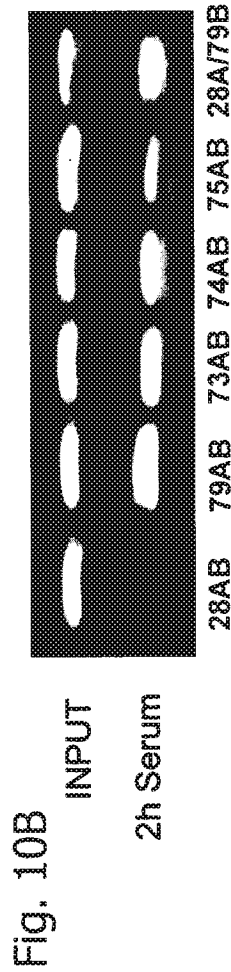


Fig. 10C

	RNA	5'	3'	Seq ID
28A	28A	5'	3'	28A
28B	28B	5'	3'	28B
29A	29A	5'	3'	29A
29B	29B	5'	3'	29B
73A	73A	5'	3'	73A
73B	73B	5'	3'	73B
74A	74A	5'	3'	74A
74B	74B	5'	3'	74B
75A	75A	5'	3'	75A
75B	75B	5'	3'	75B
79A	79A	5'	3'	79A
79B	79B	5'	3'	79B
28A	28A	5'	3'	28A
79B	79B	5'	3'	79B
79A	79A	5'	3'	79A
28B	28B	5'	3'	28B
75A	75A	5'	3'	75A
79B	79B	5'	3'	79B

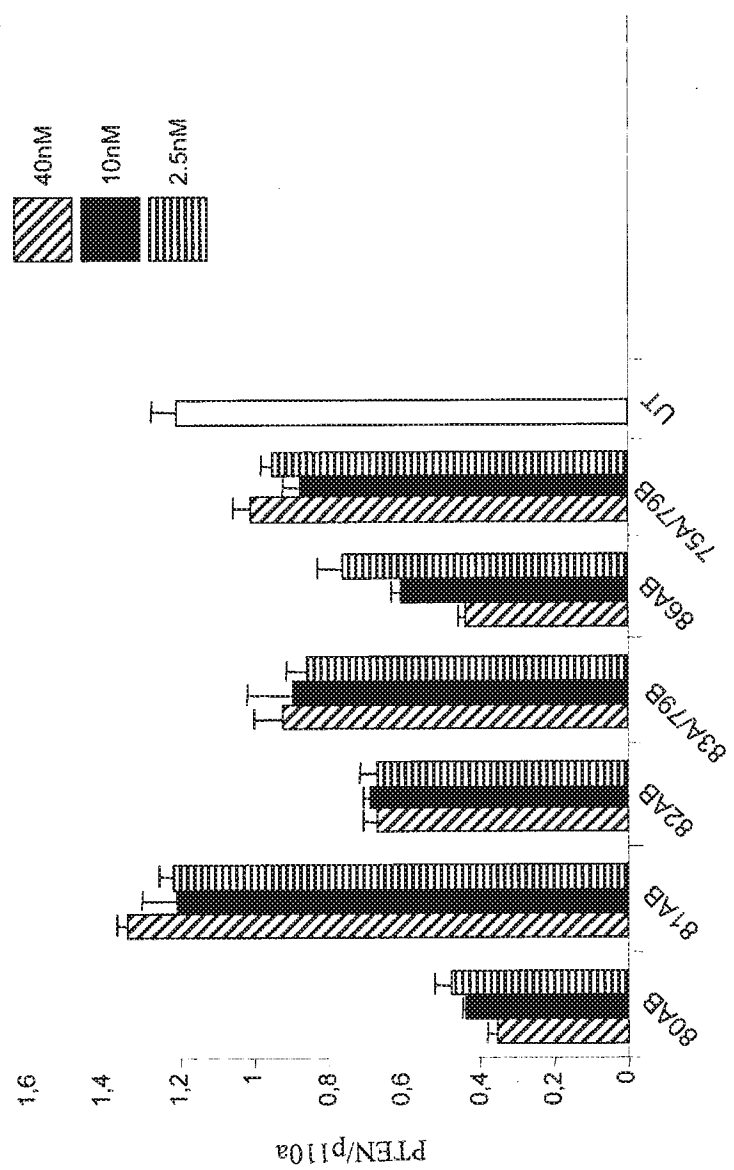


Fig. 11A

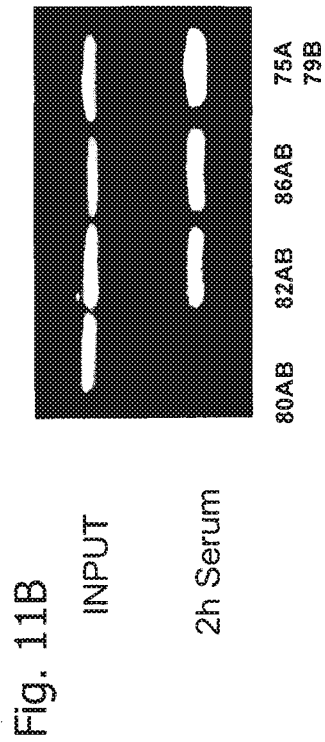
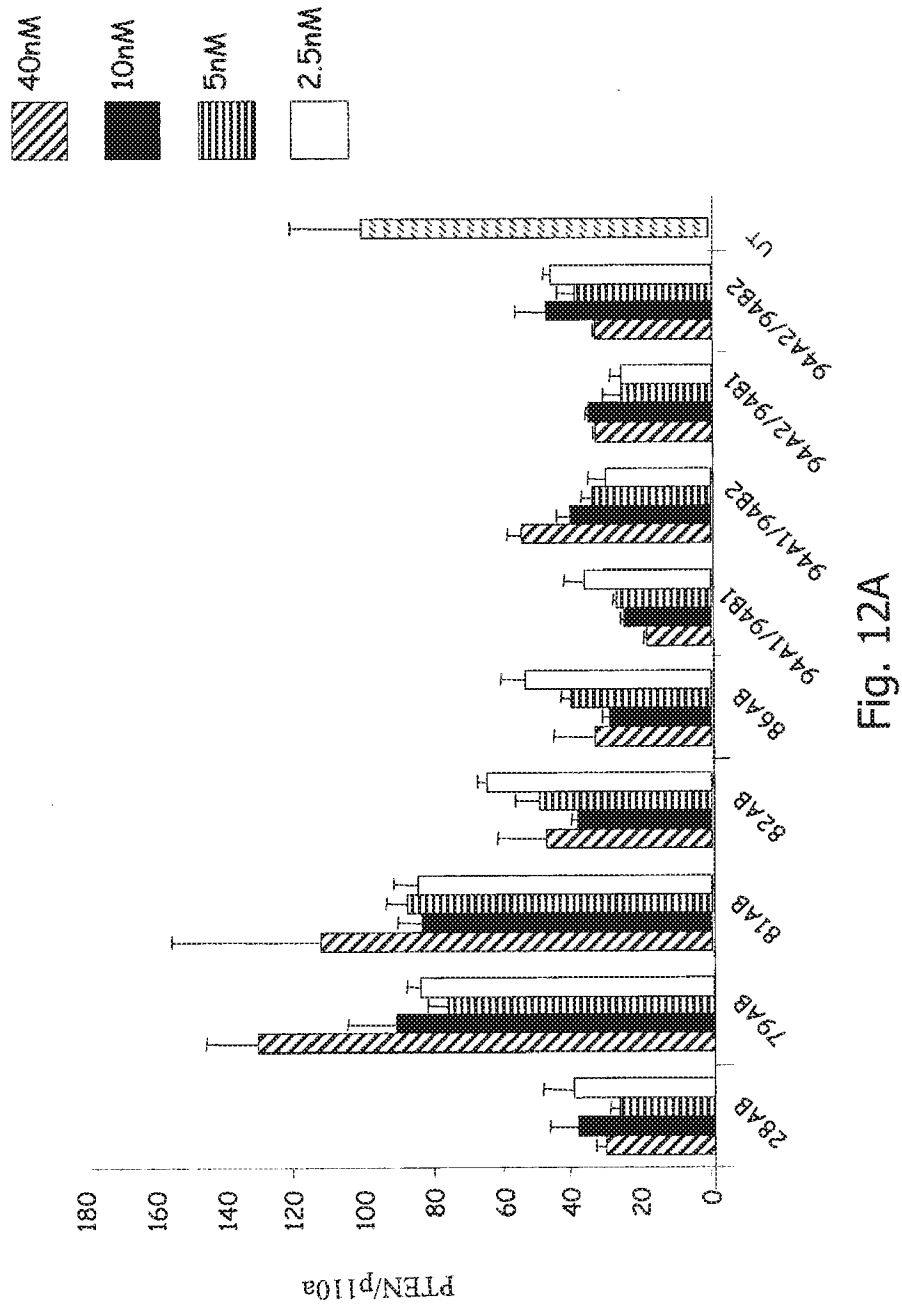


Fig. 11C

RNA	Bold Italics represents 2'O-methyl
80A	5'- <i>cuccuuuuguuucugcuaacg</i> - (SEQ ID NO:91)
80B	3'- <i>-gaggaaaaacaaagacgaungc</i> - (SEQ ID NO:92)
81A	5'- <i>cuccuuuuguuucugcuaacg</i> - (SEQ ID NO:93)
81B	3'- <i>-gaggaaaaacaaagacgaungc</i> - (SEQ ID NO:94)
82A	5'- <i>cuccuuuuguuucugcuaacg</i> - (SEQ ID NO:95)
82B	3'- <i>gaggaaaaacaaagacgaungc</i> - (SEQ ID NO:96)
83A	5'- <i>cuccuuuuguuucugcuaacg</i> - (SEQ ID NO:97)
79B	3'- <i>-gaggaaaaacaaagacgaungc</i> - (SEQ ID NO:90)
86A	5'- <i>cuccuuuuguuucugcuaacg</i> - (SEQ ID NO:98)
86B	3'- <i>-gaggaaaaacaaagacgaungc</i> - (SEQ ID NO:99)
75A	5'- <i>cuccuuuuguuucugcuaacg</i> - (SEQ ID NO:100)
79B	3'- <i>-gaggaaaaacaaagacgaungc</i> - (SEQ ID NO:90)



RNA Bold Italics represents 2' O-methyl		
28A	5'- cuccuunuguuucugcuaacg-	(SEQ ID NO:101)
28B	3'- gaggaataacaaagacgaugc-	(SEQ ID NO:102)
79A	5'- cuccuunuguuucugcuaacg-	(SEQ ID NO:103)
79B	3'- gaggaataacaaagacgaugc-	(SEQ ID NO:104)
81A	5'- cuccuunuguuucugcuaacg-	(SEQ ID NO:105)
81B	3'- gaggaataacaaagacgaugc-	(SEQ ID NO:106)
82A	5'- cuccuunuguuucugcuaacg-	(SEQ ID NO:107)
82B	3'- gaggaataacaaagacgaugc-	(SEQ ID NO:108)
86A	5'- cuccuunuguuucugcuaacg-	(SEQ ID NO:109)
86B	3'- gaggaataacaaagacgaugc-	(SEQ ID NO:110)
94A1	5'- cuccuunuguuucugcuaacg-	(SEQ ID NO:111)
94B1	3'- gaggaataacaaagacgaugc-	(SEQ ID NO:112)
94A1	5'- cuccuunuguuucugcuaacg-	(SEQ ID NO:113)
94B2	3'- gaggaataacaaagacgaugc-	(SEQ ID NO:114)
94A2	5'- cuccuunuguuucugcuaacg	(SEQ ID NO:115)
94B1	3'- gaggaataacaaagacgaugc-	(SEQ ID NO:116)
94A2	5'- cuccuunuguuucugcuaacg	(SEQ ID NO:117)
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Fig. 12B

Fig. 12C

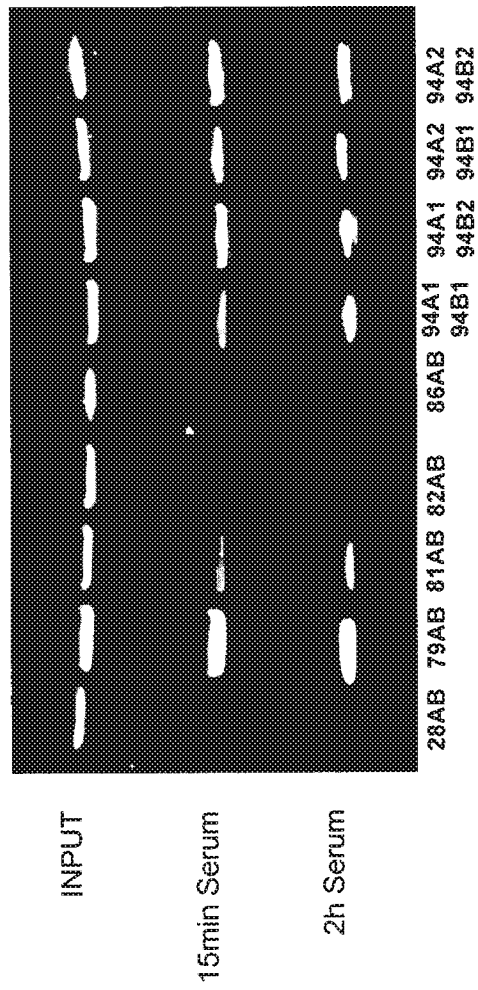
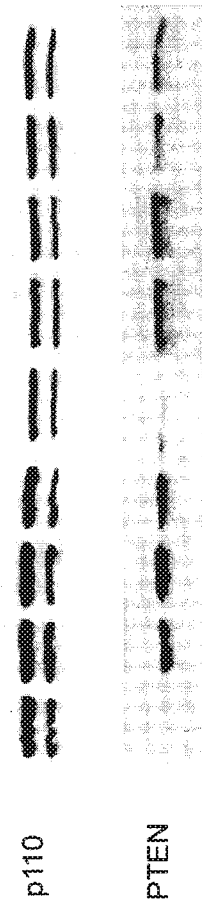


Fig. 12D



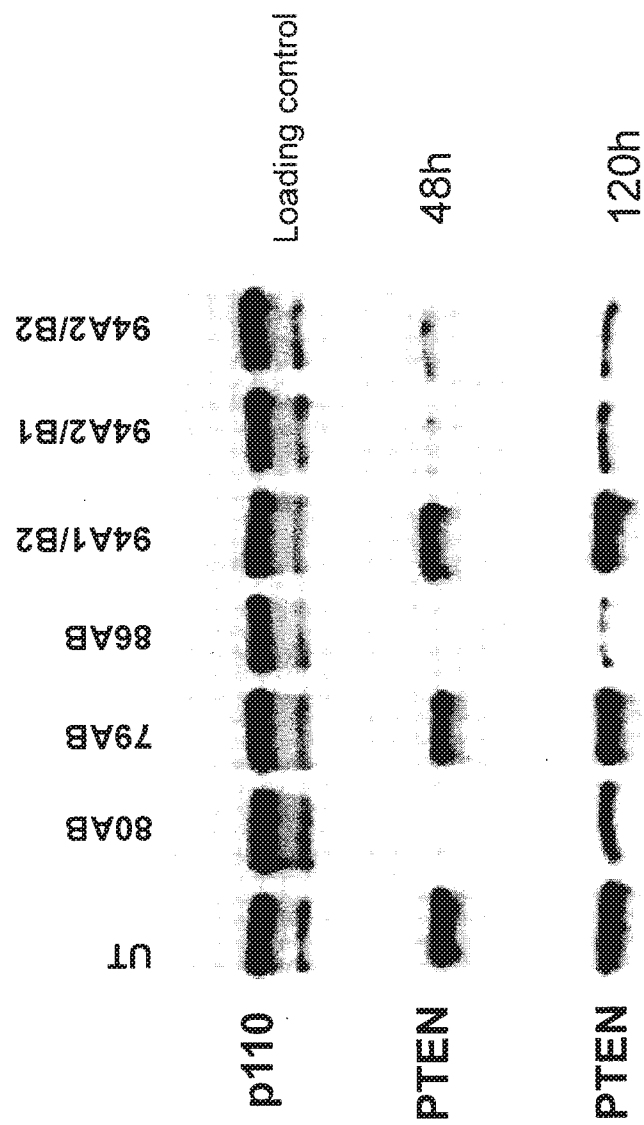


Fig. 13

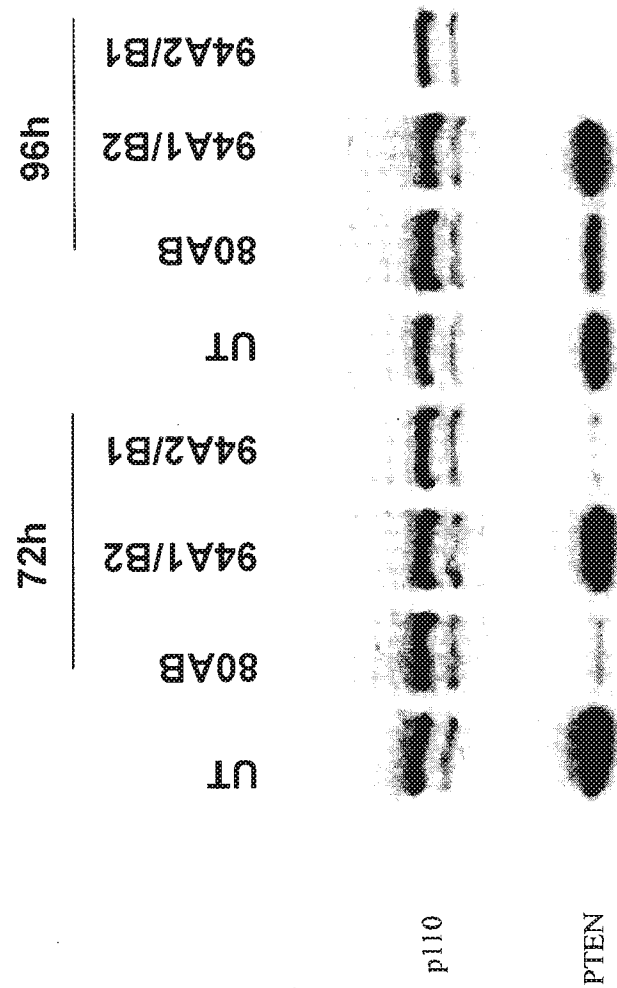
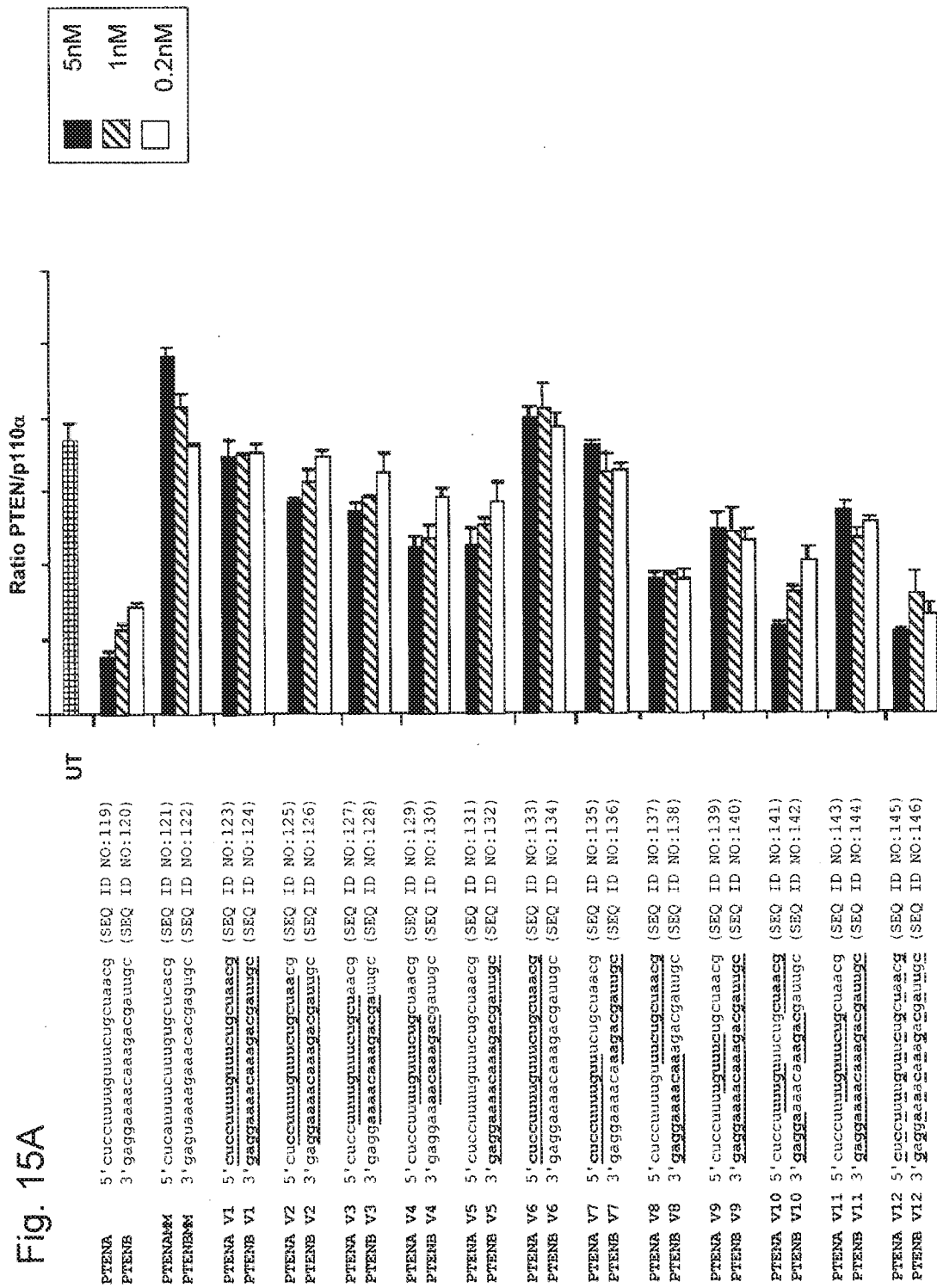


Fig. 14



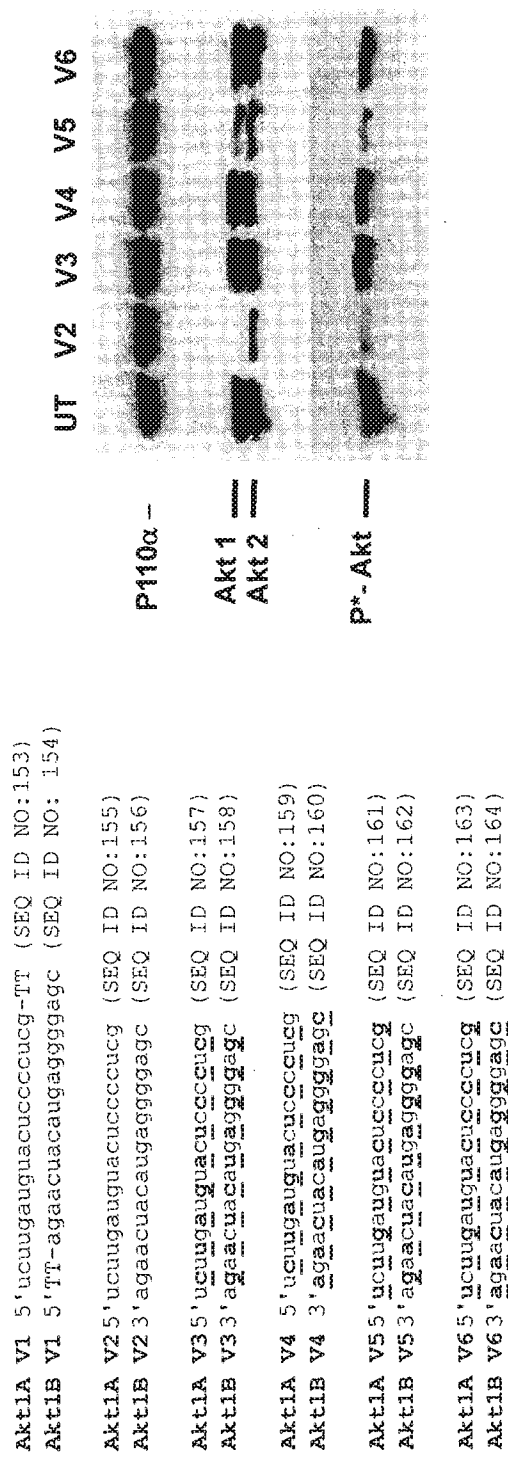


Fig. 16A

V1 V2 V3 V4 V5 V6

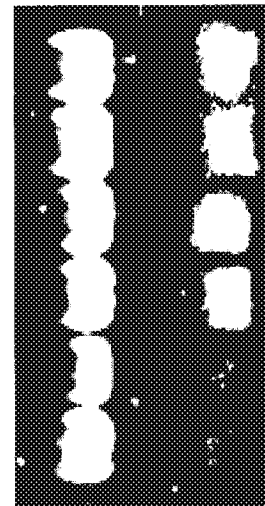


Fig. 16B

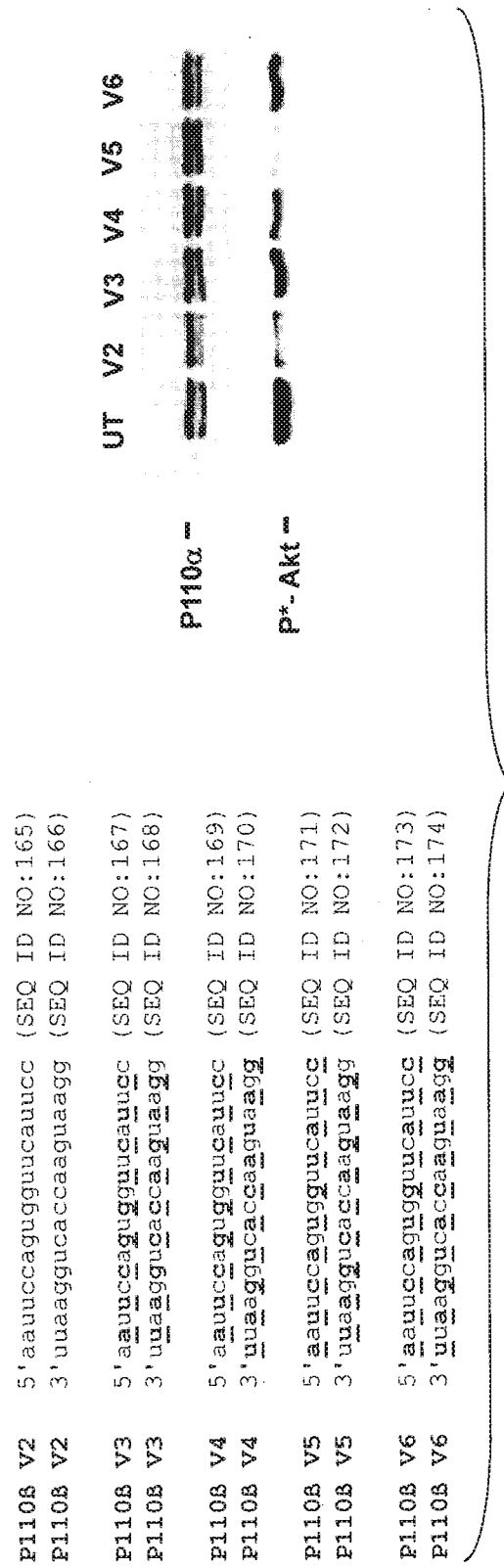


Fig. 16C

Fig. 17A

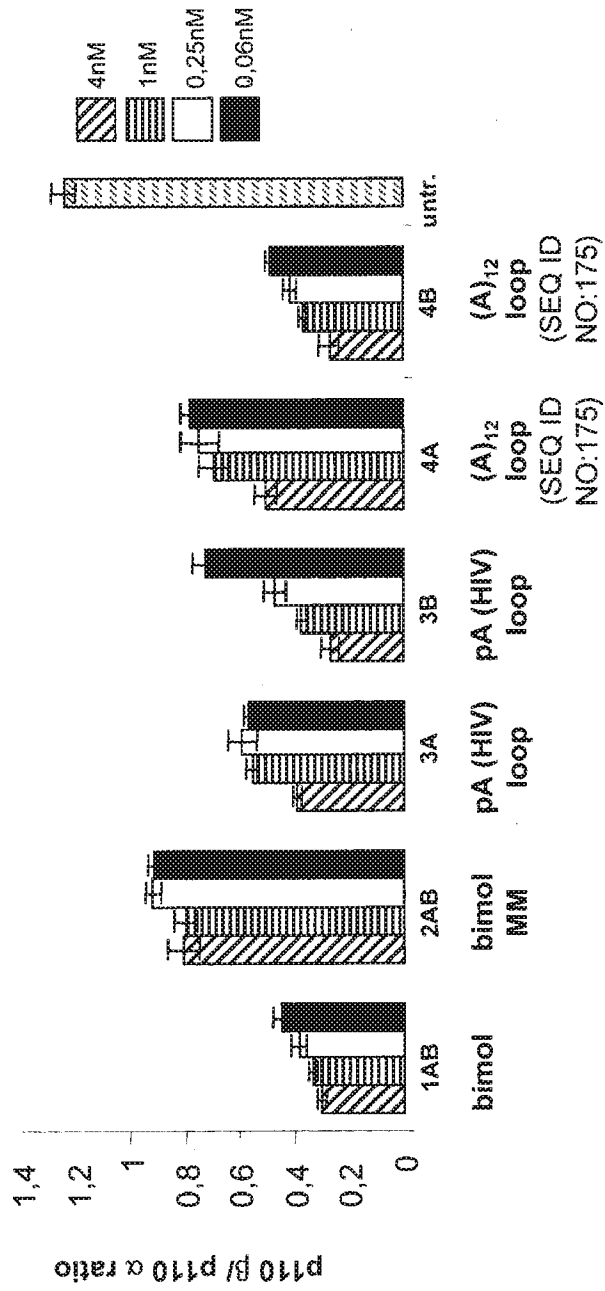


Fig. 17B

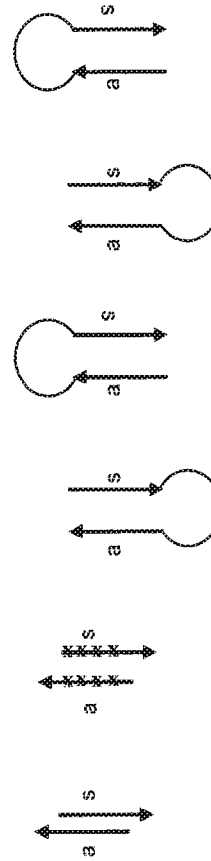


Fig. 18A

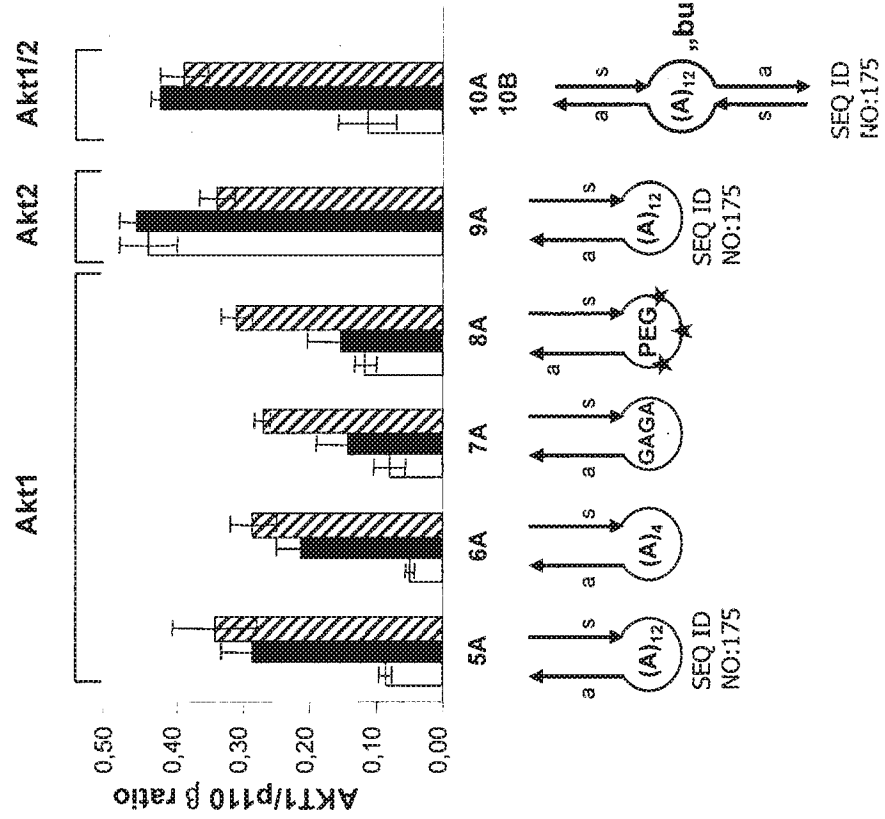
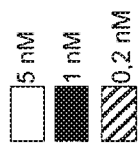
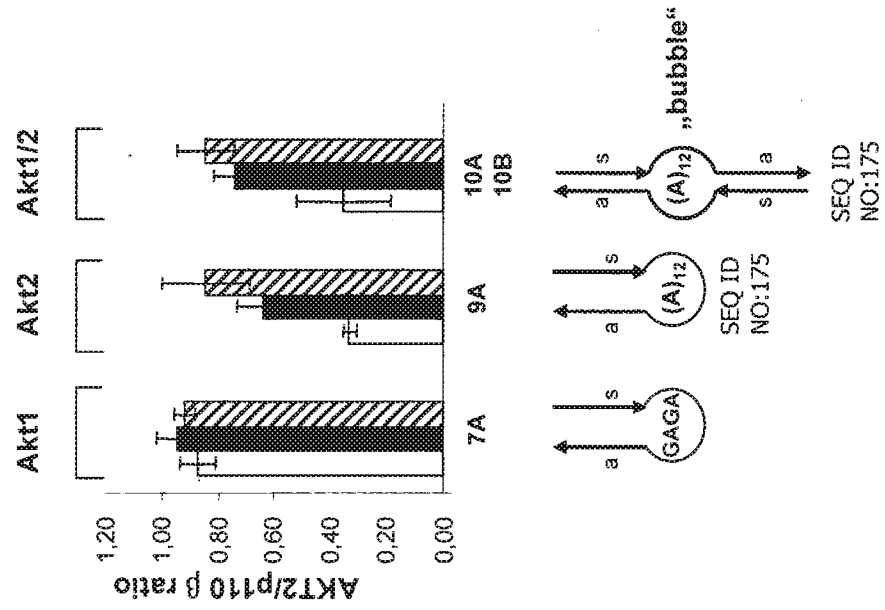


Fig. 18B



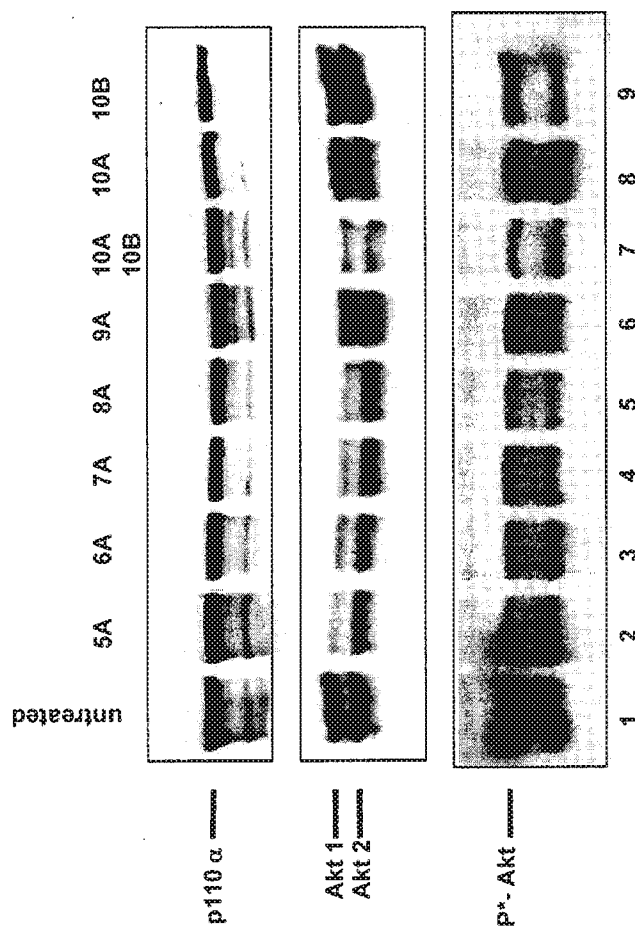


Fig. 18C

Fig. 19A

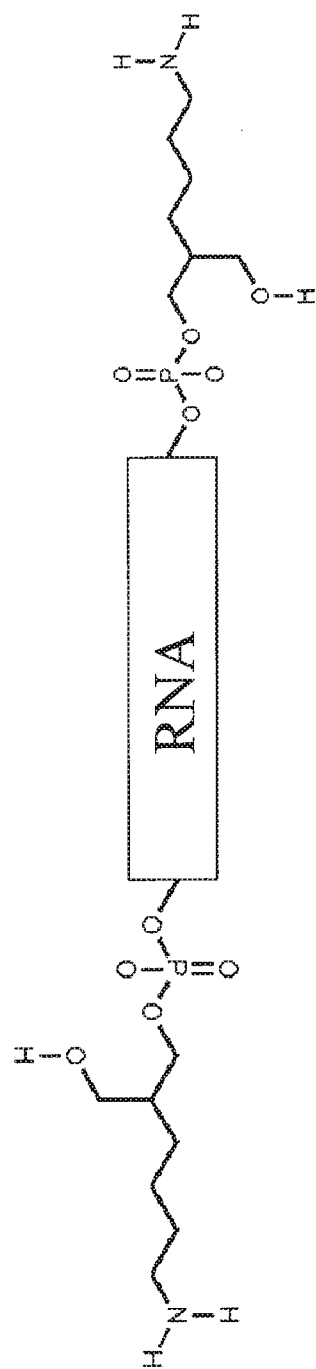
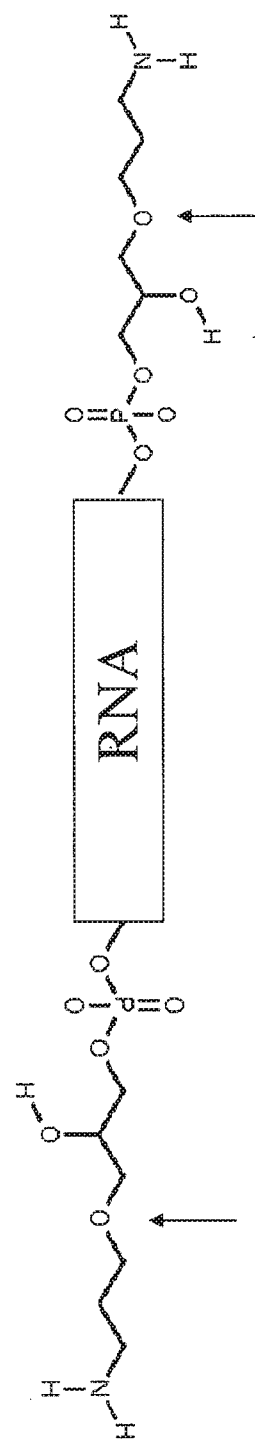


Fig. 19B



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Sequence Listing was accepted.

See attached Validation Report.

If you need help call the Patent Electronic Business Center at (866)
217-9197 (toll free).

Reviewer: Anjum, Durreshwar

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cguuagcaga aacaaaagga gtt

23

<210> 7

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oligonucleotide

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oligonucleotide

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oligonucleotide

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oligonucleotide

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cgugagcaca aagaaaauga gtt 23

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<213> Artificial Sequence

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oligonucleotide

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<210> 14

<211> 21

<212> RNA

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<210> 15

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<212> RNA

<213> Artificial Sequence

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oligonucleotide

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<223> 29A(MM) (Fig. 4B)

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oligonucleotide

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<210> 18
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oligonucleotide

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23

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<210> 20

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ucuugaugua cucccccucgu u

21

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 oligonucleotide

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<210> 32

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 <400> 46
 gaggggagua caucaagac 19

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21

<210> 48

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21

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<210> 51

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<400> 51
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<210> 52

<211> 23

<212> DNA

<213> Artificial Sequence

<220>

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APPLICATION NUMBER	FILING or 371(c) DATE	GRP ART UNIT	FIL FEE REC'D	ATTY. DOCKET NO	TOT CLAIMS	IND CLAIMS
15/589,968	05/08/2017	1674	2400	680132000106	30	1

CONFIRMATION NO. 8531

FILING RECEIPT



0000000091491578

25226
MORRISON & FOERSTER LLP
755 PAGE MILL RD
PALO ALTO, CA 94304-1018

Date Mailed: 05/22/2017

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Assignment For Published Patent Application

Silence Therapeutics GMBH, Berlin, GERMANY

Power of Attorney: None

Domestic Priority data as claimed by applicant

This application is a CON of 14/977,710 12/22/2015
which is a CON of 14/578,636 12/22/2014 PAT 9222092
which is a CON of 13/692,178 12/03/2012 PAT 8933215
which is a CON of 12/986,389 01/07/2011 PAT 8324370
which is a CON of 12/200,296 08/28/2008 PAT 7893245
which is a CON of 10/633,630 08/05/2003 PAT 7452987
which claims benefit of 60/402,541 08/12/2002

Foreign Applications (You may be eligible to benefit from the **Patent Prosecution Highway** program at the USPTO. Please see <http://www.uspto.gov> for more information.)

EUROPEAN PATENT OFFICE (EPO) 03008383.6 04/10/2003

EUROPEAN PATENT OFFICE (EPO) 02017601.2 08/05/2002

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The country code and number of your priority application, to be used for filing abroad under the Paris Convention, is **US 15/589,968**

Projected Publication Date: 08/31/2017

Non-Publication Request: No

Early Publication Request: No

Title

INTERFERING RNA MOLECULES

Preliminary Class

536

Statement under 37 CFR 1.55 or 1.78 for AIA (First Inventor to File) Transition Applications: No

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Title 37, Code of Federal Regulations, 5.11 & 5.15

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PATENT APPLICATION FEE DETERMINATION RECORD Substitute for Form PTO-875						Application or Docket Number 15/589,968				
APPLICATION AS FILED - PART I										
(Column 1)		(Column 2)		SMALL ENTITY		OR OTHER THAN SMALL ENTITY				
FOR	NUMBER FILED	NUMBER EXTRA	RATE(\$)	FEE(\$)		RATE(\$)	FEE(\$)			
BASIC FEE (37 CFR 1.16(a), (b), or (c))	N/A	N/A	N/A			N/A	280			
SEARCH FEE (37 CFR 1.16(k), (l), or (m))	N/A	N/A	N/A			N/A	600			
EXAMINATION FEE (37 CFR 1.16(o), (p), or (q))	N/A	N/A	N/A			N/A	720			
TOTAL CLAIMS (37 CFR 1.16(j))	30 minus 20 =	*				x 80 =	800			
INDEPENDENT CLAIMS (37 CFR 1.16(h))	1 minus 3 =	*				x 420 =	0.00			
APPLICATION SIZE FEE (37 CFR 1.16(s))	If the specification and drawings exceed 100 sheets of paper, the application size fee due is \$310 (\$155 for small entity) for each additional 50 sheets or fraction thereof. See 35 U.S.C. 41(a)(1)(G) and 37 CFR 1.16(s).						0.00			
MULTIPLE DEPENDENT CLAIM PRESENT (37 CFR 1.16(j))							0.00			
* If the difference in column 1 is less than zero, enter "0" in column 2.				TOTAL		TOTAL	2400			
APPLICATION AS AMENDED - PART II										
(Column 1)		(Column 2)		(Column 3)		SMALL ENTITY		OR OTHER THAN SMALL ENTITY		
AMENDMENT A		CLAIMS REMAINING AFTER AMENDMENT		HIGHEST NUMBER PREVIOUSLY PAID FOR	PRESENT EXTRA	RATE(\$)	ADDITIONAL FEE(\$)		RATE(\$)	ADDITIONAL FEE(\$)
	Total (37 CFR 1.16(i))	*	Minus	**	=	x =			x =	
	Independent (37 CFR 1.16(h))	*	Minus	***	=	x =			x =	
	Application Size Fee (37 CFR 1.16(s))									
	FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM (37 CFR 1.16(j))									
						TOTAL ADD'L FEE			TOTAL ADD'L FEE	
AMENDMENT B		CLAIMS REMAINING AFTER AMENDMENT		HIGHEST NUMBER PREVIOUSLY PAID FOR	PRESENT EXTRA	RATE(\$)	ADDITIONAL FEE(\$)		RATE(\$)	ADDITIONAL FEE(\$)
	Total (37 CFR 1.16(i))	*	Minus	**	=	x =			x =	
	Independent (37 CFR 1.16(h))	*	Minus	***	=	x =			x =	
	Application Size Fee (37 CFR 1.16(s))									
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Patentanmeldung Nr. Patent application No. Demande de brevet n°

02017601.2

Der Präsident des Europäischen Patentamts;
Im Auftrag

For the President of the European Patent Office

Le Président de l'Office européen des brevets
p.o.

R C van Dijk



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Novel forms of interfering RNA molecules

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Novel forms of interfering RNA molecules

The present invention is related to a ribonucleic acid comprising a double-stranded structure whereby the double-stranded structure comprises a first strand and a second strand, whereby the first strand comprises a first stretch of contiguous nucleotides and whereby said first stretch is at least partially complementary to the target nucleic acid, and the second strand comprises a second stretch of contiguous nucleotides whereby said second stretch is at least partially identical to a target nucleic acid, the use of such ribonucleic acid, a cell and an organism, respectively, comprising such ribonucleic acid, a composition containing such ribonucleic acid, a pharmaceutical composition containing such ribonucleic acid and a method for inhibiting expression of a targeted gene.

RNA-mediated interference (RNAi) is a post-transcriptional gene silencing mechanism initiated by double stranded RNA (dsRNA) homologous in sequence to the silenced gene (Fire (1999), Trends Genet 15, 358-63, Tuschl, *et al.* (1999), Genes Dev 13, 3191-7, , Waterhouse, *et al.* (2001), Nature 411, 834-42, Elbashir, *et al.* (2001), Nature 411, 494-8, for review see Sharp (2001), Genes Dev 15, 485-90, Barstead (2001), Curr Opin Chem Biol 5, 63-6). RNAi has been used extensively to determine gene function in a number of organisms, including plants (Baulcombe (1999), Curr Opin Plant Biol 2, 109-13), nematodes (Montgomery, *et al.* (1998), Proc Natl Acad Sci U S A 95, 15502-7), *Drosophila* (Kennerdell, *et al.* (1998), Cell 95, 1017-26, Kennerdell, *et al.* (2000), Nat Biotechnol 18, 896-8). In the nematode *C. elegans* about one third of the genome has already been subjected to functional analysis by RNAi (Kim (2001), Curr Biol 11, R85-7, Maeda, *et al.* (2001), Curr Biol 11, 171-6).

Until recently RNAi in mammalian cells was not generally applicable, with the exception of early mouse development (Wianny, *et al.* (2000), Nat Cell Biol 2, 70-5). The discovery that transfection of duplexes of 21-nt into mammalian cells interfered with gene expression and did not induce a sequence independent interferon-driven anti-viral response usually obtained with long dsRNA led to new potential application in differentiated mammalian cells (Elbashir

et al. (2001), Nature 411, 494-8). Interestingly these small interfering RNAs (siRNAs) resemble the processing products from long dsRNAs suggesting a potential bypassing mechanism in differentiated mammalian cells. The Dicer complex, a member of the RNase III family, necessary for the initial dsRNA processing has been identified (Bernstein, *et al.* (2001), Nature 409, 363-6, Billy, *et al.* (2001), Proc Natl Acad Sci U S A 98, 14428-33). One of the problems previously encountered when using unmodified ribooligonucleotides was the rapid degradation in cells or even in the serum-containing medium (Wickstrom (1986), J Biochem Biophys Methods 13, 97-102, Cazenave, *et al.* (1987), Nucleic Acids Res 15, 10507-21). It will depend on the particular gene function and assay systems used whether the respective knock down induced by transfected siRNA will be maintained long enough to achieve a phenotypic change.

The problem underlying the present invention was to provide synthetic interfering RNA molecules which are both stable and active in a biochemical environment such as a living cell.

In a first aspect of the present invention the problem is solved by a ribonucleic acid comprising a double stranded structure whereby the double- stranded structure comprises a first strand and a second strand, whereby the first strand comprises a first stretch of contiguous nucleotides and whereby said first stretch is at least partially complementary to a target nucleic acid, and the second strand comprises a second stretch of contiguous nucleotides whereby said second stretch is at least partially identical to a target nucleic acid, and whereby the double stranded structure is blunt ended.

In a second aspect the problem underlying the present invention is solved by a ribonucleic acid comprising a double stranded structure whereby the double- stranded structure comprises a first strand and a second strand, whereby the first strand comprises a first stretch of contiguous nucleotides and whereby said first stretch is at least partially complementary to a target nucleic acid, and the second strand comprises a second stretch of contiguous nucleotides, whereby said second stretch is at least partially identical to a target nucleic acid, whereby the first stretch and/or the second stretch have a length of 18 or 19 nucleotides.

In an embodiment of the ribonucleic acid according to the first aspect of the invention the first stretch and/or the second stretch have a length of 18 or 19 nucleotides.

In a further embodiment of the ribonucleic acid according to the first aspect of the invention the double stranded structure is blunt ended on both sides of the double strand.

In an alternative embodiment of the ribonucleic acid according to the first aspect of the invention the double stranded structure is blunt ended on the double stranded structure which is defined by the 5'-end of the first strand and the 3'-end of the second strand.

In a further alternative embodiment of the ribonucleic acid according to the first aspect of the invention the double stranded structure is blunt ended on the double stranded structure which is defined by the 3'-end of the first strand and the 5'-end of the second strand.

In a third aspect the problem underlying the present invention is solved by a ribonucleic acid comprising a double stranded structure whereby the double- stranded structure comprises a first strand and a second strand, whereby the first strand comprises a first stretch of contiguous nucleotides and whereby said first stretch is at least partially complementary to a target nucleic acid, and the second strand comprises a second stretch of contiguous nucleotides and whereby said second stretch is at least partially identical to a target nucleic acid, and whereby at least one of the two strands has an overhang of at least one nucleotide at the 5'-end.

In an embodiment of the ribonucleic acid according to the third aspect of the present invention the overhang consists of at least one nucleotide which is selected from the group comprising ribonucleotides and desoxyribonucleotides.

In a more preferred embodiment of the ribonucleic acid according to the third aspect of the present invention the nucleotide has a modification whereby said modification is preferably selected from the group comprising nucleotides being an inverted abasic and nucleotides having an NH_2 -modification at the 2'-position.

In a preferred embodiment of the ribonucleic acid according to the third aspect of the present invention at least one of the strands has an overhang of at least one nucleotide at the 3'-end consisting of ribonucleotide or deoxyribonucleotide.

In another preferred embodiment of the ribonucleic acid according to the third aspect of the present invention the first stretch and/or the second stretch have a length of 18 or 19 nucleotides.

In an embodiment of the ribonucleic acid according to any aspect of the present invention the double-stranded structure has a length of 17 to 21 nucleotides, preferably 18 to 19 nucleotides

In an embodiment of the ribonucleic acid according to the third aspect of the present invention the overhang at the 5'-end is on the second strand.

In a preferred embodiment of the ribonucleic acid according to the third aspect of the present invention the first strand comprises also an overhang, preferably at the 5'-end.

In an embodiment of the ribonucleic acid according to the third aspect of the present invention the 3'-end of the first strand comprises an overhang.

In an alternative embodiment of the ribonucleic acid according to the third aspect of the present invention the overhang at the 5'-end is on the first strand.

In a preferred embodiment thereof the second strand also comprise an overhang, preferably at the 5'-end.

In an embodiment of the ribonucleic acid according to the third aspect of the present invention the 3'-end of the first strand comprises an overhang.

In an embodiment of the ribonucleic acid according to any aspect of the present invention at least one nucleotide of the ribonucleic acid has a modification at the 2'-position and the modification is preferably selected from the group comprising amino, fluoro, methoxy and alkyl.

In a fourth aspect the problem underlying the present invention is solved by a ribonucleic acid comprising a double stranded structure, whereby the double- stranded structure comprises a first strand and a second strand, whereby the first strand comprises a first stretch of contiguous nucleotides and whereby said first stretch is at least partially complementary to a

target nucleic acid, and the second strand comprises a second stretch of contiguous nucleotides and whereby said second stretch is at least partially identical to a target nucleic acid,

whereby

said first strand and/or said second strand comprises a plurality of groups of modified nucleotides having a modification at the 2'-position whereby within the strand each group of modified nucleotides is flanked on one or both sides by a flanking group of nucleotides whereby the flanking nucleotides forming the flanking group of nucleotides is either an unmodified nucleotide or a nucleotide having a modification different from the modification of the modified nucleotides.

In an embodiment of the ribonucleic acid according to the fourth aspect of the present invention the ribonucleic acid is the ribonucleic acid according to the first, second or third aspect of the present invention.

In a further embodiment of the ribonucleic acid according to the fourth aspect of the present invention said first strand and/or said second strand comprise said plurality of modified nucleotides.

In another embodiment of the ribonucleic acid according to the fourth aspect of the present invention said first strand comprises said plurality of groups of modified nucleotides.

In yet another embodiment of the ribonucleic acid according to the fourth aspect of the present invention said second strand comprises said plurality of groups of modified nucleotides.

In an preferred embodiment of the ribonucleic acid according to the fourth aspect of the present invention the group of nucleotides and/or the group of flanking nucleotides comprises a number of nucleotides whereby the number is selected from the group comprising one nucleotide to 10 nucleotides.

In another embodiment of the ribonucleic acid according to the fourth aspect of the present invention the pattern of modified nucleotides of said first strand is the same as the pattern of modified nucleotides of said second strand.

In a preferred embodiment of the ribonucleic acid according to the fourth aspect of the present invention the pattern of said first strand aligns with the pattern of said second strand.

In an alternative embodiment of the ribonucleic acid according to the fourth aspect of the present invention the pattern of said first strand is shifted by one or more nucleotides relative to the pattern of the second strand.

In an embodiment of the ribonucleic acid according to the fourth aspect of the present invention the modification is selected from the group comprising amino, fluoro, methoxy and alkyl.

In another embodiment of the ribonucleic acid according to the fourth aspect of the present invention the double stranded structure is blunt ended.

In an preferred embodiment of the ribonucleic acid according to the fourth aspect of the present invention the double stranded structure is blunt ended on both sides.

In another embodiment of the ribonucleic acid according to the fourth aspect of the present invention the double stranded structure is blunt ended on the double stranded structure's side which is defined by the 5'-end of the first strand and the 3'-end of the second strand.

In still another embodiment of the ribonucleic acid according to the fourth aspect of the present invention the double stranded structure is blunt ended on the double stranded structure's side which is defined by at the 3'-end of the first strand and the 5'-end of the second strand.

In another embodiment of the ribonucleic acid according to the fourth aspect of the present invention at least one of the two strands has an overhang of at least one nucleotide at the 5'-end.

In a preferred embodiment of the ribonucleic acid according to the fourth aspect of the present invention the overhang consists of at least one desoxyribonucleotide.

In a further embodiment of the ribonucleic acid according to the fourth aspect of the present invention at least one of the strands has an overhang of at least one nucleotide at the 3'-end.

In an embodiment of the ribonucleic acid according to any of the aspects of the present invention the length of the double-stranded structure has a length from about 17 to 21 and more preferably 18 or 19 bases

In another embodiment of the ribonucleic acid according to any of the aspects of the present invention the length of said first strand and/or the length of said second strand is independently from each other selected from the group comprising the ranges of from about 15 to about 23 bases, 17 to 21 bases and 18 or 19 bases.

In a preferred embodiment of the ribonucleic acid according to any of the aspects of the present invention the complementarity between said first strand and the target nucleic acid is perfect.

In an embodiment of the ribonucleic acid according to any of the aspects of the present invention the duplex formed between the first strand and the target nucleic acid comprises at least 15 nucleotides wherein there is one mismatch or two mismatches between said first strand and the target nucleic acid forming said double-stranded structure.

In a preferred embodiment of the ribonucleic acid according to any of the aspects of the present invention the target gene is selected from the group comprising structural genes, housekeeping genes, transcription factors, motility factors, cell cycle factors, cell cycle inhibitors, enzymes, growth factors, cytokines and tumor suppressors.

In a further embodiment of the ribonucleic acid according to any of the aspects of the present invention the first strand and the second strand are linked by a loop structure.

In a preferred embodiment of the ribonucleic acid according to any of the aspects of the present invention the loop structure is comprised of a non-nucleic acid polymer.

In a preferred embodiment thereof the non-nucleic acid polymer is polyethylene glycol.

In an alternative embodiment thereof the loop structure is comprised of a nucleic acid.

In an embodiment of the ribonucleic acid according to any of the aspects of the present invention the 5'-terminus of the first strand is linked to the 3'-terminus of the second strand.

In a further embodiment of the ribonucleic acid according to any of the aspects of the present invention the 3'-end of the first strand is linked to the 5'-terminus of the second strand.

In a fifth aspect the problem underlying the present invention is solved by the use of a ribonucleic acid according to any of the aspects of the present invention, for target validation.

In a sixth aspect the problem underlying the present invention is solved by the use of a ribonucleic acid according to any of the aspects of the present invention, for the manufacture of a medicament.

In a preferred embodiment of the use according to the sixth aspect of the present invention the medicament is for the treatment of a disease or of a condition which is selected from the group comprising glioblastoma, prostate cancer, breast cancer, lung cancer, liver cancer, colon cancer, pancreatic cancer and leukaemia, diabetes, obesity, cardiovascular diseases, and metabolic diseases.

In a seventh aspect the problem underlying the present invention is solved by a cell, preferably a knockdown cell, containing a ribonucleic acid according to any of the aspects of the present invention.

In an eighth aspect the problem underlying the present invention is solved by an organism, preferably a knockdown organism, containing a ribonucleic acid according to any of the aspects of the present invention.

In a ninth aspect the problem underlying the present invention is solved by a composition containing a ribonucleic acid according to any of the aspects of the present invention.

In a tenth aspect the problem underlying the present invention is solved by a pharmaceutical composition containing a ribonucleic acid according to any of the aspects of the present invention, and a pharmaceutically acceptable carrier.

In an eleventh aspect the problem underlying the present invention is solved by a method for inhibiting the expression of a target gene in a cell or derivative thereof comprising introduction of a ribonucleic acid according to any of the aspects of the present invention into the cell in an amount sufficient to inhibit expression of the target gene, wherein the target gene is the target gene of the a ribonucleic acid according to any of the aspects of the present invention.

The present invention is based on the surprising finding that small interfering RNAs can be designed such as to be both highly specific and active as well as stable under the reaction conditions typically encountered in biological systems such as biochemical assays or cellular environments. The various interfering RNAs described in the prior art such as by Tuschl et al. (international patent application WO 01/75164) provide for a length of 21 to 23 nucleotides and a modification at the 3' end of the double-stranded RNA. It has been surprisingly found by the present inventors that the problem of stability of interfering RNA, including small interfering RNA (siRNA) which is generally referred to herein in the following as RNAi, actually resides in the attack of endonucleases rather than exonucleases as thought earlier. Based on this finding several strategies have been perceived by the present inventors which are subject to the present application.

The present invention is thus related to new forms of interfering RNA. RNAi consists of a ribonucleic acid comprising a double-stranded structure. Said double-stranded structure is formed by a first strand and a second strand. Said first strand comprises a stretch of contiguous nucleotides, also referred to as first stretch of contiguous nucleotides herein, and this first stretch is at least partially complementary to a target nucleic acid. Said second strand comprises also a stretch of contiguous nucleotides whereby said second stretch is at least partially identical to a target nucleic acid. The very basic structure of this ribonucleic acid is schematically shown in Fig. 1. Said first strand and said second strand are preferably hybridised to each other and form the double-stranded structure. The hybridisation typically occurs by Watson Crick base pairing. The inventive ribonucleic acid, however, is not

necessarily limited in its length to said double-stranded structure. There might be further nucleotides added to each strand and/or to each end of any of the strands forming the RNAi. Depending on the particular sequence of the first stretch and the second stretch, the hybridisation or base pairing is not necessarily complete or perfect, which means that the first and the second stretch are not 100 % base paired due to mismatches. There might also be one or more mismatches within the duplex. Said mismatches have no effect on the RNAi activity if placed outside a stretch of 17 matching nucleotides. If mismatches are placed to yield only 15 or less contiguous matching nucleotides, the RNAi molecule typically shows a reduced activity in down regulating mRNA for a given target compared to a 17 matching nucleotide duplex.

The first stretch of contiguous nucleotides is essentially complementary to a target nucleic acid, more preferably to a part of the target nucleic acid. Complementary as used herein preferably means that the nucleotide sequence of the first strand is hybridising to a nucleic acid sequence or a part thereof of a target nucleic acid sequence. Typically, the target nucleic acid sequence is, in accordance with the mode of action of interfering ribonucleic acids, a single stranded RNA, more preferably an mRNA. Such hybridisation occurs most likely through Watson Crick base pairing, however, is not necessarily limited thereto. The extent to which said first strand and more particularly the first stretch of contiguous nucleotides of said first strand is complementary to a target nucleic acid sequence can be as high as 100% and be as little as 80%, preferably 80-100%, more preferably 85-100%, most preferably 90-100%. Optimum complementarity seems to be 95-100%. Complementarity in this sense means that the aforementioned range of nucleotides, such as, e. g., 80%-100%, depending on the particular range, of the nucleotides are perfect by Watson Crick base pairing. It is shown in one aspect of the present invention that the complementarity between said first stretch of nucleotides and the target RNA has to be 18-19 nucleotides, stretches of as little as 17 nucleotides even with two sequence specific overhangs are not functional in mediating RNAi. Accordingly, given a duplex having a length of 19 nucleotides or base pairs a minimum complementarity of 17 nucleotides or nucleotide base pairs would be acceptable allowing for a mismatch of two nucleotides. In case of a duplex consisting of 20 nucleotides or base pairs a complementarity of 17 nucleotides or nucleotide base pairs would be allowable and functionally active. The same applies to a duplex of 21 nucleotides or base pairs with a total of 17 complementary nucleotides or base pairs. Basically, the extent of complementarity required for a length of a duplex, i. e. of a double stranded structure, can also be based on the

melting temperature of the complex formed by either the double stranded structure as described herein or by the complex of the first stretch of the first strand and the target nucleic acid.

It is to be understood that all of the ribonucleic acid of the present invention are suitable to cause or being involved in RNA mediated interference such as, for example, described in international patent applications WO 99/32619, WO 00/44895 and WO 01/75164.

The first strategy according to which an interfering ribonucleic acid molecule may be designed according to the present invention is to have an optimum length of 18 or 19 nucleotides of the stretch which is complementary to the target nucleic acid. It is also within the present invention that said optimum length of 18 or 19 nucleotides is the length of the double stranded structure in the RNAi used. This length requirement is clearly different from the technical teaching of the prior art such as, for example, the international patent application WO 01/75164. It is within the present invention that any further design, both according to the present invention and as described in the prior art, can be realised in connection with an interfering ribonucleic acid having said length characteristics, i.e. a length of 18 or 19 nucleotides.

The second strategy according to which an interfering ribonucleic acid molecule may be designed is to have a free 5' hydroxyl group, also referred to herein as free 5' OH-group at the first strand. A free 5' OH-group means that the most terminal nucleotide forming the first strand is present and is thus not modified, particularly not by an end modification. Typically, the terminal 5'-hydroxy group of the second strand, respectively, is also present in an unmodified manner. In a more preferred embodiment, also the 3'-end of the first strand and first stretch, respectively, is unmodified such as to present a free OH-group which is also referred to herein as free 3'OH-group. Preferably such free OH-group is also present at the 3'-end of the second strand and second stretch, respectively. In other embodiments of the ribonucleic acid molecules according to the present invention the 3'-end of the first strand and first stretch, respectively, and/or the 3'-end of the second strand and second stretch, respectively, may have an end modification at the 3' end.

As used herein the terms free 5'OH-group and 3'OH-group also indicate that the respective most terminal nucleotide at the 5'end and the 3' end of the polynucleotide, respectively,

presents a OH-group. Such OH-group may stem from either the sugar moiety of the nucleotide, more preferably from the 5'position in case of the 5'OH-group and from the 3'position in case of the 3'OH-group, or from a phosphate group attached to the sugar moiety of the respective terminal nucleotide. The phosphate group may in principle be attached to any OH-group of the sugar moiety of the nucleotide. Preferably, the phosphate group is attached to the 5'OH-group of the sugar moiety in case of the free 5'OH-group and/or to the 3'OH-group of the sugar moiety in case of the free 3'OH-group.

As used herein with any strategy for the design of RNAi or any embodiment of RNAi disclosed herein, the term end modification means a chemical entity added to the most 5' or 3' nucleotide of the first and/or second strand. Examples for such end modifications include, but are not limited to, inverted (deoxy) abasics, amino, fluoro, chloro, bromo, CN, CF, methoxy, imidazole, caboxylate, thioate, C₁ to C₁₀ lower alkyl, substituted lower alkyl, alkaryl or aralkyl, OCF₃, OCN, O-, S-, or N-alkyl; O-, S-, or N-alkenyl; SOCH₃; SO₂CH₃; ONO₂; NO₂, N₃; heterozycloalkyl; heterozycloalkaryl; aminoalkylamino; polyalkylamino or substituted silyl, as, among others, described in European patents EP 0 586 520 B1 or EP 0 618 925 B1.

A further end modification is a biotin group. Such biotin group may preferably be attached to either the most 5' or the most 3' nucleotide of the first and/or second strand or to both ends. In a more preferred embodiment the biotin group is coupled to a polypeptide or a protein. It is also within the scope of the present invention that the polypeptide or protein is attached through any of the other aforementioned end modifications. The polypeptide or protein may confer further characteristics to the inventive nucleic acid molecules. Among others the polypeptide or protein may act as a ligand to another molecule. If said other molecule is a receptor the receptor's function and activity may be activated by the binding ligand. The receptor may show an internalization activity which allows an effective transfection of the ligand bound inventive nucleic acid molecules. An example for the ligand to be coupled to the inventive nucleic acid molecule is VEGF and the corresponding receptor is the VEGF receptor.

Various possible embodiments of the RNAi of the present invention having different kinds of end modification(s) are presented in the following table 1.

Table 1: Various embodiments of the interfering ribonucleic acid according to the present invention

	1 st strand/1 st stretch	2 nd strand/ 2 nd stretch
1.) 5'-end 3'-end	free OH free OH	free OH free OH
2.) 5'-end 3'-end	free OH end modification	free OH end modification
3.) 5'-end 3'-end	free OH free OH	free OH end modification
4.) 5'-end 3'-end	free OH end modification	free OH free OH
5.) 5'-end 3'-end	free OH free OH	end modification free OH
6.) 5'-end 3'-end	free OH end modification	end modification free OH
7.) 5'-end 3'-end	free OH free OH	end modification end modification
8.) 5'-end 3'-end	free OH end modification	end modification end modification

The various end modifications as disclosed herein are preferably located at the ribose moiety of a nucleotide of the ribonucleic acid. More particularly, the end modification may be attached to or replace any of the OH-groups of the ribose moiety, including but not limited to the 2'OH, 3'OH and 5'OH position, provided that the nucleotide thus modified is a terminal nucleotide. Inverted abasics are nucleotides, either desoxyribonucleotides or ribonucleotides which do not have a nucleobase moiety. This kind of compound is, among others, described in Sternberger et al.(2002), Antisense. Nucl. Ac. Drug Dev. in press.

Any of the aforementioned end modifications may be used in connection with the various embodiments of RNAi depicted in table 1. In connection therewith it is to be noted that any of the RNAi forms or embodiments disclosed herein with the sense strand being inactivated, preferably by having an end modification more preferably at the 5' end, are particularly advantageous. This arises from the inactivation of the sense strand which corresponds to the

second strand of the ribonucleic acids described herein, which might otherwise interfere with an unrelated single-stranded RNA in the cell. Thus the expression and more particularly the translation pattern of the transcriptome of a cell is more specifically influenced. This effect is also referred to as off-target effect. Referring to table 1 those embodiments depicted as embodiments 7 and 8 are particularly advantageous in the above sense as the modification results in an inactivation of the – target unspecific – part of the RNAi (which is the second strand) thus reducing any unspecific interaction of the second strand with single-stranded RNA in a cellular or similar system where the RNAi according to the present invention is going to be used to knock down specific ribonucleic acids and proteins, respectively.

A third strategy subject to the present invention is to realise a ribonucleic acid comprising a double-stranded structure whereby the double-stranded structure comprises a first strand and a second strand, whereby the first strand comprises a first stretch of contiguous nucleotides and whereby said first stretch is at least partially complementary to a target nucleic acid, and the second strand comprises a second stretch of contiguous nucleotides whereby said second stretch is at least partially identical to a target nucleic acid whereby the double-stranded structure is blunt-ended. As used in connection with the present description the term double-stranded structure is also referred to as duplex. This design of RNAi is thus clearly different from, e. g., the one of Tuschl et al. as specified in international patent application WO 01/75164 which discloses a 3'-overhang. As used herein overhang refers to a double-stranded structure whereby at least one end of one strand is longer than the corresponding end of the other strand forming the double-stranded structure, which is also referred to herein as the counter strand. Preferably, the first stretch is identical to the first strand and the second stretch is identical to the second strand.

Taken the effectiveness of blunt-ended RNAi and the advantages of an end modification of either the first or the second strand or both, respectively, of the respective ribonucleic acid it is preferred to have a combination of both design principles. In other words, it is within the present invention to have blunt-ended RNAi carrying any end modification scheme as depicted in table 1.

The fourth strategy subject to the present invention is to have an overhang at the 5'-end of the ribonucleic acid. More particularly, such overhang may in principle be present at either or both the first strand and second strand of the ribonucleic acid according to the present

invention. The length of said overhang may be as little as one nucleotide and as long as 2 to 8 nucleotides, preferably 2, 4, 6 or 8 nucleotides. It is within the present invention that the 5' overhang may be located on the first strand and/or the second strand of the ribonucleic acid according to the present application. The nucleotide(s) forming the overhang may be (a) desoxyribonucleotide(s), (a) ribonucleotide(s) or a continuation thereof.

The overhang preferably comprises at least one desoxyribonucleotide, whereby said one desoxyribonucleotide is preferably the most 5'-terminal one. It is within the present invention that the 3'-end of the respective counter-strand of the inventive ribonucleic acid does not have an overhang, more preferably not a desoxyribonucleotide overhang. Here again, any of the inventive ribonucleic acids may comprise an end modification scheme as outlined in connection with table 1 and/or an end modification as outlined herein.

The fifth strategy in the design of interfering ribonucleic acids subject to the present application resides in the formation of a certain pattern of modified nucleotides on at least one of the strands and more particularly on one of the stretches of contiguous nucleotides of the ribonucleic acid(s) according to the present invention. The kind of modification of said nucleotides may be the same as discussed in connection with the other strategies for designing interfering RNA as disclosed herein and more particularly the kind of modification described herein for or as an end modification, such as, e.g., inverted abasics, methoxy, or amino and the like at the ribose moiety of at least one nucleotide forming the ribonucleotide acids according to the present application. It is to be noted that the modification of said nucleotides may be any form of modification described herein, more particularly the kind of modification as described herein as end modification with the particularity that the so-called end modification is not necessarily located at terminal nucleotides. Rather the modification is occurring at a non-terminal nucleotide. Under such conditions the modification is preferably attached to the ribose moiety of the - to be - modified nucleotide and even more preferably to the 2' position of the ribose moiety.

It is also within the present invention that any ribonucleic acid designed according to this strategy may also have the features conferred to a ribonucleic acid according to the present application by any of the other design strategies disclosed herein. Accordingly, the interfering ribonucleic acid having a pattern of modified nucleotides may have an end modification, an

end modification scheme, may be blunt ended or may have a 5' overhang or any combination of two or more of these elements or characteristics.

Apart from the aforementioned modifications which may be presented either as end modifications or as modification pattern, the ribonucleic acid backbone as such may be further modified by forming different links between the nucleotides. Such different links are, among others, described in European patent EP 0 586 520 B1 and European patent EP 0 618 925 B1. Of particular interest here are internal modification(s) of the ribonucleic acid backbone which have been shown to confer higher nuclease resistance of ribooligonucleotides. In a preferred embodiment the modification of the modified nucleotide is a methoxylation of the 2'-OH-group of the ribose moiety of the nucleotide.

In a preferred embodiment, both strands, and more particularly both the first stretch and the second stretch show this kind of modification of the nucleotides forming said strands and stretches, respectively. However, it is also within the present invention that either the first strand and first stretch, respectively, or the second strand and second stretch, respectively, show this particular pattern of modification of the nucleotides. As used herein, the term group of modified nucleotide or flanking group of nucleotide may comprise or represent as little nucleotides as one nucleotide.

Taken the stretch of contiguous nucleotides there is a pattern of modification of the nucleotides forming the stretch may be realised such that a single nucleotide or group of nucleotides which are covalently linked to each other via standard phosphodiester bonds or, at least partially, through phosphorothioate bonds, show such kind of modification. In case such nucleotide or group of nucleotides is not forming the 5'-end or 3'-end of said stretch a nucleotide or group of nucleotides follows on both sides of the nucleotide which does not have the modification of the preceding nucleotide or group of nucleotides. It is to be noted that this kind of nucleotide or group of nucleotides, however, may have a different modification. This sequence of modified nucleotide and group of nucleotides, respectively, and unmodified or differently modified nucleotide or group of nucleotides may be repeated one or several times. Preferably, the sequence is repeated more than one time. For reason of clarity the pattern is discussed in more detail in the following, generally referring to a group of modified nucleotides or a group of unmodified nucleotides whereby each of said group may actually comprise as little as a single nucleotide. Unmodified nucleotide as used herein

means either not having any of the afore-mentioned modifications at the nucleotide forming the respective nucleotide or group of nucleotides, or having a modification which is different from the one of the modified nucleotide and group of nucleotides, respectively.

It is also within the present invention that the modification of the unmodified nucleotide(s) wherein such unmodified nucleotide(s) is/are actually modified in a way different from the modification of the modified nucleotide(s), can be the same or even different for the various nucleotides forming said unmodified nucleotides.

The pattern of modified and unmodified nucleotides may be such that the 5'-terminal nucleotide of the strand or of the stretch starts with a modified group of nucleotides or starts with an unmodified group of nucleotides. However, in an alternative embodiment it is also possible that the 5'-terminal nucleotide is formed by an unmodified group of nucleotides.

This kind of pattern may be realised either on the first stretch or the second stretch of the interfering RNA or on both. It has to be noted that a 5' phosphate on the target-complementary strand of the siRNA duplex is required for siRNA function, suggesting that cells check the authenticity of siRNAs through a free 5' OH (which can be phosphorylated) and allow only such bona fide siRNAs to direct target RNA destruction (Nykanen, *et al.* (2001), Cell 107, 309-21).

Preferably, the first stretch shows a kind of pattern of modified and unmodified groups of nucleotides, whereas the second stretch does not show this kind of pattern. This may be useful insofar as the first stretch is actually the more important one for the target-specific degradation process underlying the interference phenomenon of RNA so that for specificity reasons the second stretch can be chemically modified so it is not functional in mediating RNA interference.

However, it is also within the present invention that both the first stretch and the second stretch have this kind of pattern. Preferably, the pattern of modification and non-modification is the same for both the first stretch and the second stretch.

In a preferred embodiment the group of nucleotides forming the second stretch and corresponding to the modified group of nucleotides of the first stretch are also modified

whereas the unmodified group of nucleotides of or forming the second stretch correspond to the unmodified group of nucleotides of or forming the first stretch. This possibility is schematically depicted in Fig. 2A. Another alternative is that there is a phase shift of the pattern of modification of the first stretch and first strand, respectively, relative to the pattern of modification of the second stretch and second strand, respectively. Preferably, the shift is such that the modified group of nucleotides of the first strand corresponds to the unmodified group of nucleotides of the second strand and vice versa. This possibility is shown in Fig. 2B. It is also within the present invention that the phase shift of the pattern of modification is not complete but overlapping as illustrated in Fig. 2C.

In a preferred embodiment the second nucleotide at the terminus of the strand and stretch, respectively, is an unmodified nucleotide or the beginning of group of unmodified nucleotides. Preferably, this unmodified nucleotide or unmodified group of nucleotides is located at the 5'-end of the first and second strand, respectively, and even more preferably of the first strand. In an even more preferred embodiment the unmodified nucleotide or unmodified group of nucleotide is located at the 5'-end of the first strand and first stretch, respectively. In a preferred embodiment the pattern consists of alternating single modified and unmodified nucleotides.

Although not limited thereto, the double-stranded structure of the inventive ribonucleic acid, which is also referred to as duplex, is formed by the first strand and second strand, respectively, or the first and second stretch of contiguous nucleotides. The length of the first stretch and second stretch, respectively, is typically about 15 to about 23, preferably 17 to 21, and more preferably 18 or 19 bases. In connection with this it is to be noted that a length of less than 30 nucleotides, preferably less than 21 nucleotides does not cause any biological system which is basically capable of showing RNA interference and also interferon response, to develop an interferon response. The reason for this resides in the observation that a given cell is experiencing profound physiological changes when double-stranded DNA longer than 30 base pairs binds and activates the protein kinase PKR and 2',5'-oligoadenylate synthetase. Activated PKR stalls translation via phosphorylation of eIF2a, activated 2',5'-AS causes mRNA degradation. These effects are not desired in target validation and animal models because they overrule the effect of the target specific knockdown on the phenotype.

According to a sixth strategy in the design of interfering ribonucleic acids subject to the present application the ribonucleic acid comprises a double-stranded structure whereby the double-stranded structure comprises a first strand and a second strand, whereby the first strand comprises a first stretch of contiguous nucleotides and whereby said first stretch is at least partially complementary to a target nucleic acid, and the second strand comprises a second stretch of contiguous nucleotides whereby said second stretch is at least partially identical to a target nucleic acid whereby one terminus of the first strand and one terminus of the second strand are linked by a loop structure.

In an embodiment the loop structure is comprised of a non-nucleic acid polymer. Such non-nucleic acid polymer may be polyethylene glycol or similar polymers. The non-nucleic acid polymers may in principle be chosen from the group comprising polymers which do not comprise a polynucleotide and allow that the two strands to be linked may actually hybridize to each other. To allow for such hybridization the molecule or moiety of the molecule linking the two stretches hybridizing with each other, has to have a certain molecular structure or molecular flexibility to allow the bending of the molecule so as to allow that both stretches get in close contact and in a three-dimensional orientation which permits hybridization. Such molecule or moiety factually acts as a hinge. In principle any molecule which complies with this requirement may be used in connection with the present invention. Besides polyethylene glycol amino acid based molecules may be used. Such amino acid based molecules may be either homopolymers or heteropolymers. A useful example is a homopolymer consisting of seven glycine residues which allows the generation of a hinge as required to bring the two stretches to hybridize in the close proximity as needed. This glycine based hinge is described, e. g., in Guan K. L. and Dixon J. E. (1991), *Anal Biochem.* 192, 262. In another embodiment the hinge may be formed by crown ethers known in the art.

In an alternative the loop is comprised of a nucleic acid. As used herein, LNA as described in Elayadi and Corey (2001) *Curr Opin Investig Drugs.* 2(4):558-61. Review; Orum and Wengel (2001) *Curr Opin Mol Ther.* 3(3):239-43; and PNA are regarded as nucleic acids and may also be used as loop forming polymers. Basically, the 5'-terminus of the first strand may be linked to the 3'-terminus of the second strand. As an alternative, the 3'-end of the first strand may be linked to the 5'-terminus of the second strand. The nucleotide sequence forming said loop structure is regarded as not being critical. However, the length of the nucleotide sequence forming such loop seems to be critical for sterical reasons. Accordingly, a minimum

length of four nucleotides seems to be appropriate to form the required loop structure. In principle, the maximum number of nucleotides forming the hinge or the link between both stretches to be hybridized is not limited. However, the longer a polynucleotide is, the more likely secondary and tertiary structures are formed and thus the required orientation of the stretches affected. Preferably, a maximum number of nucleotides forming the hinge is about 12 nucleotides. It is within the disclosure of this application that any of the designs described above may be combined with the present sixth strategy, i. e. by linking the two strands covalently in a manner that back folding (loop) can occur through a loop structure.

The present inventors have surprisingly found that if the loop is placed 3' of the antisense strand, i. e. the first strand of the ribonucleic acid(s) according to the present invention, the activities of this kind of RNAi are higher compared to the placement of the loop 5' of the antisense strand. Accordingly, the particular arrangement of the loop relative to the antisense strand and sense strand, i. e. the first strand and the second strand, respectively, is crucial and is thus in contrast to the understanding as expressed in the prior art where the orientation is said to be of no relevance. However, this seems not true given the experimental results presented herein. The understanding as expressed in the prior art is based on the assumption that any RNAi, is subject to a processing during which non-loop linked RNAi is generated. However, if this was the case, the clearly observed increased activity of those structures having the loop placed 3' of the antisense could not be explained. Insofar a preferred arrangement in 5' → 3' direction of this kind of small interfering RNAi is second strand - loop - first strand. The respective constructs may be incorporated into suitable vector systems. Preferably the vector comprises a promoter for the expression of RNAi. Preferably the respective promoter is pol III and more preferably the promoters are the U6, H1, 7SK promoter as described in Good et al. (1997) *Gene Ther*, 4, 45-54.

Because of the general applicability of the concept of interfering RNA and thus the knockdown or knockout for a coding nucleotide such as an mRNA any gene producing such RNA may be modified in its expression by using the ribonucleic acid molecule according to the present invention. Because of this basic and generally applicable mechanism any application based thereon can be realised which imply the knockdown or knockout of a gene. A preferred application is the use of the inventive ribonucleic acid for target validation. As used herein, target validation shall mean a process that involves taking steps to prove that a DNA, RNA, or protein molecule is directly involved in a biological process, preferably in a

process – causally – involved in a disease or non-standard condition and is therefore a suitable target for development of a new therapeutic compound. Sequence homology studies have successfully classified genes into target families. The enormous task of deciphering which of these targets are key players in diseases and which should be used for subsequent drug development needs to be addressed in an efficient manner. Therefore, the knockdown of gene expression should be reduced by 50-100%, preferably by 90% to see significant effects on the phenotype. In other cases depending on the gene, a knockdown of as little as 20% might be sufficient to yield a phenotype. A phenotype will be defined by comparison of cells containing functional RNAi molecules with cells containing non functional RNAi molecules. This will ensure a significant readout even under conditions where the protein function is inhibited only partially. Generally there is no linear correlation between degree of mRNA reduction and the extent of the phenotype. It has to be acknowledged that for some proteins a reduction of about 20 % of the protein is sufficient to create a change in the phenotype whereas in case of other genes and mRNA, respectively, as little as 5 to 10 % remaining protein is sufficient to maintain the observed phenotype.

A further use of the ribonucleic acid molecules according to the present invention is its use for the manufacture of a medicament or its use as a medicament. Such medicament could either be used for diseases or conditions such as any type of cancer where a gene and its product have been linked to the onset, cause or progression of this disease. Additionally, such medicament could be used to treat diseases where the presence or overexpression of a gene product is causing a pathological phenotype. In a preferred embodiment the disease is characterised by a gain of function and may be remedied through application or administration of the corresponding, biologically active RNAi. Diseases or conditions which may be treated by the medicament comprising a ribonucleic acid as disclosed herein, may be selected from the group comprising cancer, heart diseases, metabolic diseases, dermatological diseases, inflammatory diseases, immune system disorders and autoimmune disorders. The various forms of cancer include, but are not limited to, solid tumors and tumors of the hematopoietic system, such as glioblastoma, prostate cancer, breast cancer, lung cancer, liver cancer, pancreatic cancer and leukaemia. Metabolic diseases include, but are not limited to, obesity and diabetes. Dermatological diseases include, but are not limited to, psoriasis. In another aspect the ribonucleic acid molecules according to the present invention may be used as diagnostics, preferably for those diseases as specified in connection with the above-mentioned diseases and conditions. Such diagnosis could be based on the observation that

upon applying the ribonucleic acid molecules according to the present invention to a sample a change in the expression pattern of the sample occurs. Preferably such sample comprises cells from a subject from whom it is assumed that it may exhibit said disease or condition to be treated.

A further application of the nucleic acids according to the present invention resides in their use in the screening of pharmaceutically active compounds and lead optimization. The latter is done such as to compare the effect of candidate drugs such as small molecules and compare the effect created by said candidate drugs with the effect observed upon administering specific RNAi designed on the basis of the principles disclosed herein. In doing so candidate drugs having off-target effects may be eliminated from the screening process whereas those candidate drugs which create a similar or identical phenotype are deemed as highly relevant lead compound or may even be a pharmaceutically active compound themselves. In this approach, the highly specific RNAi molecules act as gold standard against which candidate drugs are measured.

In a further aspect the invention is related to a cell, preferably a knockdown cell, which contains a ribonucleic acid as disclosed herein. Such cell is preferably a cell which is either isolated or contained in a tissue or even organ which again preferably is not contained in an organism. However, the cell may also be contained in an organism. The cell is preferably a cell which is involved in the disease or condition which is to be treated by the inventive ribonucleic acids. This kind of knock-down cells may be used to generate an expression profile based on, e. g., mRNA or protein, in order to elucidate functional relationship and to determine downstream targets.

In a further aspect the invention is related to an organism containing a ribonucleic acid as disclosed herein. Preferably such organism is a vertebrate organism and more preferably the vertebrate organism is a mammal. A mammal as used herein is, among others and not limited thereto, an ape, a dog, a cat, a goat, a sheep, a pig, a guinea pig, a rabbit, a mouse, a rat and a human being. In a further aspect the present invention is related to a composition containing a ribonucleic acid according to the present invention. Preferably such composition comprises negative and positive controls either in combination with the effective ribonucleic acid or separated therefrom. Such composition may further comprise a solvent, preferably a buffer.

In a further aspect the present invention is related to a pharmaceutical composition containing a ribonucleic acid according to the present invention and a pharmaceutically acceptable carrier. Pharmaceutically acceptable carriers are known to the one skilled in the art and comprise, among others, diluent, buffers and the like. The pharmaceutical composition may comprise further pharmaceutically active compounds. In case the disease or condition to be treated using the ribonucleic acid molecules according to the present invention are preferably those which are already now used in connection with the treatment of said diseases or conditions. Due to the different mode of action of the ribonucleic acid molecules according to the present invention and the medicaments used for the treatment of said diseases and conditions according to the prior art, synergistic effects will happen.

The invention is now further illustrated by reference to the figures and examples from which further features, embodiments and advantages of the present invention may be taken.

Fig. 1 shows a schematic illustration defining the terminology as used herein. The upper of the two strand is the first strand and the antisense strand of the targeted nucleic acid such as mRNA. The second strand is the one which essentially corresponds in its sequence to the targeted nucleic acid and thus forms the sense strand. Both, the first strand and second strand form a double-stranded structure, typically through Watson Crick base pairing.

Fig. 2 illustrates some embodiments of the ribonucleic acid molecules of the present invention with patterns of modified and unmodified groups of nucleotides which is also referred to herein as pattern of modification. In Fig. 2A the modified and unmodified groups of nucleotides on both the first stretch and the second stretch are located on corresponding parts of the stretches whereas in Fig. 2B the pattern realised on the first strand is also realised on the second strand, however, with a phase shift such that the modified group of nucleotides of the first stretch is base pairing with an unmodified group of nucleotides of the second stretch and vice versa. In Fig. 2C a further possibility of arranging the modified and unmodified groups of nucleotides is realised. It is also within the present invention that the pattern of the first stretch is independent from the pattern of the second stretch and that both patterns partially overlap in terms of relative position to each other in the double-stranded structure defined by base pairing.

Fig. 3 shows the result of a knockdown experiment using RNAi molecules with different end protection groups. More particularly Fig. 3A shows that the various forms of end protected RNAi molecules are functional on the knockdown of PTEN mRNA.

Fig. 3B shows the sequence of the different RNAi molecules used in the experiment the result of which is depicted in Fig. 3A. Fig. 3C shows the result of an immunoblot analysis of PTEN protein after treatment with modified RNAi molecules in comparison to PTEN specific antisense constructs.

Fig. 4 shows that the 3' overhang of RNAi molecules is not important for RNA interference. More particularly, Fig. 4A shows a dose response curve of different RNAi molecules and Fig. 4B shows the sequence of the RNAi molecules used in the experiment the result of which is shown in Fig. 4A.

Fig. 5 shows that duplex length of the RNAi molecules has to be at least 18-19 nucleotide. More particularly, Fig. 5B shows the sequence of the PTEN specific RNAi molecules used in the experiment the result of which is depicted in Fig. 5A as dose response curve.

Fig. 6 shows that four terminal mismatched nucleotides in RNAi molecules with a length of 19 nucleotides are still functional in mediating Akt1 knockdown. More particularly, Fig. 6B shows the sequence of the RNAi molecules used in the experiment the result of which is depicted in Fig. 6A.

Fig. 7 shows the result of studies on the stability in serum conferred to RNAi molecules by 2'-O-methylation and that end modifications have no beneficial effects on RNAi stability. More particularly, Fig. 7A shows the result of a gel electrophoresis of the various RNAi molecules depicted in Fig. 7B being subject to incubation with fetal calf serum.

Fig. 8 shows that an amino end modification results in loss of activity. Fig. 8B shows the particular RNAi molecules used in the experiments the result of which is shown in Fig. 8A. Fig. 8C shows the design principles which may be deduced from the results depicted in Fig. 8A. As used in Fig. 8C the term functional means functionally active in the particular assay system as described in the example and "not functional" means not functionally active in said system.

Fig. 9 shows that 2'-O-Alkyl (methyl) modifications stabilize RNAi molecules but also result in reduction of their activity. More particularly, Fig. 9C shows the sequence of the RNAi molecules used in the experiment the result of which is depicted as a dose response curve in Fig. 9A. Fig. 9B shows the result of a gel electrophoresis of the various RNAi molecules depicted in Fig. 9C being subject to a two hour incubation in fetal calf serum.

Fig. 10 shows the result of an experiment on the efficacy of RNAi molecules with blocks of 2'-O-methyl modifications with Fig. 10A graphically depicting the results of said experiments as a dose response curve and with Fig. 10C showing the sequences of the particular RNAi molecules used in said experiments. Fig. 10B shows the result of a gel electrophoresis of the various RNAi molecules depicted in Fig. 10C being subject to a two hour incubation in fetal calf serum.

Fig. 11 shows that alternating 2'-O-methyl modification result in activity of the modified RNAi molecules compared to unmodified forms. More particularly, Fig. 11B shows the sequence of the RNAi molecules used in this experiment the result of which is depicted in Fig. 11A. Fig. 11C shows the stability of said RNAi molecules following incubation in serum for two hours, whereas Fig. 11D shows an immunoblot for PTEN protein upon application of different RNAi molecules to HeLa cells. As may be taken therefrom RNAi molecules with alternating modifications are stabilized against endonuclease degradation and active in mediating a PTEN protein knock down.

Fig. 12 shows the result of a Western Blot analysis to determine the time course of PTEN protein knock down. Cells were continuously transfected with 2'-O-Methyl modified versus unmodified RNAi molecules using cationic lipids for 72 h. Protein lysates were prepared and analysed by immunoblot after 48 and 120 h. For the 96 h and 120 h timepoints the cells were splitted, replated and incubated in the absence of RNAi molecules for additional 24 and 48 h. .

Fig. 13 shows a Western Blot depicting the protein knock down of PTEN being persistent using alternating modified RNAi molecules versus unmodified RNAi molecules. Transfection were performed for only 5 h and new medium without transfection reagents was added. Lysates were analysed by immunoblot 72h and 96h post transfection with the indicated RNAi molecules. .

Fig. 14 shows the efficacy of various RNAi molecules with hairpin structures as dose response curve while Fig. 14B shows the structure of said RNAi molecules the result of which is depicted in Fig. 14A. Synthetic siRNAs with different loops are functional in reducing the p110 β , Akt1 and Akt 2 expression. (14A) Inhibition of p110 β mRNA expression in siRNA transfected HeLa cells. Samples were analyzed in parallel for the level of p110 β mRNA expression 24h after transfection of the indicated siRNAs. The transfected bimolecular siRNAs (21mer with 3' TT overhangs, molecule 1AB) or the monomolecular siRNAs with loop structures are schematically shown. Note that the position of the loops (HIV derived pA-loop; (A)₁₂-loop) relative to the antisense sequence is reversed in 3A, 4A relative to 3B, 4B. The 2AB siRNA molecule contains 6 mismatches in the 21mer duplex and serves as a negative control together with the untreated sample. RNA was prepared and subjected to real time RT-PCR (Taqman) analysis. p110 β mRNA levels are shown relative to the mRNA levels of p110 α , which serve as an internal reference. Each bar represents triplicate transfections (\pm standard deviation). HeLa cells were transfected at 50% confluency (2500 cells per 96 well) with siRNAs at the indicated concentrations in growth medium.

Fig. 15 shows the efficacy of various RNAi molecules with intermolecular and intramolecular loop structures as dose response curves. (15A) Inhibition of Akt1 mRNA expression in siRNA transfected HeLa cells. Samples were analysed in parallel for the level of Akt1 and Akt2 mRNA expression 24h after transfection of the indicated siRNAs. The different loops (A-loops; GAGA-loop and a polyethyleneglycol (PEG)-linker) and their putative secondary structure are shown schematically. The siRNA molecule 9A is specific for Akt2 and serves as a negative control. Note that 10A and 10B do not contain self-complementary sequences and are transfected in combination. Akt1 mRNA levels is shown relative to the mRNA levels of p110 β , which served as internal control. (15B) Inhibition of Akt2 mRNA expression in HeLa cells transfected with the indicated siRNA molecules. Akt2 mRNA levels is shown relative to the mRNA levels of p110 β . The Akt1 specific molecule 7A serves here as a negative control.

Fig. 15 C shows a Western Blot analysis on Akt protein depicting the functionality of synthetic siRNAs with different loops in specifically reducing the Akt1 and Akt 2 expression. Inhibition of Akt1 and Akt2 protein expression were analysed by immunoblot. The cells were harvested 48h after transfection of the indicated hairpin siRNAs (20nM). Cell extracts were separated by SDS-PAGE and analysed by immunoblotting using anti-p110 antibody, anti Akt

1/2. Similar results were obtained with an antibody specific for the phosphorylated form of Akt1. The positions of p110 α , another catalytic subunit of PI 3-kinase, which was used as a loading control, and of Akt1, Akt2 and phosphorylated Akt (P*-Akt) are indicated on the left.

Fig. 16 shows an NH₂ modification, also referred to herein as amino modification, which may be present at either the 3'-OH terminal nucleotide or the 5' terminal nucleotide. The amino group is attached to the phosphate which in turn is attached to the OH group of the sugar moiety, through an alkyl group comprising an alkyl chain of 1 to 8, preferably 6 C atoms, whereby the second C atom close to the phosphate group has a CH₂OH group attached thereto. As an alternative the linker may be formed by an ether whereby the ether is comprised of two alcohols whereby one alcohol is an amino alcohol and the other is a dialcohol with one alcohol group involved in the formation of the ether group and the other one being an OH group located at either of the C atoms, preferably at the second C atom relative to the phosphate group.

Example 1: Dose response of synthetic duplex RNAi molecules

In this example the impact of NH₂ end protection groups on the activity of duplex RNAi molecules was investigated. Synthetic siRNAs were purchased from Biospring (Frankfurt, Germany). The ribo-oligonucleotides were resuspended in RNase free TE to a final concentration of 50 μ M. In the case of bimolecular siRNA molecules equal aliquots (100 μ M) were combined to a final concentration of 50 μ M. For the formation of intramolecular duplexes the siRNAs were incubated at 50 °C for 2 min in annealing buffer (25mM NaCl; 5mM MgCl₂) and were cooled down to RT. Transfections were carried out in 96 well or 10-cm plates (at 30% to 50% confluency) by using various cationic lipids such as Oligofectamine, Lipofectamine (Life Technologies), NC388 (Ribozyme Pharmaceuticals, Inc., Boulder, CO), or FuGene 6 (Roche) according to the manufacturer's instructions. RNAi molecules were transfected by adding pre-formed 5x concentrated complex of annealed RNAi and lipid in serum-free medium to cells in complete medium. Prior to transfection 2500 HeLa cells were plated per well 15 – 18 hours before transfection for the 96 well format.

The total transfection volume was 100 μ l for cells plated in 96-wells and 10 ml for cells in 10 cm plates. The final lipid concentration was 0.8 to 1.2 μ g/ml depending on cell density; the RNAi concentration is indicated in each experiment.

Complex formation was allowed to take place for 30min at 37°C. Complexes were added to cells to yield a final 1x concentration of both lipid and RNAi. Depending on the analysis performed following transfection, cells were lysed using a standard cell lysis buffer for protein extraction (Klippel, A., Escobedo, J. A., Hirano, M. and Williams, L. T. (1994). *Mol Cell Biol* 14, 2675-2685) or a denaturing buffer for RNA isolation according to the RNA isolation kit (Invitex, Berlin (Germany) 24 to 48 hours post transfection for RNA analysis and 48 to 72 hours post transfection for protein analysis by Western Blot.

Determination of the relative amounts of RNA levels by Taqman analysis:

24h post transfection the RNA of cells transfected in 96-wells was isolated and purified using the Invisorb RNA HTS 96 kit (Invitex GmbH, Berlin). Inhibition of PTEN mRNA expression was detected by real time RT-PCR (Taqman) analysis using 300 nM PTEN 5' primer CACCGCCAAATTAACTGCAGA, 300 nM PTEN 3' primer AAGGGTTTGATAAGTTCTAGCTGT and 100 nM of the PTEN Taqman probe Fam-TGCACAGTATCCTTTTGAAGACCATAACCCA-Tamra in combination with 40 nM β -actin 5' primer GTTTGAGACCTTCAACACCCCA, 40 nM β -actin 3' primer GACCAGAGGCATACAGGGACA and 100 nM of the β -actin Taqman probe Vic-CCATGTACGTAGCCATCCAGGCTGTG-Tamra. The Akt primers and probes are determined in Sternberger et al. (Sternberger, a.a.O.) and are used according to the manufacturer's instructions (Applied Biosystem; use of Amplicon Set). Also said primers and probes may be designed using the software program Primer Express (Applied Biosystem). The reaction was carried out in 50 μ l and assayed on the ABI PRISM 7700 Sequence detector (Applied Biosystems) according to the manufacturer's instructions under the following conditions: 48°C for 30 min, 95°C for 10 min, followed by 40 cycles of 15 sec at 95°C and 1 min at 60°C.

RNA knockdown is shown by real time RT-PCR analysis of HeLa cells transfected with 21 nt long siRNA duplex molecules unmodified and modified with either NH₂ or inverted Abasics groups at the 5'-end at a lipid carrier concentration of 1.0 μ g/ml. Cell density was 2000 cells/well. Modifications on the 3'-end are either RNA overhangs, RNA overhangs with amino groups or DNA overhangs.

Preparation of cell extracts and immunoblotting. Cells were washed twice with cold phosphate-buffered saline and lysed at 4°C in lysis buffer containing 20 mM Tris (pH 7.5), 137 mM NaCl, 15% (vol/vol) glycerol, 1% (vol/vol) Nonidet P-40 (NP-40), 2 mM phenylmethylsulfonyl fluoride, 10 mg aprotinin per ml, 20 mM leupeptin, 2 mM benzamidine, 1 mM sodium vanadate, 25 mM β -glycerol phosphate, 50 mM NaF and 10 mM NaPPi. Lysates were cleared by centrifugation at 14,000 x g for 5 minutes and aliquots of the cell extracts containing equal amounts of protein were analyzed for protein expression by Western-blotting: Samples were separated by SDS-PAGE and transferred to nitrocellulose-filters (Schleicher & Schuell). Filters were blocked in TBST buffer (10 mM Tris-HCl (pH 7.5), 150 mM NaCl, 0.05% (vol/vol) Tween 20, 0.5% (wt/vol) sodium azide) containing 5% (wt/vol) dried milk. The respective antibodies were added in TBST at appropriate dilutions. Bound antibody was detected using anti-mouse- or anti-rabbit-conjugated horse radish peroxidase (Transduction Laboratories) in TBST, washed, and developed using the SuperSignal West Dura (Pierce) or ECL (Amersham) chemoluminescence substrates (c.f. Sternberger et al. (2002). *Antisense. Nucl. Ac. Drug Dev. in press.*).

Antibodies. The murine monoclonal anti-p110 antibody U3A and the murine monoclonal anti-p85 antibody N7B have been described (Klippel et al., 1994, aaO). Rabbit polyclonal anti-Akt and anti-phospho Akt (S473) antibodies were obtained from Cell Signaling Technology. The murine monoclonal anti-PTEN antibody was from Santa Cruz Biotechnology. The PTEN 53 specific antisense molecule, i. e. geneBloc, is described in Sternberg et al. [Sternberger, supra] having the following sequence (ucuccuuTTGTTTCTGcuaacga), whereby the nucleotide depicted in lower case are ribonucleotides whereas the nucleotide in capital letters are desoxyribonucleotides. This antisense molecule is also identical to RNAi 1A without TT.

The results are shown in Fig. 3A and the respective RNAi molecules in Fig. 3B which are directed to the mRNA of PTEN. The nucleotides written in lower case letters represent ribonucleotides whereas capital letters represent desoxyribonucleotides. The term NH₂ indicates that the 3' '-position of the ribonucleotide was modified by an amino group. The RNAi molecules used in this and other examples disclosed herein are also referred to as small interfering RNA molecules siRNA. It is to be noted that in any of the figures contained herein the upper strand is the antisense or first strand, whereas the lower strand is the sense or second strand of the interfering RNA molecule.

As can be taken from Fig. 3A amino end modifications such as amino modification and inverted abasics modification of the terminal OH group of the nucleic acid are as potent as unmodified ends when the modification is located at the 3' end of the antisense strand (see also Fig. 8A; 8B). Therefore chemical modification to stabilize or with other beneficial properties (delivery) will be tolerated without activity loss when located at the 3' OH; especially when the 3'OH is located on an overhanging nucleotide.

For the experiment shown in Fig. 3C similar conditions as outlined above were used. The first strand and the second strand of the RNAi were either modified by a NH₂ group at the 3'-position of the ribose moiety or by an inverted abasic at said positions. The first construct is designated as siRNA-NH₂ (3A3B) the second as siRNA-iB (4A4B). The sequence of both molecules is depicted in Fig. 3B. The term 3A3B indicates that the interfering ribonucleic acid consists of strand 3A as the antisense strand and strand 3B as the sense strand. For reason of comparison an antisense oligonucleotide designated GB53 (Steinberger et al., supra) was generated which was directed against the PTEN mRNA as well. The particularities of this latter experiment were as follows.

As may be taken from Fig. 3C end protected RNAi molecules depicted in Fig.3B are functional in yielding a PTEN protein knockdown.

From this example it can be taken that both end protection groups render RNAi molecules active in knocking down PTEN protein. This inhibition is as efficient as inhibition with antisense constructs but at lower concentrations used which is a clear advantage over the already very powerful antisense technology.

Example 2: Overhang requirements for RNAi duplex activity in vivo

The experimental procedures were the same as depicted in connection with example 1 except that the PTEN mRNA targeting interfering RNAi molecules were differently designed. The results are shown in Fig. 4A as dose response curves with Fig. 4B showing the particular sequence and modifications of the interfering RNAi molecules used to generate the data depicted in Fig. 4A. The nomenclature is such that, e. g., RNAi 18 is composed of strand 18A as antisense strand and strand 18B as sense strand.

Blunt ended molecules are compared to molecules with 3'-overhangs (RNAi 18) and 5'-overhangs (RNAi 30 and RNAi 31) in their activity to knockdown PTEN mRNA in HeLa cells. The activity of blunt ended molecules (RNAi 28) and molecules with 5'-overhangs is comparable to the activity of molecules with 3'-overhangs. This shows that 3'-overhangs are not essential for RNAi activity.

Example 3: Duplex length requirements of interfering RNA molecules for RNAi activity in vivo

The experimental approach was similar to the one outlined in connection with example 1 except that the interfering RNA molecules were directed against the mRNA of Akt1. The negative control to show the specificity of the RNAi molecules was again p110 mRNA. The experimental results are shown in Fig. 5A with the particularities of the interfering RNAi molecules used being represented in Fig. 5B.

Taqman analysis on Akt RNA from HeLa cells transfected with different RNAi molecules shows that the doublestrand duplex of the siRNA molecules has to be longer than 17 base pairs to show activity whereas molecules with 17 base pair long duplexes or shorter are not functional even if sequence-specific overhangs are added. The shortest RNAi molecules successfully tested were 18 to 19 nucleotides or base pairs in length. It is to be noted that the design of the interfering RNA molecule 51A/51B referred to as RNAi 51 corresponds to the one as described in international patent application WO 01/75164. The RNAi molecule 55A/55B comprises a stretch of 17 nucleotides and has a clearly decreased activity in terms of degradation of Akt1 mRNA.

Given the experimental results, the minimum requirement for optimum RNAi mediated interference is thus a duplex length of 18 or 19 nucleotides, independent of the further design of the RNAi molecules such as blunt end or 5'-overhang or any other form as disclosed herein, but generally applicable to RNAi molecules. However, it has to be acknowledged that the particular design of the RNAi molecules may confer further advantages to said molecules, such as, e. g., increased efficiency and increased stability, respectively.

Example 4: Target –antisense homology requirements for RNAi in vivo

The experimental set-up was similar to the one described in example 1, whereby the RNAi is specific for Akt1. In addition, a PTEN specific interfering RNA molecule was designed and used as negative control. The results are shown in Fig. 6A and Fig. 6B.

Having established the minimal duplex length of 18 or more than 18 nucleotides for functional siRNA molecules we have asked the question how many matching nucleotides between target mRNA and siRNA are necessary for silencing activity. As shown by Taqman analysis on Akt1 RNA a stretch of 19 to 15 nucleotides perfectly matching to the target RNA, in the present case Akt 1, is sufficient to mediate RNAi activity. A PTEN specific RNAi molecule does not reduce RNA amounts of Akt1 thus confirming the specificity of this approach. Mismatches of one or two nucleotides at any or both ends of a strand are functional suggesting that a homolog stretch of 15 nt between a target mRNA and RNAi is sufficient for gene silencing. It can be concluded from these data that unspecific gene silencing can occur by chance through unspecific binding to unrelated targets. This is based on the understanding that a stretch of 15 to 17 matching base pairs is not specific for a single gene and will occur by chance considering the complexity and size of the genome or transcriptome of vertebrates.

Based on this observation the present invention is reducing this off-target problem of siRNA by two approaches. First by reducing the molecule length of the siRNA molecules to the minimal requirements (18-19 nt) and thereby reducing the chance of homology to off-targets. Second, by inactivation of the sense strand to prevent a unwanted RNA silencing caused by accidental complementarity of the sense strand to a unrelated target RNA (see also Example 6).

Example 5: Stability of modified RNAi molecules in serum

Oligonucleotides were incubated in human serum for 15 min and two hours and loaded on 10% polyacrylamide gel with untreated controls. The results are shown in Fig. 7A. The various RNAi molecules used are shown and described in more detail in Fig. 7B.

From this example it can be taken that the RNAi duplex of RNA molecules with all nucleotides modified with 2'-O-methyl groups (RNAi molecules 79A79B and 28A28B) have higher stability in serum. It is also shown that a blunt duplex is more stable than the duplex molecule with overhangs. From this the conclusion may be drawn that end protection (e.g. iB or Amino) is not increasing the stability in serum.

In addition, it can also be concluded that in contrast to the understanding in the art before the filing of this application endonucleases rather than exonucleases are more important in the protection of RNAi molecules.

In view of this, in addition to the various modifications or designs of the inventive RNAi molecules as disclosed in this application a further or additional modification of the nucleotides may be the use of a phosphorothioate backbone of the RNAi molecules which may be either complete or partial in order to inhibit endonuclease function. A complete phosphorothioate backbone means that any of the nucleotides exhibits a phosphorothioate group whereas a partial phosphorothioate backbone means that not all of the nucleotides forming the RNAi molecule have a phosphorothioate modification. This modification is suitable to increase the lifetime of RNAi molecules irrespective of the further design of RNAi molecules. In this regard, a partially or completely phosphorothioate modified RNAi is subject to the present invention which may be realized in connection with the different strategies for the design of interfering RNA molecules as disclosed herein or with any of the designs known in the art.

Example 6: Inactivation of the sense strand by NH₂ end protection groups on the 5' and 3' ends

The experimental set-up was similar to the one described in connection with example 1 with the target nucleic acid sequence being PTEN mRNA. The concentration of Hela cells was 2,000 cells per well. RNA of PTEN was analysed in Taqman assays after transfection of differently modified RNAi molecules. The different interfering RNA molecules used are depicted in Fig. 8B whereas the experimental results are shown in Fig. 8A.

As may be taken from the dose response curves of various RNAi molecules depicted in Fig. 8A RNAi molecules are functional when the sense strand, i. e. the second strand, is modified on both ends with amino groups. Particularly effective are RNAi molecules 20A26B, 18A26B, and 28A26B. The lowest activity is shown by RNAi molecule 26A26B which corresponds to end modification on all 4 ends of the duplex (Tuschl is 18AB) .

However, RNAi activity is also achieved when the antisense strand, i. e. the first strand, is modified only at the 3' end leaving a free OH group at the 5' end (RNAi constructs 22A26B; 20A26B). There is no activity when the antisense strand is modified with amino groups on both the 5' and the 3' end (26A26B). This leads to the conclusion that any end of the antisense strand and more particularly the 5' end of the antisense should be kept without modifications. Additionally, it is worth stating that the NH₂ end modification can be used to inactivate the sense strand on the 5' and 3' end and therefore reduce off-target effects mediated by an otherwise functional sense strand which results in a significantly increased specificity of the RNAi molecule which is advantageous for target validation as well as for any medical use of the RNAi molecule.

The further generalisation of the results from this experiment is depicted in Fig. 8C. Functionally active RNAi are accordingly those not having an amino modification at the antisense strand or having an amino modification only at the 3' end of the antisense strand whereas an amino modification at both ends of the antisense strand is not functional, i. e. does not result in a knockdown of the target mRNA.

Example 7: Impact of 2'-O-methyl modification of RNAi molecules for endonuclease protection.

RNA knockdown is again shown using the real time RT-PCR analysis on Hela cells transfected with RNAi duplex molecules directed against the PTEN mRNA as represented in Fig. 9A. Experimental procedures were basically the same as specified in example 1. The structure of the RNAi molecules investigated and their dose responses, which are depicted in Fig. 9A, are shown in Fig. 9C. The nucleotides printed in bold are those having a 2'-O-methyl modification.

It is illustrated by the dose response curves shown for various RNAi molecules in Fig. 9A that internal 2'-O-alkyl groups are reducing RNAi activity. Preferably such 2'-O-alkyl groups are 2'-O-methyl or 2'-O-ethyl groups. However, molecules with unmodified nucleotides in combination with 2'-O-alkyl modification show significant activity. As is also depicted in Fig. 9A there is no activity when the antisense strand is all modified with 2'-O-methyl groups and the sense strand is not modified (c. f., e. g., RNAi molecule 79A28B). Taken the results of a stability test such as incubation of the various RNAi molecules in serum, as depicted in Fig. 9B, shows that 2'-O-alkyl modifications stabilize RNAi molecules against degradation. This clearly beneficial effect, however, is at least to a certain degree counterbalanced by the effect that 2'-O-alkyl modifications generally result in a reduced knockdown activity. Accordingly, the design of RNAi molecules has to balance stability against activity which makes it important to be aware of the various design principles as disclosed in the present application.

Example 8: Impact of blocks of internal 2'-O-methyl modifications on the stability of RNAi molecules in serum.

The experimental approach in connection with this study was actually the same as depicted in example 1. Again, PTEN RNA is analysed by real time RT-PCR on Hela cells at a density of 2000 cells/well which were transfected with different doses of RNAi molecules. RNAi molecules were incubated in serum for two hours and analysed on a 10% polyacrylamide gel. The results of this study are illustrated in Figs. 10A to 10C, whereby Fig. 10A shows the dose response of the various RNAi molecules depicted in Fig. 10C and Fig. 10B shows the result of a stability test using some of the RNAi molecules depicted in Fig. 10C. It is to be acknowledged that the nucleotides written in bold in Fig. 10C are the ones carrying a modification which is in the present case of a 2'-O-methyl modification of the ribose moiety of the nucleotides.

There is a dose dependent inhibition by the unmodified RNAi molecules. It is also shown that the 2'-O-methyl modification of the core 9 nucleotides makes the RNAi stable in serum and allows activity of the duplex in the meaning of mediating the interference phenomenon leading to a degradation of the PTEN mRNA. Total modification of the sense strand makes the RNAi molecule stable in serum and allows certain activity.

Alternating blocks of 5 nucleotides with 2'-O-methyl modification renders the RNAi molecule stable in serum and allows activity on PTEN RNA as shown by incubating the RNAi duplex in serum for two hours and loading the samples on a 10% polyacrylamide gel. As may be taken from Fig. 10B the duplex comprising strands 80A and 80B is strongly degraded after incubation in serum for two hours. The duplex consisting of strands 82A and 82B confirms the result that the 5'-end of the first strand which comprises the antisense strand should not be modified at the 5'-terminal nucleoties (compare 82A82B with the reverse orientated 81A81B). This is also confirmed by the results obtained having the duplex consisting of the strands 86A and 86B which is both active and stabilised in serum. It is noteworthy that molecules with unmodified blocks at the terminal 5' of the antisense strand are more active whereby the 5' terminal OH group is preferably not derivatized.

Further experiments were carried out using different modification patterns of 2'-O-methyl modification of the nucleotides. The results thereof are shown in Fig. 11A to 11C and further discussed herein in example 9.

Example 9: The impact of alternating internal 2'-O-alkyl modification on serum stability of RNAi molecules.

The experimental set-up for performing this kind of study was the same as used in connection with the studies reported in example 1 and example 8, respectively, with the targeted nucleic acid being again PTEN mRNA. Hela cells were transfected with the different RNAi molecules depicted in Fig. 11B and RNA knockdown was demonstrated using real time RT-PCR on PTEN RNA in a dose-dependent manner (Fig. 11A). The stability of the various RNAi molecules after 15 min and two hours in serum at 37°C is depicted in Fig. 11C and a Western Blot for p110 and PTEN as the target-protein of the various RNAi molecules is depicted in Fig. 11D with the RNAi molecules tested being the same in both the experiments underlying Fig. 11C and Fig. 11D.

As illustrated in Fig. 11A and Fig. 11C nucleotides modified with 2'-O-methyl groups alternating with unmodified nucleotides render RNAi molecules stable in serum while still allowing them to be active in the sense of interfering with the target mRNA. It is shown that

incubation of RNAi duplex molecules for 15 min and two hours in serum will degrade the unmodified duplex and the duplex where the 10 most 5'-positioned nucleotides are unmodified.

In the RNAi molecules represented in Fig. 11B various patterns of modified and unmodified nucleotides are realised. The RNAi molecule 94A1/94B1 comprises a structure wherein a modified nucleotide is flanked by an unmodified nucleotide with the unmodified nucleotide being located at the 5' end of the first strand. The RNAi molecule comprised of strands 94A2 and 94B2 is another example where the modified nucleotides and the unmodified nucleotides of the first and the second strand are located at opposing sites. In contrast to this the RNAi molecule comprised of strands 94A1 and 94B2 have the same pattern of modified and unmodified nucleotides. However, there is a phase shift such that the modified nucleotide forms base pairing with an unmodified nucleotide. The two RNAi molecules comprised of strands 94A1 and 94B1 and strands 94A2 and 94B2 differ from each other such that in the first case the first strand starts with an unmodified nucleotide and the corresponding first nucleotide of the second strand, i. e. the nucleotide at the 3' end of the second strand, starts with an unmodified nucleotide with the arrangement being opposite to this in the RNAi molecule comprised of 94A2 and 94B2.

Additionally, alternatingly modified RNAi molecules as depicted in Fig. 11B are functional in mediating a PTEN protein knock down as shown in Fig. 11D but only when the second 5' and second 3' terminal nucleotide is not modified (see 94A294B1 and 94A294B2). Taken together these data show that the most stable and most active RNAi molecules do have alternating 2' Alkyl modified and unmodified nucleotide residues. It should be noted that these molecules do show a very similar mRNA reduction when compared to unmodified siRNA molecules which being stable in serum allows increased or easier handling.

Example 10: Functional Protein Knockdown mediated by internally modified RNAi molecules

The experimental approach was similar to the one outlined in connection with example 1.

Western Blots were performed on HeLa cells harvested at various time points following transfection (48, 72, 96 and 120 hours) with alternatingly modified RNAi molecules as depicted in Fig. 11B. For experimental reasons it is noteworthy that at the 96 hour time point cells have been split and half the population was replated. A total of 40 nM of the various RNAi molecules were applied to the cells. Cells were continuously transfected for 72h with cationic lipids as described in example 1; then replated in the absence of transfection reagents.

Transfections were carried out in 96 well or 10-cm plates (at 30% to 50% confluency) by using various cationic lipids such as Oligofectamine, Lipofectamine (Life Technologies), NC388, L8 (Atugen, Berlin), RNAi were transfected by adding pre-formed 5x concentrated complex of siRNAs and lipid in serum-free medium to cells in complete medium. The total transfection volume was 100 μ l for cells plated in 96-wells and 10 ml for cells in 10 cm plates. The final lipid concentration was 0.8 to 1.2 μ g/ml depending on cell density; the siRNA concentration is indicated in each experiment.

The result of the Western Blot analysis is depicted in Fig. 12. As can be taken from this Figure, modified RNAi molecules of the 94A2B1 and 94A2B2 version yield a longer lasting PTEN protein knock down as unmodified molecules. The lack of protein knock down also seen in Fig.11 with molecules of the 94A1B1 and 94A1B2 version is confirmed in this experiment. Unmodified molecules (80AB) are not as potent in supporting long lasting protein knock down when the cells are not continuously transfected.

Example 11: Persistent PTEN protein knock down with alternating 2'-O-methyl modifications of RNAi molecules

The experimental approach was similar to the one outlined in connection with example 10 with the exception that the transfection was terminated after 5 h by replacing the transfection medium with new medium. The protocol was slightly modified such that for each of the RNAi molecules a 40 nM concentration was realized using a stock solution of 1 μ g RNAi / ml cationic lipid as described in connection with example 1. 5 hours after transfection the medium was withdrawn and fresh EMEM added. The cells were split after 72, with half of the cells being lysed and the other half newly plated and lysed 24 h later (96h post transfection). The result of a Western Blot analysis using 3 different RNAi molecules (80AB, 94A1/B2,

94A2/B1 are depicted in Fig. 13. As a positive control untreated cells were used. Fig. 13 shows the expression of PTEN after 72 h and 96 h, respectively. Taken the structural particularities of the various RNAi molecules it can be taken from Fig. 13 that protein knockdown is persistent with alternating molecules of the 94A2B1 kind even over 96 h after splitting and replating cells compared to unmodified RNAi molecules (such as 80AB) and RNAi molecule 94A1B2.

Example 12: Different loop structures are functional in mediating RNA interference

To test whether RNAi molecules, preferably synthetic RNAi molecules with self complementary structures can inhibit gene expression as efficiently as standard double stranded siRNA molecules, HeLa cells were transfected with p110 β specific synthetic siRNAs. Transfections were carried out in 96 well or 10-cm plates (at 30% to 50% confluency) by using various cationic lipids such as Oligofectamine, Lipofectamine (Life Technologies), GeneBlocs were transfected by adding pre-formed 5x concentrated complex of GB and lipid in serum-free medium to cells in complete medium. The total transfection volume was 100 μ l for cells plated in 96-wells and 10 ml for cells in 10 cm plates. The final lipid concentration was 0.8 to 1.2 μ g/ml depending on cell density; the RNAi molecule concentration is indicated in each experiment.

A dose dependent titration showed no significant difference in efficiency of mRNA knock-down achieved by the standard bimolecular double strand 21mer and the corresponding monomolecular molecules as analysed by realtime PCR (Taqman) (Fig. 14A). Two different loop structures a (A)₁₂ loop and a HIV derived pA-loop were tested in parallel with similar results. A comparison of the relative position of the antisense sequence and the loop structure revealed an improved knock-down efficiency with the antisense sequence being located 3' to the loop (Fig. 14B; compare construct 3A, 3B and 4A, 4B).

Example 13: Studies on the efficacy of different intramolecular hairpin loops and intermolecular “bubbles”

The influence of different loop structures on inhibition of mRNA and protein expression was tested. For these experiments Akt1 and Akt2 were chosen as targets. The experimental approach was similar to the one described in example 12.

Significantly, the reduction in Akt1 mRNA as depicted in Fig. 15A and Fig. 15B as well as Akt1 protein levels as depicted in Fig. 15C was completely independent of the loop structure tested (compare molecules 5A, 6A, 7A, 8A) (the structure of the RNAi molecule tested is always depicted underneath the bar diagram). Even a molecule containing a rather unphysiological structure such as a polyethylenglycol linker (PEG) as a loop efficiently reduced Akt1 expression indicating that size and nucleotide sequence of the loop is not crucial (Fig. 15A; molecule 8A). A synthetic siRNA molecule specific for Akt2 (9A) was used to control for specificity, and had no effect on Akt1 levels as shown in Fig. 15A. This molecule, however, efficiently silenced Akt2 expression (Fig. 15B; Fig. 15C). Self-complementary RNA molecules with loop structures have the possibility to anneal as doublestrands in monomolecular or bimolecular structures under physiological hybridization conditions (Fig. 15B, loop or bubble structure). To address the question of whether the siRNA molecules exert their function via adapting an intra-molecular loop or an inter-molecular “bubble” (schematically shown in Fig. 15B) two molecules not capable of folding back on themselves were transfected. These constructs contained Akt1- and Akt2-specific sequences within the same molecule (Fig. 15B, constructs 10A, 10B) and were designed to be restricted to form a bimolecular duplex (“bubble”). Surprisingly, this molecule efficiently mediated both Akt1 and Akt2 mRNA knock-down as well as protein knock-down when transfected after annealing of both strands.

Whether loop and bubble structures are indeed substrates for RNA processing enzymes, e.g. *Dicer*, is not clear at this point. A recent study by Paddison and coworkers suggests that hairpin containing siRNAs are more dependent on *Dicer* activity than double stranded siRNAs. However, these data demonstrating RNA interference activity using a PEG linker molecule indicate that the linker sequence is likely to be irrelevant.

The features of the present invention disclosed in the specification, the claims and/or the drawings may both separately and in any combination thereof be material for realizing the invention in various forms thereof.

Claims

1. A ribonucleic acid comprising a double stranded structure whereby the double-stranded structure comprises a first strand and a second strand, whereby the first strand comprises a first stretch of contiguous nucleotides and whereby said first stretch is at least partially complementary to a target nucleic acid, and the second strand comprises a second stretch of contiguous nucleotides whereby said second stretch is at least partially identical to a target nucleic acid, characterised in that the double stranded structure is blunt ended.
2. A ribonucleic acid comprising a double stranded structure whereby the double-stranded structure comprises a first strand and a second strand, whereby the first strand comprises a first stretch of contiguous nucleotides and whereby said first stretch is at least partially complementary to a target nucleic acid, and the second strand comprises a second stretch of contiguous nucleotides whereby said second stretch is at least partially identical to a target nucleic acid, characterised in that the first stretch and/or the second stretch have a length of 18 or 19 nucleotides.
3. The ribonucleic acid according to claim 1, wherein the first stretch and/or the second stretch have a length of 18 or 19 nucleotides.
4. The nucleic acid according to claim 1 or 3, wherein the double stranded structure is blunt ended on both sides of the double strand.
5. The nucleic acid according to claim 1 or 3, wherein the double stranded structure is blunt ended on the double stranded structure which is defined by the 5'-end of the first strand and the 3'-end of the second strand.

6. The nucleic acid according to claim 1 or 3, wherein the double stranded structure is blunt ended on the double stranded structure which is defined by the 3'-end of the first strand and the 5'-end of the second strand.
7. A ribonucleic acid comprising a double stranded structure whereby the double-stranded structure comprises a first strand and a second strand, whereby the first strand comprises a first stretch of contiguous nucleotides and whereby said first stretch is at least partially complementary to a target nucleic acid, and the second strand comprises a second stretch of contiguous nucleotides and whereby said second stretch is at least partially identical to a target nucleic acid, characterised in that at least one of the two strands has an overhang of at least one nucleotide at the 5'-end.
8. The ribonucleic acid according to claim 7, wherein the overhang consists of at least one nucleotide which is selected from the group comprising ribonucleotides and desoxyribonucleotides.
9. The ribonucleic acid according to claim 8, wherein the nucleotide has a modification whereby said modification is preferably selected from the group comprising nucleotides being an inverted abasic and nucleotides having an NH_2 -modification at the 2'-position.
10. The ribonucleic acid according to any of claims 7 to 9, wherein at least one of the strands has an overhang of at least one nucleotide at the 3'-end consisting of ribonucleotide or deoxyribonucleotide.
11. The ribonucleic acid according to any of claims 7 to 10, wherein the first stretch and/or the second stretch have a length of 18 or 19 nucleotides.
12. The ribonucleic acid according to any of claims 1 to 11, wherein the double-stranded structure has a length of 17 to 21 nucleotides, preferably 18 to 19 nucleotides
13. The ribonucleic acid according to any of claims 7 to 12, wherein the overhang at the 5'-end is on the second strand.

14. The ribonucleic acid according to claim 13, characterised in that the first strand comprises also an overhang, preferably at the 5'-end.
15. The ribonucleic acid according to any of claims 7 to 14, wherein the 3'-end of the first strand comprises an overhang.
16. The ribonucleic acid according to any of claims 7 to 12, wherein the overhang at the 5'-end is on the first strand.
17. The ribonucleic acid according to claim 16, characterised in that the second strand also comprise an overhang, preferably at the 5'-end.
18. The ribonucleic acid according to any of claims 7 to 12 and 16 to 17, wherein the 3'-end of the first strand comprises an overhang.
19. The ribonucleic acid according to any of claims 1 to 18, wherein at least one nucleotide of the ribonucleic acid has a modification at the 2'-position and the modification is preferably selected from the group comprising amino, fluoro, methoxy and alkyl.
20. A ribonucleic acid comprising a double stranded structure, whereby the double-stranded structure comprises a first strand and a second strand, whereby the first strand comprises a first stretch of contiguous nucleotides and whereby said first stretch is at least partially complementary to a target nucleic acid, and the second strand comprises a second stretch of contiguous nucleotides and whereby said second stretch is at least partially identical to a target nucleic acid,

characterised in

that said first strand and/or said second strand comprises a plurality of groups of modified nucleotides having a modification at the 2'-position whereby within the strand each group of modified nucleotides is flanked on one or both sides by a flanking group of nucleotides whereby the flanking nucleotides forming the flanking group of

nucleotides is either an unmodified nucleotide or a nucleotide having a modification different from the modification of the modified nucleotides.

21. The ribonucleic acid according to claim 20, wherein the ribonucleic acid is the ribonucleic acid according to any of claims 1 to 19.
22. The ribonucleic acid according to claims 20 or 21, wherein said first strand and/or said second strand comprise said plurality of modified nucleotides.
23. The ribonucleic acid according to claims 20 or 21, wherein said first strand comprises said plurality of groups of modified nucleotides.
24. The ribonucleic acid according to claims 20 or 21, wherein said second strand comprises said plurality of groups of modified nucleotides.
25. The ribonucleic acid according to claims 20 or 24, wherein the group of nucleotides and/or the group of flanking nucleotides comprises a number of nucleotides whereby the number is selected from the group comprising one nucleotide to 10 nucleotides.
26. The ribonucleic acid according to any of claims 20 to 25, wherein the pattern of modified nucleotides of said first strand is the same as the pattern of modified nucleotides of said second strand.
27. The ribonucleic acid according to any of claims 20 to 26, wherein the pattern of said first strand aligns with the pattern of said second strand.
28. The ribonucleic acid according to any of claims 20 to 26, wherein the pattern of said first strand is shifted by one or more nucleotides relative to the pattern of the second strand.
29. The ribonucleic acid according to any of claims 20 to 28, wherein the modification is selected from the group comprising amino, fluoro, methoxy and alkyl.

30. The ribonucleic acid according to any of claims claim 20 to 29, wherein the double stranded structure is blunt ended.
31. The ribonucleic acid according to any of claims 20 to 30, wherein the double stranded structure is blunt ended on both sides.
32. The ribonucleic acid according to any of claims 20 to 31, wherein the double stranded structure is blunt ended on the double stranded structure which is defined by the 5'-end of the first strand and the 3'-end of the second strand.
33. The nucleic acid according to any of claims 20 to 32, wherein the double stranded structure is blunt ended on the double stranded structure which is defined by at the 3'-end of the first strand and the 5'-end of the second strand.
34. The ribonucleic acid according to claim 20 or 33, wherein at least one of the two strands has an overhang of at least one nucleotide at the 5'-end.
35. The nucleic acid according to claim 34, wherein the overhang consists of at least one desoxyribonucleotide.
36. The ribonucleic acid according to claim 34 or 35, wherein at least one of the strands has an overhang of at least one nucleotide at the 3'-end.
37. The ribonucleic acid according to any of claims 1 to 36, wherein the length of the double-stranded structure has a length from about 17 to 21 and more preferably 18 or 19 bases
38. The ribonucleic acid according to any of claims 1 to 37, wherein the length of said first strand and/or the length of said second strand is independently from each other selected from the group comprising the ranges of from about 15 to about 23 bases, 17 to 21 bases and 18 or 19 bases.
39. The ribonucleic acid according to any of claims 1 to 38, wherein the complementarity between said first strand and the target nucleic acid is perfect.

40. The ribonucleic acid according to any of claims 1 to 39, wherein the duplex formed between the first strand and the target nucleic acid comprises at least 15 nucleotides wherein there is one mismatch or two mismatches between said first strand and the target nucleic acid forming said double-stranded structure.
41. The ribonucleic acid according to any of claims 1 to 40, wherein the target gene is selected from the group comprising structural genes, housekeeping genes, transcription factors, motility factors, cell cycle factors, cell cycle inhibitors, enzymes, growth factors, cytokines and tumor suppressors.
42. The ribonucleic acid according to any of claims 1 to 41, whereby the first strand and the second strand are linked by a loop structure.
43. The ribonucleic acid according to claim 42, wherein the loop structure is comprised of a non-nucleic acid polymer.
44. The ribonucleic acid according to claim 43, characterised in that the non-nucleic acid polymer is polyethylene glycol.
45. The ribonucleic acid according to any of claims 42 to 44, wherein the loop structure is comprised of a nucleic acid.
46. The ribonucleic acid according to any of claims 42 to 45, characterised in that the 5'-terminus of the first strand is linked to the 3'-terminus of the second strand.
47. The ribonucleic acid according to any of claims 42 to 46, characterised in that the 3'-end of the first strand is linked to the 5'-terminus of the second strand.
48. Use of a ribonucleic acid according to any of claims 1 to 47, for target validation.
49. Use of a ribonucleic acid according to any of claims 1 to 47 for the manufacture of a medicament.

50. The use according to claim 49, wherein the medicament is for the treatment of a disease or of a condition which is selected from the group comprising glioblastoma, prostate cancer, breast cancer, lung cancer, liver cancer, colon cancer, pancreatic cancer and leukaemia, diabetes, obesity, cardiovascular diseases, and metabolic diseases.
51. A cell, preferably a knockdown cell, containing a ribonucleic acid according to any of claims 1 to 47.
52. An organism, preferably a knockdown organism, containing a ribonucleic acid according to any of claims 1 to 47.
53. A composition containing a ribonucleic acid according to any of claims 1 to 47.
54. A pharmaceutical composition containing a ribonucleic acid according to any of claims 1 to 47 and a pharmaceutically acceptable carrier.
55. A method for inhibiting the expression of a target gene in a cell or derivative thereof comprising introduction of a ribonucleic acid according to any of claims 1 to 47 into the cell in an amount sufficient to inhibit expression of the target gene, wherein the target gene is the target gene of the a ribonucleic acid according to any of claims 1 to 47.

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05. Aug. 2002

Abstract

The present invention is related to a ribonucleic acid comprising a double stranded structure whereby the double- stranded structure comprises a first strand and a second strand, whereby the first strand comprises a first stretch of contiguous nucleotides and whereby said first stretch is at least partially complementary to a target nucleic acid, and the second strand comprises a second stretch of contiguous nucleotides whereby said second stretch is at least partially identical to a target nucleic acid, and whereby the double stranded structure is blunt ended.

1/19

Fig. 1:

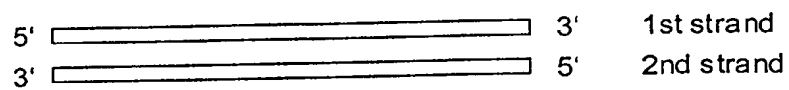


Fig. 2:

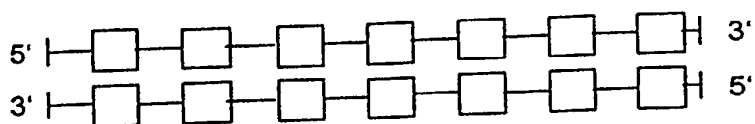


Fig. 2B

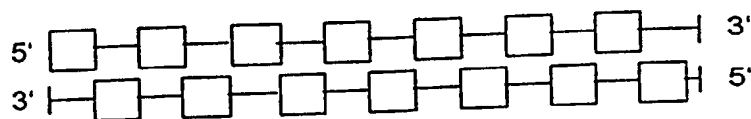
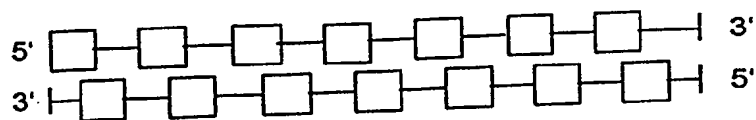


Fig. 2C

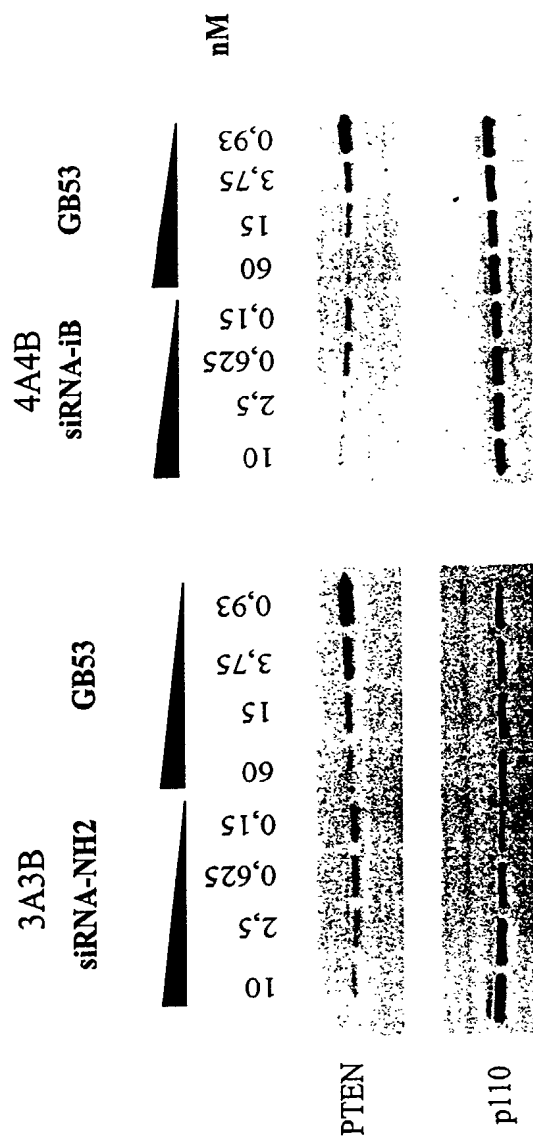


3A



ONLY RNA WITH 2 DEOXY AT EACH END

5MM	5' -	cucuuuuuuugugcucacg-tt
5BMM	3' -tt-	gaguaaaaaagaacacgagugc



3C

4B

18A	5' -	cuccuuuuuuuucugcuaacg -TT
18B	3' -TT	-gaggaaaaacaaagacgaugc
19A (MM)	5' -	cucauuuuuuuugucucacg -TT
19B (MM)	3' -TT	-gaguaaaaagaaacacgagugc
28A	5' -	cuccuuuuuuuucugcuaacg -
28B	3' -	-gaggaaaaacaaagacgaugc -
29A (MM)	5' -	cucauuuuuuuugucucacg -
29B (MM)	3' -	-gaguaaaaagaaacacgagugc
30A	5' -TT	-cuccuuuuuuuucugcuaacg -
30B	3' -	-gaggaaaaacaaagacgaugc -TT
31A (MM)	5' -TT	-cucauuuuuuuugucucacg -
31B (MM)	3' -	-gaguaaaaagaaacacgagugc -TT

4A

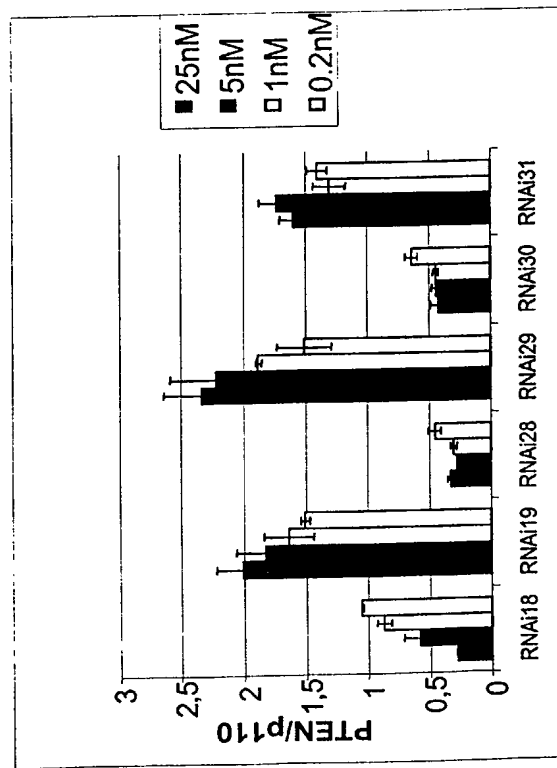
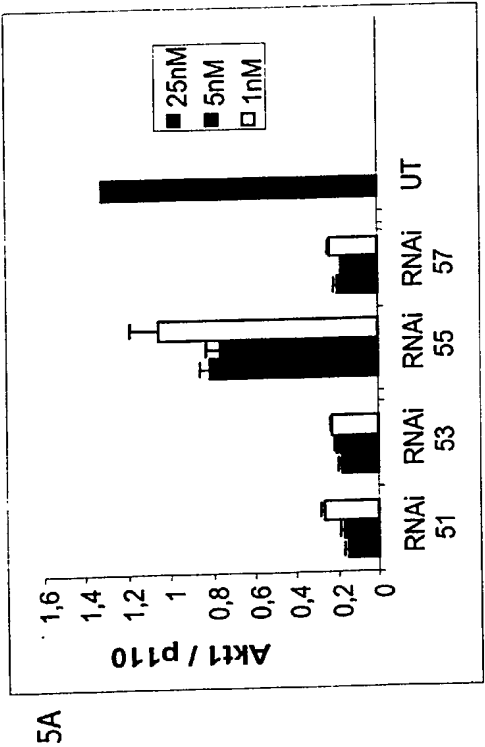


Fig. 4

Fig. 5



5B

51A	5' -	-ucuugauguacuucccucg-uu	19nt 3'uu
51B	3' -	uu-agaacuacaugaggggagc-	
53A	5' -	-ucuugauguacuucccucg-tt	19nt 3'seq.specific RNA
53B	3' -	cc-agaacuacaugaggggagc-	
55A	5' -	-cuugauguacuucccucg-gt	17nt 3'seq.specific RNA
55B	3' -	ca-gaacuacaugaggggag-	
57A	5' -	-ucuugauguacuucccucg-tt	19nt 3'seq.specific DNA
57B	3' -	CC-agaacuacaugaggggagc-	

Fig. 6

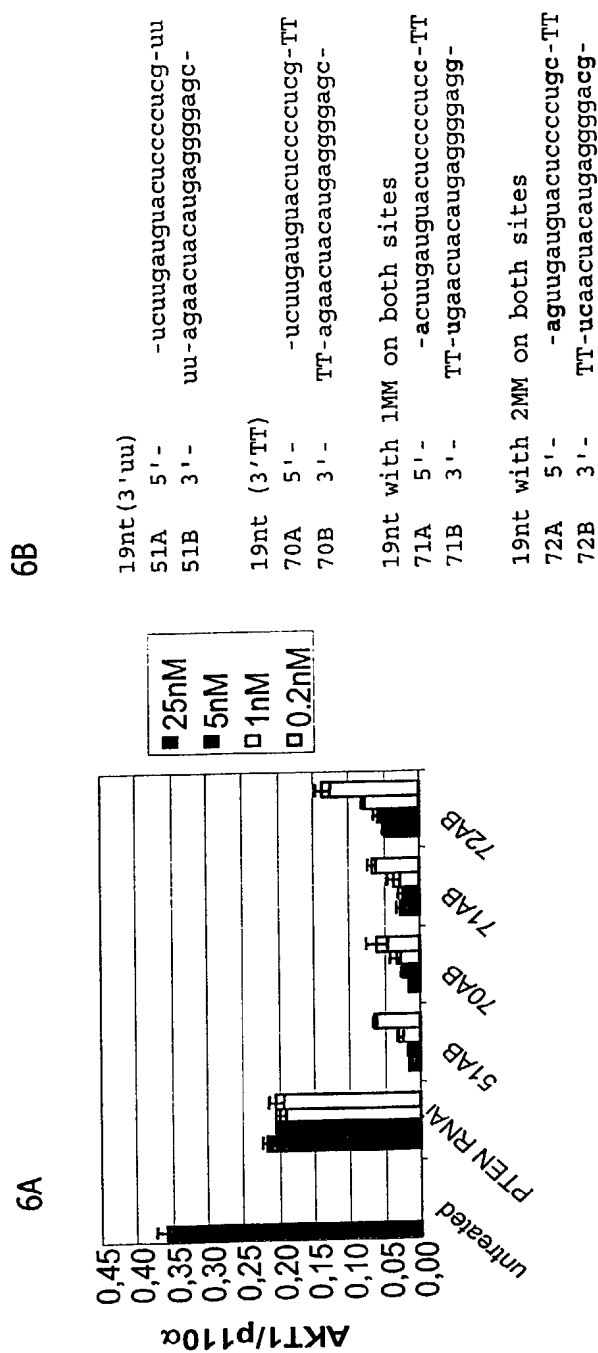


Fig. 7

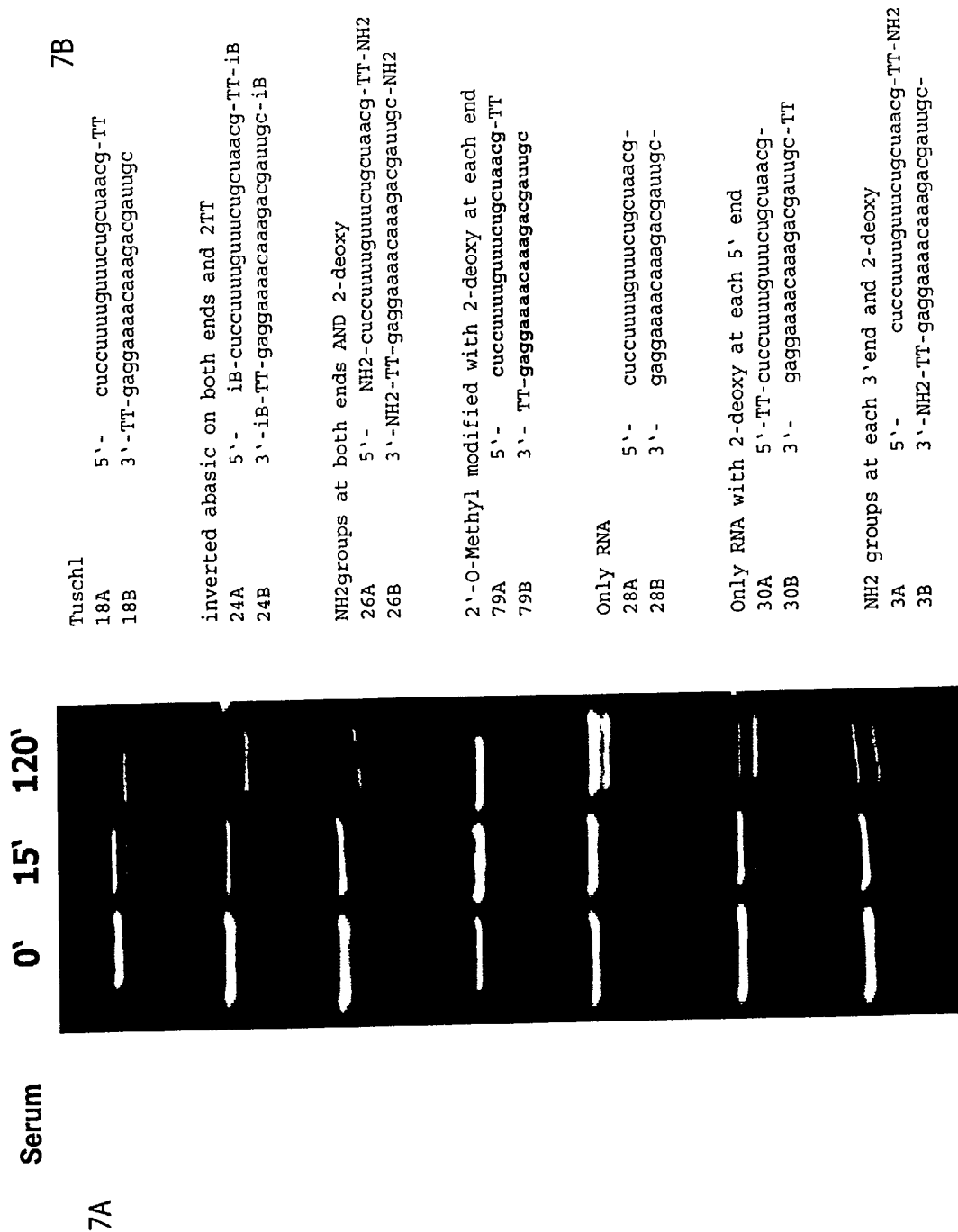
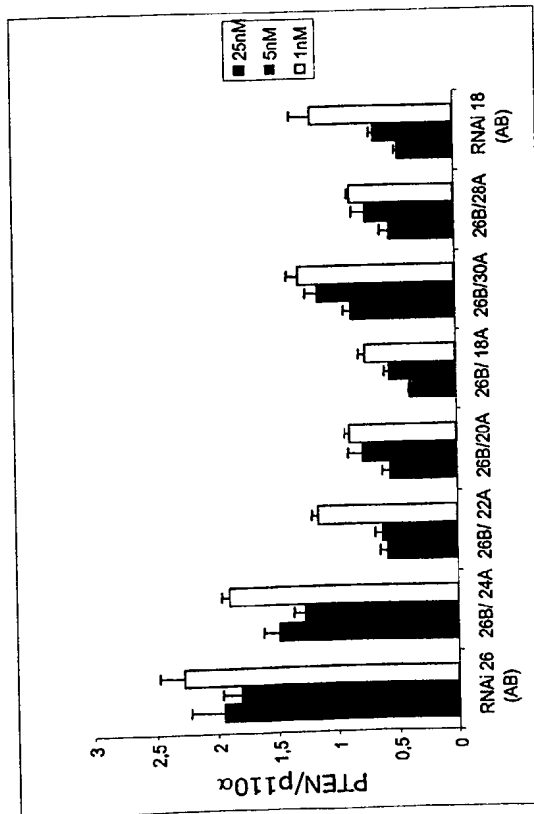


Fig. 8

8A



8B

26A	5' - NH2-cuccuuuuuuuucugcuaacg-TT-NH2
26B	3' -NH2-TT-gaggaaaaaagacgaugc-NH2
24A	5' - iB-cuccuuuuuuuucugcuaacg-TT-iB
26B	3' -NH2-TT-gaggaaaaaagacgaugc-NH2
22A	5' - cuccuuuuuuuucugcuaacg-TT-iB
26B	3' -NH2-TT-gaggaaaaaagacgaugc-NH2
20A	5' - cuccuuuuuuuucugcuaacg-TT-NH2
26B	3' -NH2-TT-gaggaaaaaagacgaugc-NH2
18A	5' - cuccuuuuuuuucugcuaacg-TT
26B	3' -NH2-TT-gaggaaaaaagacgaugc-NH2
30A	5' - TT-cuccuuuuuuuucugcuaacg-
26B	3' -NH2-TT-gaggaaaaaagacgaugc-NH2
28A	5' - cuccuuuuuuuucugcuaacg-
26B	3' -NH2-TT-gaggaaaaaagacgaugc-NH2
18A	5' - cuccuuuuuuuucugcuaacg-TT
18B	3' - TT-gaggaaaaaagacgaugc

8C

-nnnnnnnnnnnnnnnnnn-tt	functional
NH2-nnnnnnnnnnnnnnnnn-NH2	functional
-nnnnnnnnnnnnnnnnnn-tt-NH2	functional
NH2-nnnnnnnnnnnnnnnnn-NH2	functional
NH2-nnnnnnnnnnnnnnnnn-tt-NH2	not functional
tt-nnnnnnnnnnnnnnnnn-tt	not functional

Fig. 9

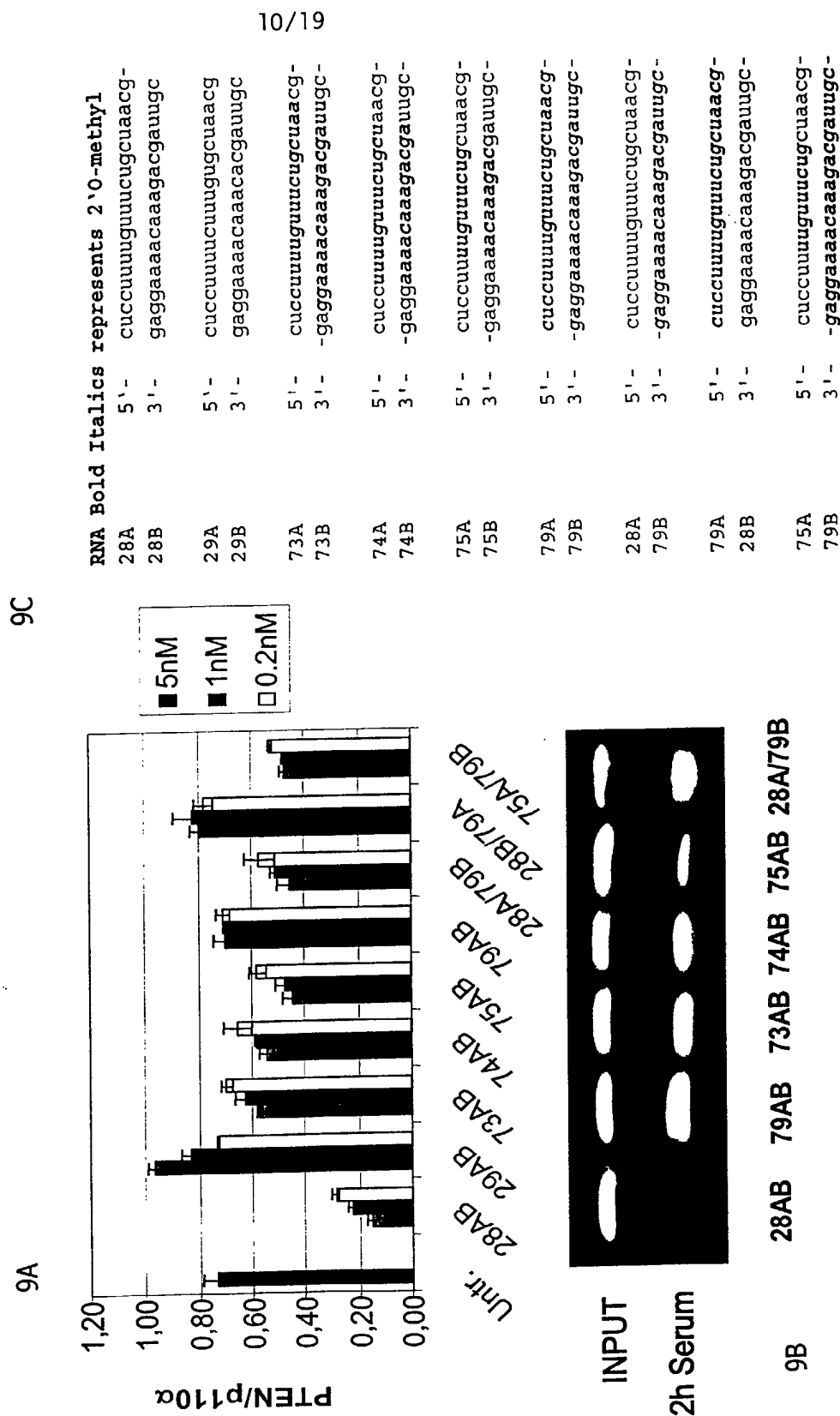


Fig. 10

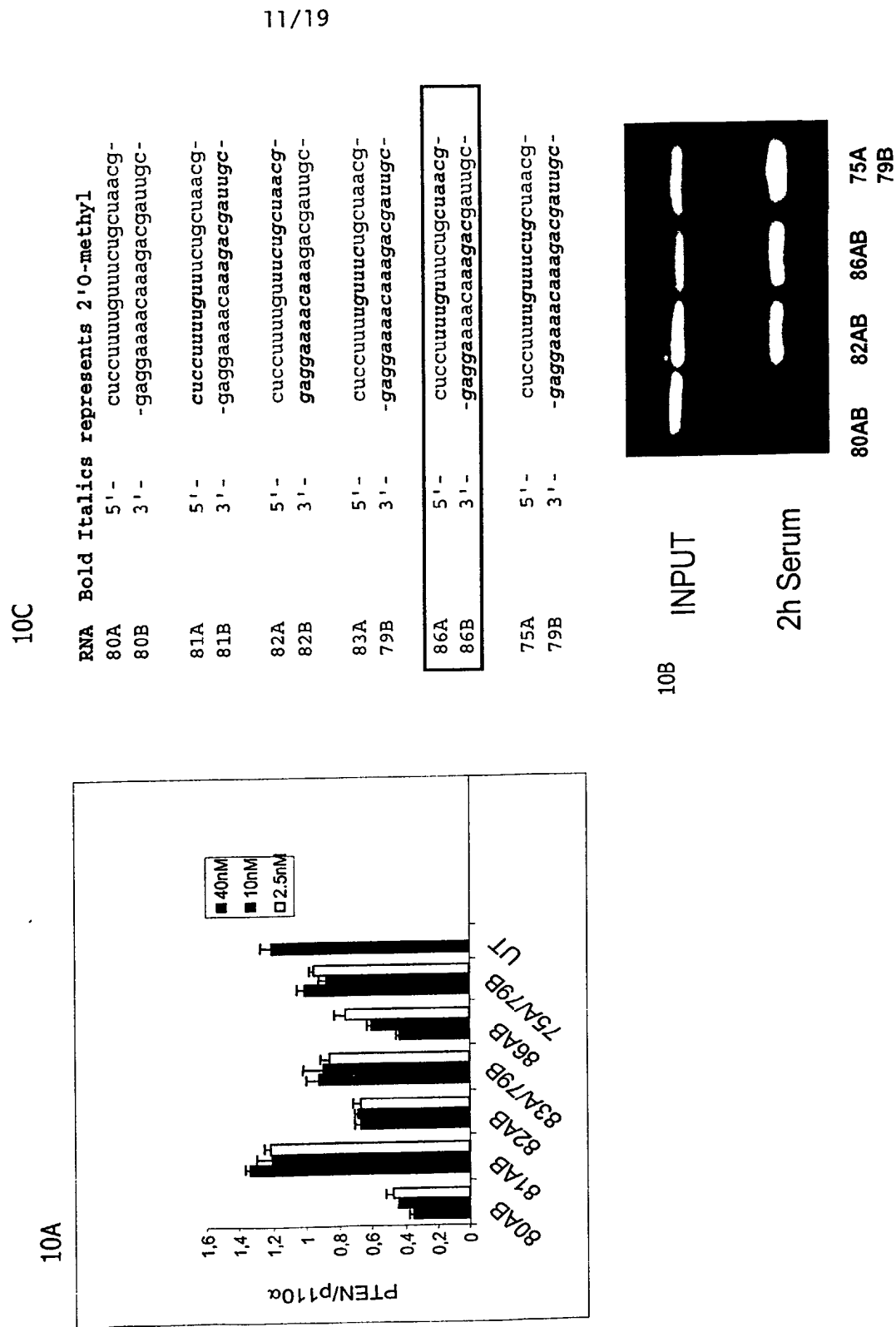


Fig. 11

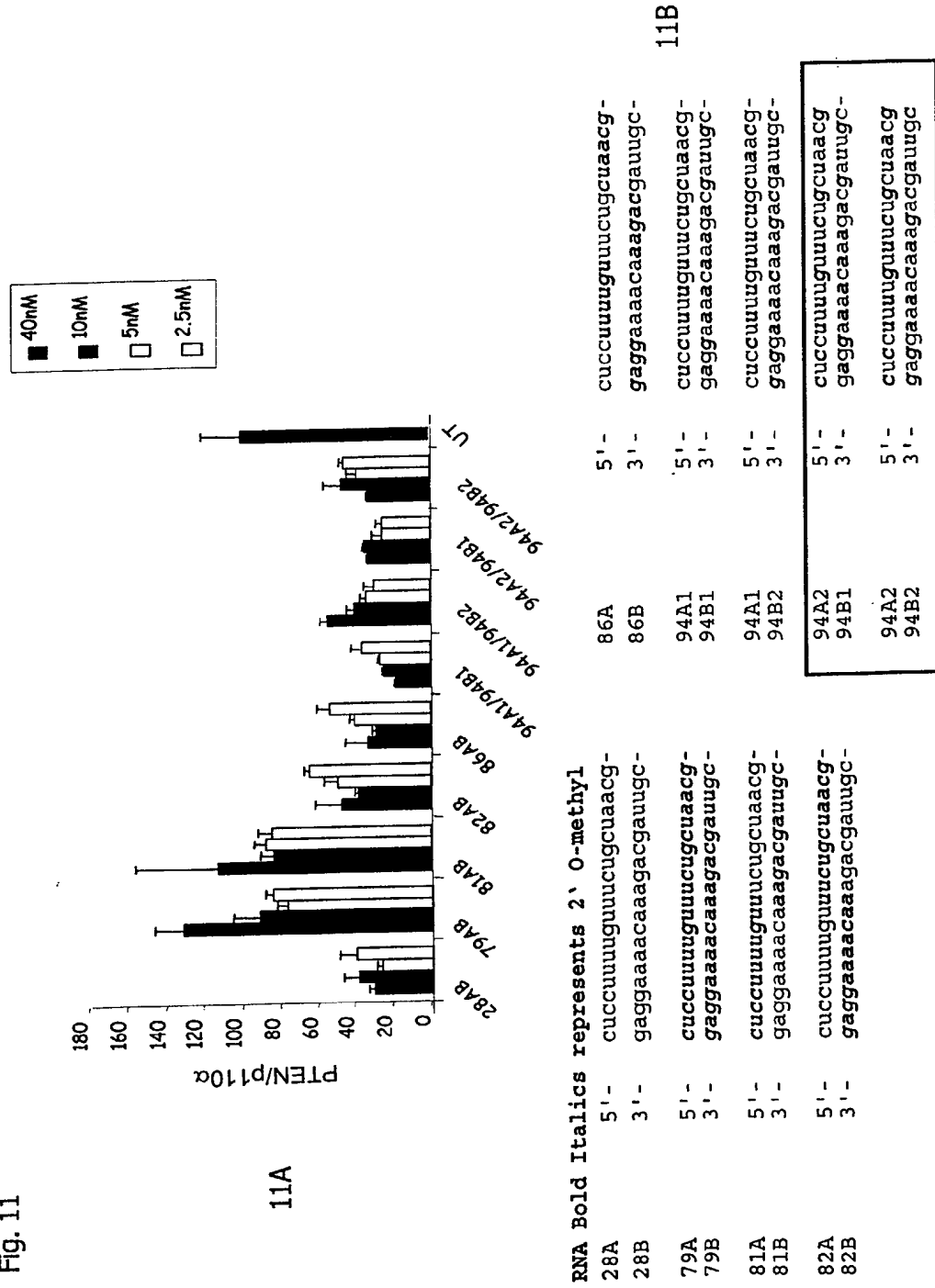
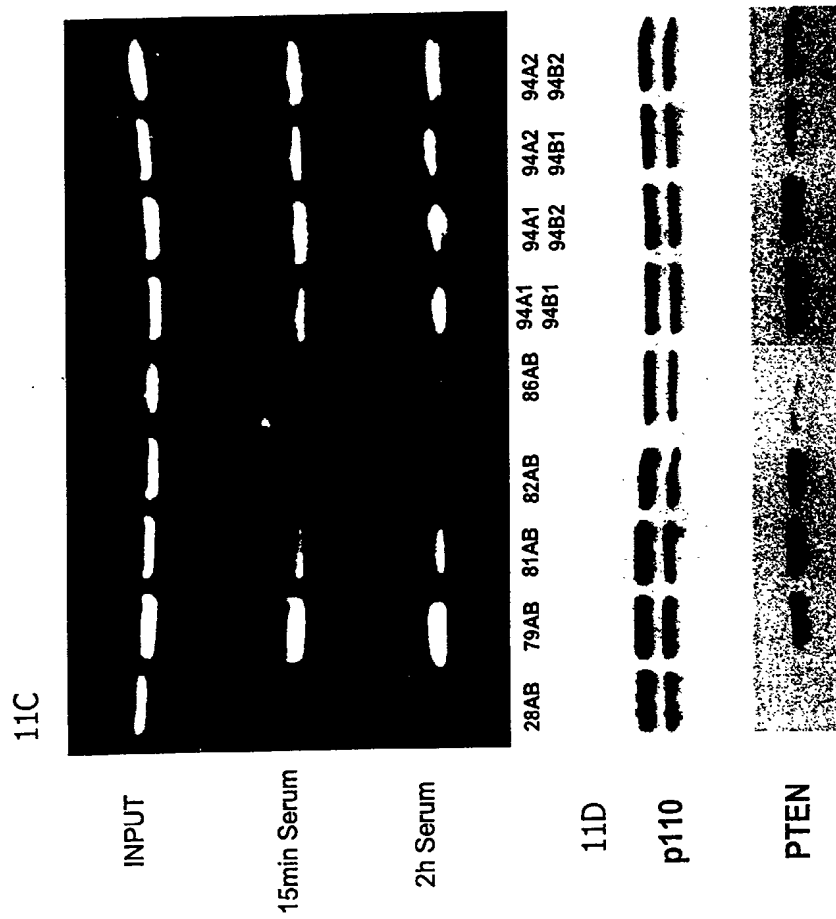
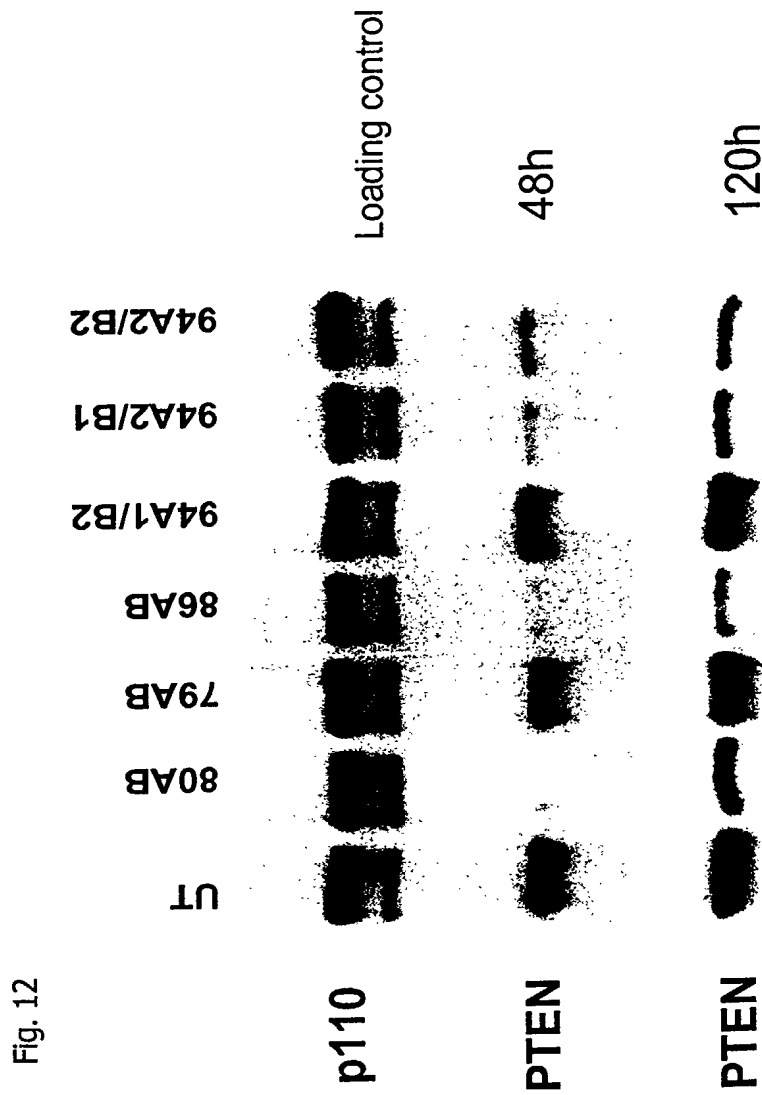


Fig. 11





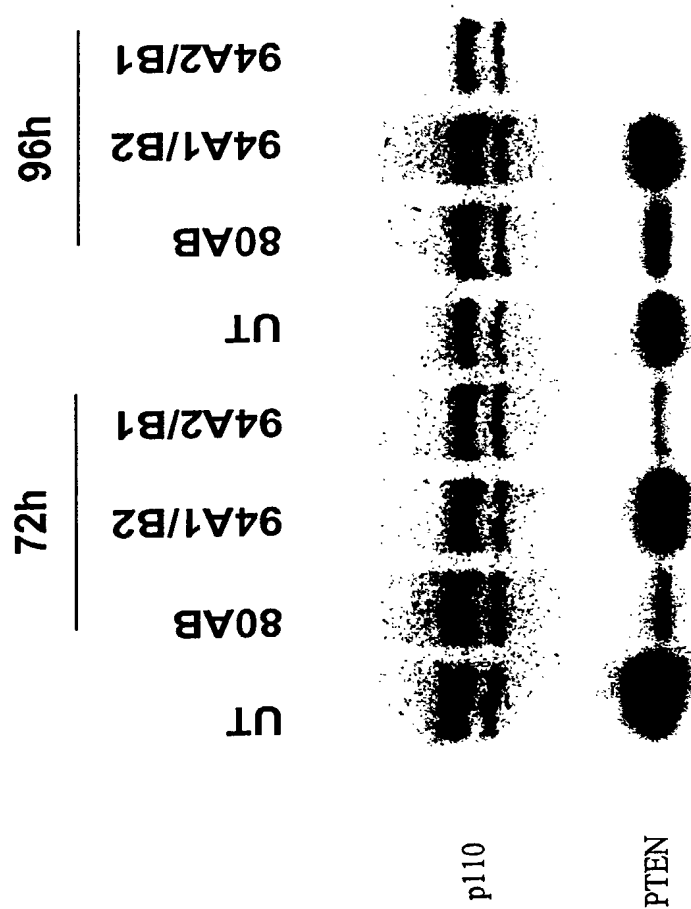


Fig. 13

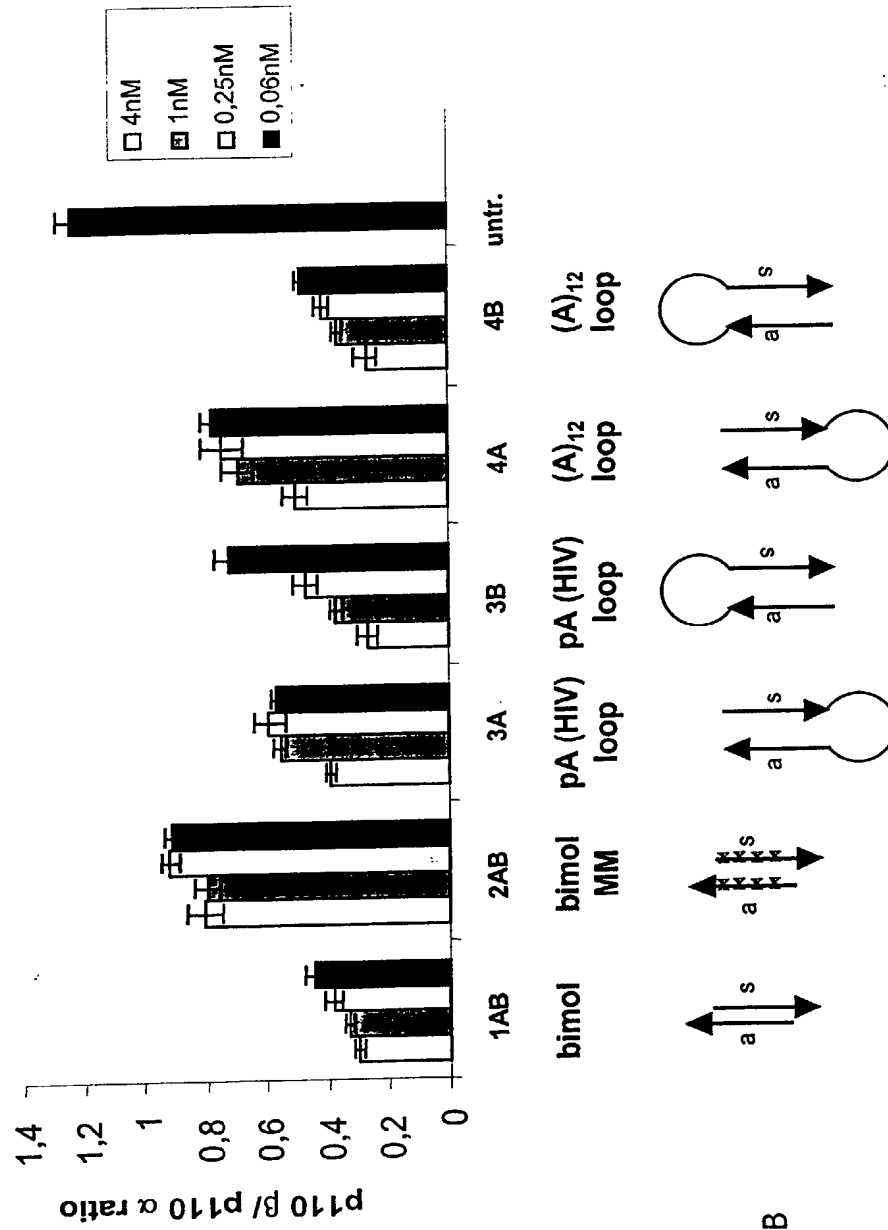
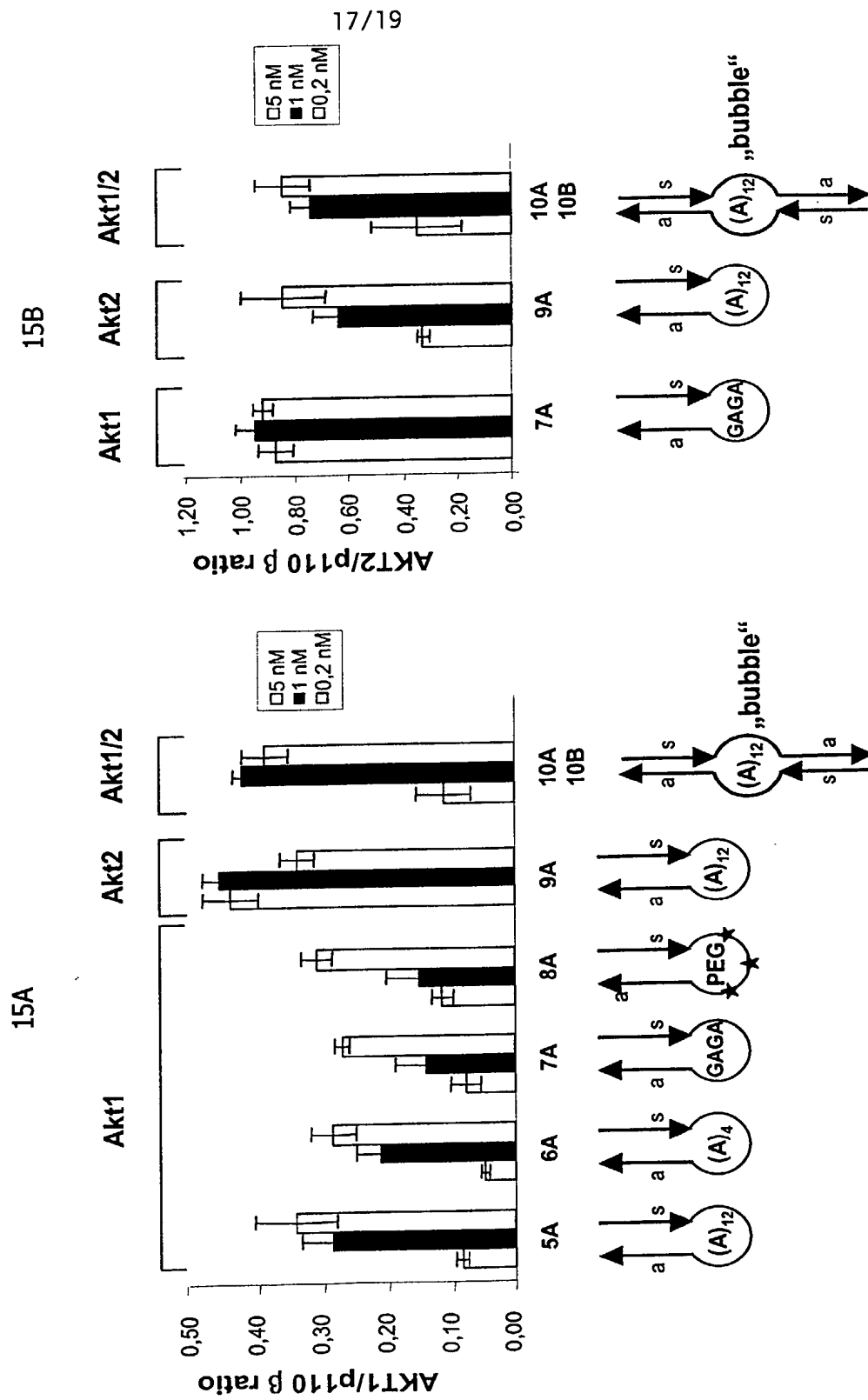


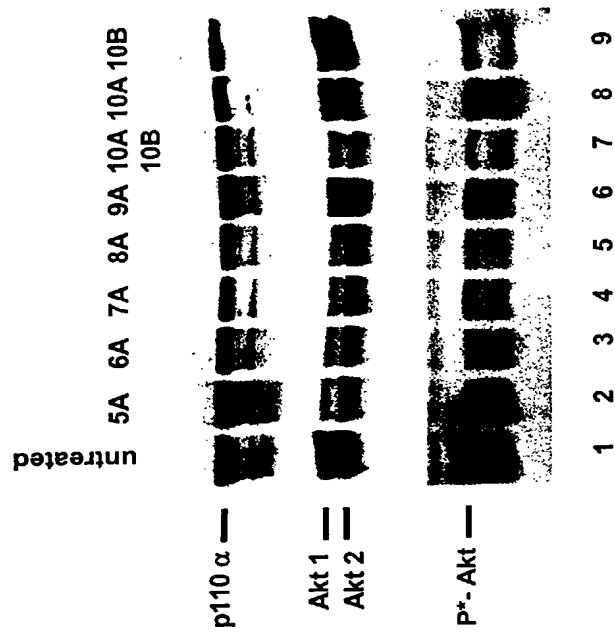
Fig.14

14A

14B

Fig. 15





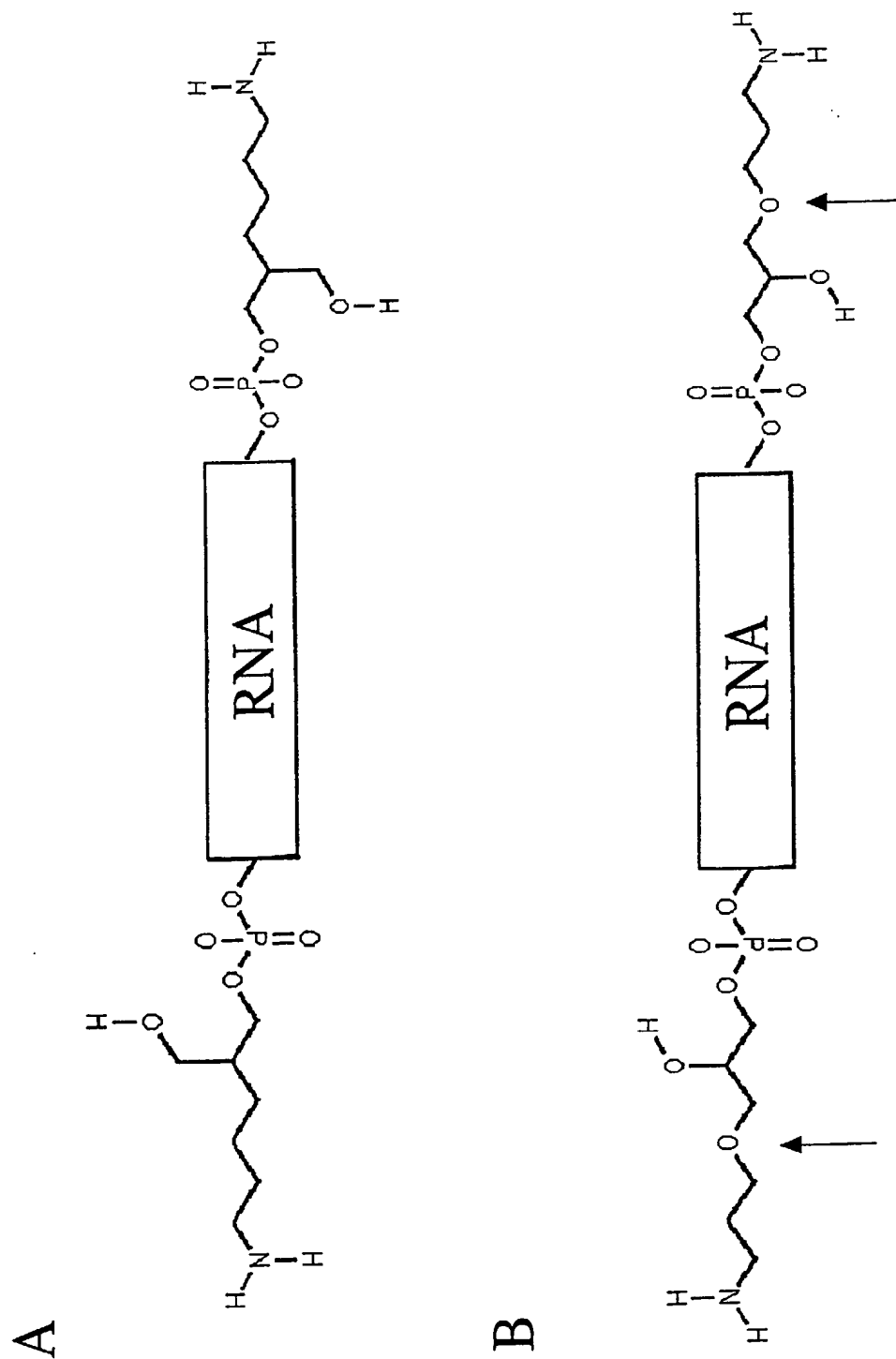


Fig. 16



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Die angehefteten Unterlagen stimmen mit der ursprünglich eingereichten Fassung der auf dem nächsten Blatt bezeichneten europäischen Patentanmeldung überein.

The attached documents are exact copies of the European patent application described on the following page, as originally filed.

Les documents fixés à cette attestation sont conformes à la version initialement déposée de la demande de brevet européen spécifiée à la page suivante.

Patentanmeldung Nr. Patent application No. Demande de brevet n°

03008383.6

Der Präsident des Europäischen Patentamts;
Im Auftrag

For the President of the European Patent Office

Le Président de l'Office européen des brevets
p.o.

R C van Dijk



Anmeldung Nr:
Application no.: 03008383.6
Demande no:

Anmeldetag:
Date of filing: 10.04.03
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Anmelder/Applicant(s)/Demandeur(s):

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ALLEMAGNE

Bezeichnung der Erfindung/Title of the invention/Titre de l'invention:
(Falls die Bezeichnung der Erfindung nicht angegeben ist, siehe Beschreibung.
If no title is shown please refer to the description.
Si aucun titre n'est indiqué se referer à la description.)

Further novel forms of interfering RNA molecules

In Anspruch genommene Priorität(en) / Priority(ies) claimed /Priorité(s)
revendiquée(s)
Staat/Tag/Aktenzeichen/State/Date/File no./Pays/Date/Numéro de dépôt:

Internationale Patentklassifikation/International Patent Classification/
Classification internationale des brevets:

C12N15/00

Am Anmeldetag benannte Vertragstaaten/Contracting states designated at date of
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AT BE BG CH CY CZ DE DK EE ES FI FR GB GR HU IE IT LU MC NL
PT SE SI SK TR LI

Further novel forms of interfering RNA molecules

The present invention is related to a ribonucleic acid comprising a double-stranded structure whereby the double-stranded structure comprises a first strand and a second strand, whereby the first strand comprises a first stretch of contiguous nucleotides and whereby said first stretch is at least partially complementary to the target nucleic acid, and the second strand comprises a second stretch of contiguous nucleotides whereby said second stretch is at least partially identical to a target nucleic acid, the use of such ribonucleic acid, a cell and an organism, respectively, comprising such ribonucleic acid, a composition containing such ribonucleic acid, a pharmaceutical composition containing such ribonucleic acid and a method for inhibiting expression of a targeted gene.

RNA-mediated interference (RNAi) is a post-transcriptional gene silencing mechanism initiated by double stranded RNA (dsRNA) homologous in sequence to the silenced gene (Fire (1999), Trends Genet 15, 358-63, Tuschl, *et al.* (1999), Genes Dev 13, 3191-7, , Waterhouse, *et al.* (2001), Nature 411, 834-42, Elbashir, *et al.* (2001), Nature 411, 494-8, for review see Sharp (2001), Genes Dev 15, 485-90, Barstead (2001), Curr Opin Chem Biol 5, 63-6). RNAi has been used extensively to determine gene function in a number of organisms, including plants (Baulcombe (1999), Curr Opin Plant Biol 2, 109-13), nematodes (Montgomery, *et al.* (1998), Proc Natl Acad Sci U S A 95, 15502-7), *Drosophila* (Kennerdell, *et al.* (1998), Cell 95, 1017-26, Kennerdell, *et al.* (2000), Nat Biotechnol 18, 896-8). In the nematode *C. elegans* about one third of the genome has already been subjected to functional analysis by RNAi (Kim (2001), Curr Biol 11, R85-7, Maeda, *et al.* (2001), Curr Biol 11, 171-6).

Until recently RNAi in mammalian cells was not generally applicable, with the exception of early mouse development (Wianny, *et al.* (2000), Nat Cell Biol 2, 70-5). The discovery that transfection of duplexes of 21-nt into mammalian cells interfered with gene expression and did not induce a sequence independent interferon-driven anti-viral response usually obtained with long dsRNA led to new potential application in differentiated mammalian cells (Elbashir

et al. (2001), Nature 411, 494-8). Interestingly these small interfering RNAs (siRNAs) resemble the processing products from long dsRNAs suggesting a potential bypassing mechanism in differentiated mammalian cells. The Dicer complex, a member of the RNase III family, necessary for the initial dsRNA processing has been identified (Bernstein, *et al.* (2001), Nature 409, 363-6, Billy, *et al.* (2001), Proc Natl Acad Sci U S A 98, 14428-33). One of the problems previously encountered when using unmodified ribooligonucleotides was the rapid degradation in cells or even in the serum-containing medium (Wickstrom (1986), J Biochem Biophys Methods 13, 97-102, Cazenave, *et al.* (1987), Nucleic Acids Res 15, 10507-21). It will depend on the particular gene function and assay systems used whether the respective knock down induced by transfected siRNA will be maintained long enough to achieve a phenotypic change.

The problem underlying the present invention was to provide synthetic interfering RNA molecules which are both stable and active in a biochemical environment such as a living cell.

In a first aspect of the present invention the problem is solved by a ribonucleic acid comprising a double stranded structure whereby the double- stranded structure comprises a first strand and a second strand, whereby the first strand comprises a first stretch of contiguous nucleotides and whereby said first stretch is at least partially complementary to a target nucleic acid, and the second strand comprises a second stretch of contiguous nucleotides whereby said second stretch is at least partially identical to a target nucleic acid, and whereby the double stranded structure is blunt ended.

In a second aspect the problem underlying the present invention is solved by a ribonucleic acid comprising a double stranded structure whereby the double- stranded structure comprises a first strand and a second strand, whereby the first strand comprises a first stretch of contiguous nucleotides and whereby said first stretch is at least partially complementary to a target nucleic acid, and the second strand comprises a second stretch of contiguous nucleotides, whereby said second stretch is at least partially identical to a target nucleic acid, whereby the first stretch and/or the second stretch have a length of 18 or 19 nucleotides.

In an embodiment of the ribonucleic acid according to the first aspect of the invention the first stretch and/or the second stretch have a length of 18 or 19 nucleotides.

In a further embodiment of the ribonucleic acid according to the first aspect of the invention the double stranded structure is blunt ended on both sides of the double strand.

In an alternative embodiment of the ribonucleic acid according to the first aspect of the invention the double stranded structure is blunt ended on the double stranded structure which is defined by the 5'-end of the first strand and the 3'-end of the second strand.

In a further alternative embodiment of the ribonucleic acid according to the first and the second aspect of the invention the double stranded structure is blunt ended on the double stranded structure which is defined by the 3'-end of the first strand and the 5'-end of the second strand.

In a third aspect the problem underlying the present invention is solved by a ribonucleic acid comprising a double stranded structure whereby the double-stranded structure comprises a first strand and a second strand, whereby the first strand comprises a first stretch of contiguous nucleotides and whereby said first stretch is at least partially complementary to a target nucleic acid, and the second strand comprises a second stretch of contiguous nucleotides and whereby said second stretch is at least partially identical to a target nucleic acid, and whereby at least one of the two strands has an overhang of at least one nucleotide at the 5'-end.

In an embodiment of the ribonucleic acid according to the third aspect of the present invention the overhang consists of at least one nucleotide which is selected from the group comprising ribonucleotides and desoxyribonucleotides.

In a more preferred embodiment of the ribonucleic acid according to the third aspect of the present invention the nucleotide has a modification whereby said modification is preferably selected from the group comprising nucleotides being an inverted abasic and nucleotides having an NH_2 -modification at the 2'-position.

In a preferred embodiment of the ribonucleic acid according to the third aspect of the present invention at least one of the strands has an overhang of at least one nucleotide at the 3'-end consisting of ribonucleotide or deoxyribonucleotide.

In another preferred embodiment of the ribonucleic acid according to the third aspect of the present invention the first stretch and/or the second stretch have a length of 18 or 19 nucleotides.

In an embodiment of the ribonucleic acid according to any aspect of the present invention the double-stranded structure has a length of 17 to 21 nucleotides, preferably 18 to 19 nucleotides

In an embodiment of the ribonucleic acid according to the third aspect of the present invention the overhang at the 5'-end is on the second strand.

In a preferred embodiment of the ribonucleic acid according to the third aspect of the present invention the first strand comprises also an overhang, preferably at the 5'-end.

In an embodiment of the ribonucleic acid according to the third aspect of the present invention the 3'-end of the first strand comprises an overhang.

In an alternative embodiment of the ribonucleic acid according to the third aspect of the present invention the overhang at the 5'-end is on the first strand.

In a preferred embodiment thereof the second strand also comprise an overhang, preferably at the 5'-end.

In an embodiment of the ribonucleic acid according to the third aspect of the present invention the 3'-end of the first strand comprises an overhang.

In an embodiment of the ribonucleic acid according to any aspect of the present invention at least one nucleotide of the ribonucleic acid has a modification at the 2'-position and the modification is preferably selected from the group comprising amino, fluoro, methoxy, alkoxy and alkyl.

In a fourth aspect the problem underlying the present invention is solved by a ribonucleic acid comprising a double stranded structure, whereby the double-stranded structure comprises a first strand and a second strand, whereby the first strand comprises a first stretch of contiguous nucleotides and whereby said first stretch is at least partially complementary to a

target nucleic acid, and the second strand comprises a second stretch of contiguous nucleotides and whereby said second stretch is at least partially identical to a target nucleic acid,

whereby

said first strand and/or said second strand comprises a plurality of groups of modified nucleotides having a modification at the 2'-position whereby within the strand each group of modified nucleotides is flanked on one or both sides by a flanking group of nucleotides whereby the flanking nucleotides forming the flanking group of nucleotides is either an unmodified nucleotide or a nucleotide having a modification different from the modification of the modified nucleotides.

In an embodiment of the ribonucleic acid according to the fourth aspect of the present invention the ribonucleic acid is the ribonucleic acid according to the first, second or third aspect of the present invention.

In a further embodiment of the ribonucleic acid according to the fourth aspect of the present invention said first strand and/or said second strand comprise said plurality of modified nucleotides.

In another embodiment of the ribonucleic acid according to the fourth aspect of the present invention said first strand comprises said plurality of groups of modified nucleotides.

In yet another embodiment of the ribonucleic acid according to the fourth aspect of the present invention said second strand comprises said plurality of groups of modified nucleotides.

In an preferred embodiment of the ribonucleic acid according to the fourth aspect of the present invention the group of modified nucleotides and/or the group of flanking nucleotides comprises a number of nucleotides whereby the number is selected from the group comprising one nucleotide to 10 nucleotides.

In another embodiment of the ribonucleic acid according to the fourth aspect of the present invention the pattern of modified nucleotides of said first strand is the same as the pattern of modified nucleotides of said second strand.

In a preferred embodiment of the ribonucleic acid according to the fourth aspect of the present invention the pattern of said first strand aligns with the pattern of said second strand.

In an alternative embodiment of the ribonucleic acid according to the fourth aspect of the present invention the pattern of said first strand is shifted by one or more nucleotides relative to the pattern of the second strand.

In an embodiment of the ribonucleic acid according to the fourth aspect of the present invention the modification is selected from the group comprising amino, fluoro, methoxy, alkoxy and alkyl.

In another embodiment of the ribonucleic acid according to the fourth aspect of the present invention the double stranded structure is blunt ended.

In an preferred embodiment of the ribonucleic acid according to the fourth aspect of the present invention the double stranded structure is blunt ended on both sides.

In another embodiment of the ribonucleic acid according to the fourth aspect of the present invention the double stranded structure is blunt ended on the double stranded structure's side which is defined by the 5'-end of the first strand and the 3'-end of the second strand.

In still another embodiment of the ribonucleic acid according to the fourth aspect of the present invention the double stranded structure is blunt ended on the double stranded structure's side which is defined by at the 3'-end of the first strand and the 5'-end of the second strand.

In another embodiment of the ribonucleic acid according to the fourth aspect of the present invention at least one of the two strands has an overhang of at least one nucleotide at the 5'-end.

In a preferred embodiment of the ribonucleic acid according to the fourth aspect of the present invention the overhang consists of at least one desoxyribonucleotide.

In a further embodiment of the ribonucleic acid according to the fourth aspect of the present invention at least one of the strands has an overhang of at least one nucleotide at the 3'-end.

In an embodiment of the ribonucleic acid according to any of the aspects of the present invention the length of the double-stranded structure has a length from about 17 to 21 and more preferably 18 or 19 bases

In another embodiment of the ribonucleic acid according to any of the aspects of the present invention the length of said first strand and/or the length of said second strand is independently from each other selected from the group comprising the ranges of from about 15 to about 23 bases, 17 to 21 bases and 18 or 19 bases.

In a preferred embodiment of the ribonucleic acid according to any of the aspects of the present invention the complementarity between said first strand and the target nucleic acid is perfect.

In an embodiment of the ribonucleic acid according to any of the aspects of the present invention the duplex formed between the first strand and the target nucleic acid comprises at least 15 nucleotides wherein there is one mismatch or two mismatches between said first strand and the target nucleic acid forming said double-stranded structure.

In an embodiment of the ribonucleic acid according to any of the aspects of the present invention, wherein both the first strand and the second strand each comprise at least one group of modified nucleotides and at least one flanking group of nucleotides, whereby each group of modified nucleotides comprises at least one nucleotide and whereby each flanking group of nucleotides comprising at least one nucleotide; with each group of modified nucleotides of the first strand being aligned with a flanking group of nucleotides on the second strand, whereby the most terminal 5' nucleotide of the first strand is a nucleotide of the group of modified nucleotides, and the most terminal 3' nucleotide of the second strand is a nucleotide of the flanking group of nucleotides.

In a preferred embodiment of the ribonucleic acid according to the fourth aspect, wherein each group of modified nucleotides consists of a single nucleotide and/or each flanking group of nucleotides consists of a single nucleotide.

In a further embodiment of the ribonucleic acid according to the fourth aspect, wherein on the first strand the nucleotide forming the flanking group of nucleotides is an unmodified nucleotide which is arranged in a 3' direction relative to the nucleotide forming the group of modified nucleotides, and wherein on the second strand the nucleotide forming the group of modified nucleotides is a modified nucleotide which is arranged in 5' direction relative to the nucleotide forming the flanking group of nucleotides.

In a another embodiment of the ribonucleic acid according to the fourth aspect, wherein the first strand comprises eight to twelve, preferably nine to eleven, groups of modified nucleotides, and wherein the second strand comprises seven to eleven, preferably eight to ten, groups of modified nucleotides.

In a preferred embodiment of the ribonucleic acid according to any of the aspects of the present invention the target gene is selected from the group comprising structural genes, housekeeping genes, transcription factors, motility factors, cell cycle factors, cell cycle inhibitors, enzymes, growth factors, cytokines and tumor suppressors.

In a further embodiment of the ribonucleic acid according to any of the aspects of the present invention the first strand and the second strand are linked by a loop structure.

In a preferred embodiment of the ribonucleic acid according to any of the aspects of the present invention the loop structure is comprised of a non-nucleic acid polymer.

In a preferred embodiment thereof the non-nucleic acid polymer is polyethylene glycol.

In an alternative embodiment thereof the loop structure is comprised of a nucleic acid.

In an embodiment of the ribonucleic acid according to any of the aspects of the present invention the 5'-terminus of the first strand is linked to the 3'-terminus of the second strand.

In a further embodiment of the ribonucleic acid according to any of the aspects of the present invention the 3'-end of the first strand is linked to the 5'-terminus of the second strand.

In a fifth aspect the problem underlying the present invention is solved by the use of a ribonucleic acid according to any of the aspects of the present invention, for target validation.

In a sixth aspect the problem underlying the present invention is solved by the use of a ribonucleic acid according to any of the aspects of the present invention, for the manufacture of a medicament.

In a preferred embodiment of the use according to the sixth aspect of the present invention the medicament is for the treatment of a disease or of a condition which is selected from the group comprising glioblastoma, prostate cancer, breast cancer, lung cancer, liver cancer, colon cancer, pancreatic cancer and leukaemia, diabetes, obesity, cardiovascular diseases, and metabolic diseases.

In a seventh aspect the problem underlying the present invention is solved by a cell, preferably a knockdown cell, containing a ribonucleic acid according to any of the aspects of the present invention.

In an eighth aspect the problem underlying the present invention is solved by an organism, preferably a knockdown organism, containing a ribonucleic acid according to any of the aspects of the present invention.

In a ninth aspect the problem underlying the present invention is solved by a composition containing a ribonucleic acid according to any of the aspects of the present invention.

In a tenth aspect the problem underlying the present invention is solved by a pharmaceutical composition containing a ribonucleic acid according to any of the aspects of the present invention, and a pharmaceutically acceptable carrier.

In an eleventh aspect the problem underlying the present invention is solved by method for inhibiting the expression of a target gene in a cell or derivative thereof comprising introducing of a ribonucleic acid according to any of the aspects of the present invention into the cell in an

amount sufficient to inhibit expression of the target gene, wherein the target gene is the target gene of the a ribonucleic acid according to any of the aspects of the present invention.

The present invention is based on the surprising finding that small interfering RNAs can be designed such as to be both highly specific and active as well as stable under the reaction conditions typically encountered in biological systems such as biochemical assays or cellular environments. The various interfering RNAs described in the prior art such as by Tuschl et al. (international patent application WO 01/75164) provide for a length of 21 to 23 nucleotides and a modification at the 3' end of the double-stranded RNA. It has been surprisingly found by the present inventors that the problem of stability of interfering RNA, including small interfering RNA (siRNA) which is generally referred to herein in the following as RNAi, actually resides in the attack of endonucleases rather than exonucleases as thought earlier. Based on this finding several strategies have been perceived by the present inventors which are subject to the present application.

The present invention is thus related to new forms of interfering RNA. RNAi consists of a ribonucleic acid comprising a double-stranded structure. Said double-stranded structure is formed by a first strand and a second strand. Said first strand comprises a stretch of contiguous nucleotides, also referred to as first stretch of contiguous nucleotides herein, and this first stretch is at least partially complementary to a target nucleic acid. Said second strand comprises also a stretch of contiguous nucleotides whereby said second stretch is at least partially identical to a target nucleic acid. The very basic structure of this ribonucleic acid is schematically shown in Fig. 1. Said first strand and said second strand are preferably hybridised to each other and form the double-stranded structure. The hybridisation typically occurs by Watson Crick base pairing. The inventive ribonucleic acid, however, is not necessarily limited in its length to said double-stranded structure. There might be further nucleotides added to each strand and/or to each end of any of the strands forming the RNAi. Depending on the particular sequence of the first stretch and the second stretch, the hybridisation or base pairing is not necessarily complete or perfect, which means that the first and the second stretch are not 100 % base paired due to mismatches. There might also be one or more mismatches within the duplex. Said mismatches have no effect on the RNAi activity if placed outside a stretch of preferably 15, 16 or 17 matching nucleotides. If mismatches are placed to yield only 15 or less contiguous matching nucleotides, the RNAi molecule typically

shows a reduced activity in down regulating mRNA for a given target compared to a 17 matching nucleotide duplex.

The first stretch of contiguous nucleotides of the first strand is essentially complementary to a target nucleic acid, more preferably to a part of the target nucleic acid. Complementary as used herein preferably means that the nucleotide sequence of the first strand is hybridising to a nucleic acid sequence of a target nucleic acid sequence or a part thereof. Typically, the target nucleic acid sequence or target nucleic acid is, in accordance with the mode of action of interfering ribonucleic acids, a single stranded RNA, more preferably an mRNA. Such hybridisation occurs most likely through Watson Crick base pairing, however, is not necessarily limited thereto. The extent to which said first strand and more particularly the first stretch of contiguous nucleotides of said first strand is complementary to a target nucleic acid sequence can be as high as 100% and be as little as 80%, preferably 80-100%, more preferably 85-100%, most preferably 90-100%. Optimum complementarity seems to be 95-100%. Complementarity in this sense means that the aforementioned range of nucleotides, such as, e. g., 80%-100%, depending on the particular range, of the nucleotides are perfect by Watson Crick base pairing. It is shown in one aspect of the present invention that the complementarity between said first stretch of nucleotides and the target RNA has to be 18-19 nucleotides, stretches of as little as 17 nucleotides even with two sequence specific overhangs are not functional in mediating RNAi. Accordingly, given a duplex having a length of 19 nucleotides or base pairs a minimum complementarity of 17 nucleotides or nucleotide base pairs would be acceptable allowing for a mismatch of two nucleotides. In case of a duplex consisting of 20 nucleotides or base pairs a complementarity of 17 nucleotides or nucleotide base pairs would be allowable and functionally active. The same applies to a duplex of 21 nucleotides or base pairs with a total of 17 complementary nucleotides or base pairs. Basically, the extent of complementarity required for a length of a duplex, i. e. of a double stranded structure, can also be based on the melting temperature of the complex formed by either the double stranded structure as described herein or by the complex of the first stretch of the first strand and the target nucleic acid.

It is to be understood that all of the ribonucleic acids of the present invention are suitable to cause or being involved in RNA mediated interference such as, for example, described in international patent applications WO 99/32619, WO 00/44895 and WO 01/75164.

The first strategy according to which an interfering ribonucleic acid molecule may be designed according to the present invention is to have an optimum length of 18 or 19 nucleotides of the stretch which is complementary to the target nucleic acid. It is also within the present invention that said optimum length of 18 or 19 nucleotides is the length of the double stranded structure in the RNAi used. This length requirement is clearly different from the technical teaching of the prior art such as, for example, international patent application WO 01/75164. It is within the present invention that any further design, both according to the present invention and as described in the prior art, can be realised in connection with an interfering ribonucleic acid having said length characteristics, i.e. a length of 18 or 19 nucleotides.

The second strategy according to which an interfering ribonucleic acid molecule may be designed is to have a free 5' hydroxyl group, also referred to herein as free 5' OH-group, at the first strand. A free 5' OH-group means that the most terminal nucleotide forming the first strand is present and is thus not modified, particularly not by an end modification. Typically, the terminal 5'-hydroxy group of the second strand, respectively, is also present in an unmodified manner. In a more preferred embodiment, also the 3'-end of the first strand and first stretch, respectively, is unmodified such as to present a free OH-group which is also referred to herein as free 3'OH-group, whereby the design of the 5' terminal nucleotide is the one of any of the afore-described embodiments. Preferably such free OH-group is also present at the 3'-end of the second strand and second stretch, respectively. In other embodiments of the ribonucleic acid molecules as described previously according to the present invention the 3'-end of the first strand and first stretch, respectively, and/or the 3'-end of the second strand and second stretch, respectively, may have an end modification at the 3' end.

As used herein the terms free 5'OH-group and 3'OH-group also indicate that the respective most terminal nucleotide at the 5'end and the 3' end of the polynucleotide, respectively, presents an OH-group. Such OH-group may stem from either the sugar moiety of the nucleotide, more preferably from the 5'position in case of the 5'OH-group and from the 3'position in case of the 3'OH-group, or from a phosphate group attached to the sugar moiety of the respective terminal nucleotide. The phosphate group may in principle be attached to any OH-group of the sugar moiety of the nucleotide. Preferably, the phosphate group is attached to the 5'OH-group of the sugar moiety in case of the free 5'OH-group and/or to the

3'OH-group of the sugar moiety in case of the free 3'OH-group still providing what is referred to herein as free 5' or 3' OH-group.

As used herein with any strategy for the design of RNAi or any embodiment of RNAi disclosed herein, the term end modification means a chemical entity added to the most 5' or 3' nucleotide of the first and/or second strand. Examples for such end modifications include, but are not limited to, inverted (deoxy) abasics, amino, fluoro, chloro, bromo, CN, CF, methoxy, imidazole, caboxylate, thioate, C₁ to C₁₀ lower alkyl, substituted lower alkyl, alkaryl or aralkyl, OCF₃, OCN, O-, S-, or N-alkyl; O-, S-, or N-alkenyl; SOCH₃; SO₂CH₃; ONO₂; NO₂, N₃; heterozycloalkyl; heterozycloalkaryl; aminoalkylamino; polyalkylamino or substituted silyl, as, among others, described in European patents EP 0 586 520 B1 or EP 0 618 925 B1.

As used herein, alkyl or any term comprising "alkyl" means any carbon atom chain comprising 1 to 12, preferably 1 to 6 and more, preferably 1 to 2 C atoms.

A further end modification is a biotin group. Such biotin group may preferably be attached to either the most 5' or the most 3' nucleotide of the first and/or second strand or to both ends. In a more preferred embodiment the biotin group is coupled to a polypeptide or a protein. It is also within the scope of the present invention that the polypeptide or protein is attached through any of the other aforementioned end modifications. The polypeptide or protein may confer further characteristics to the inventive nucleic acid molecules. Among others the polypeptide or protein may act as a ligand to another molecule. If said other molecule is a receptor the receptor's function and activity may be activated by the binding ligand. The receptor may show an internalization activity which allows an effective transfection of the ligand bound inventive nucleic acid molecules. An example for the ligand to be coupled to the inventive nucleic acid molecule is VEGF and the corresponding receptor is the VEGF receptor.

Various possible embodiments of the RNAi of the present invention having different kinds of end modification(s) are presented in the following table 1.

Table 1: Various embodiments of the interfering ribonucleic acid according to the present invention

	1 st strand/1 st stretch	2 nd strand/ 2 nd stretch
1.) 5'-end 3'-end	free OH free OH	free OH free OH
2.) 5'-end 3'-end	free OH end modification	free OH end modification
3.) 5'-end 3'-end	free OH free OH	free OH end modification
4.) 5'-end 3'-end	free OH end modification	free OH free OH
5.) 5'-end 3'-end	free OH free OH	end modification free OH
6.) 5'-end 3'-end	free OH end modification	end modification free OH
7.) 5'-end 3'-end	free OH free OH	end modification end modification
8.) 5'-end 3'-end	free OH end modification	end modification end modification

The various end modifications as disclosed herein are preferably located at the ribose moiety of a nucleotide of the ribonucleic acid. More particularly, the end modification may be attached to or replace any of the OH-groups of the ribose moiety, including but not limited to the 2'OH, 3'OH and 5'OH position, provided that the nucleotide thus modified is a terminal nucleotide. Inverted abasics are nucleotides, either desoxyribonucleotides or ribonucleotides which do not have a nucleobase moiety. This kind of compound is, among others, described in Sternberger et al.(2002), Antisense. Nucl. Ac. Drug Dev. in press.

Any of the aforementioned end modifications may be used in connection with the various embodiments of RNAi depicted in table 1. In connection therewith it is to be noted that any of the RNAi forms or embodiments disclosed herein with the sense strand being inactivated, preferably by having an end modification more preferably at the 5' end, are particularly advantageous. This arises from the inactivation of the sense strand which corresponds to the

second strand of the ribonucleic acids described herein, which might otherwise interfere with an unrelated single-stranded RNA in the cell. Thus the expression and more particularly the translation pattern of the transcriptome of a cell is more specifically influenced. This effect is also referred to as off-target effect. Referring to table 1 those embodiments depicted as embodiments 7 and 8 are particularly advantageous in the above sense as the modification results in an inactivation of the – target unspecific – part of the RNAi (which is the second strand) thus reducing any unspecific interaction of the second strand with single-stranded RNA in a cellular or similar system where the RNAi according to the present invention is going to be used to knock down specific ribonucleic acids and proteins, respectively.

A third strategy subject to the present invention is to realise a ribonucleic acid comprising a double-stranded structure whereby the double-stranded structure comprises a first strand and a second strand, whereby the first strand comprises a first stretch of contiguous nucleotides and whereby said first stretch is at least partially complementary to a target nucleic acid, and the second strand comprises a second stretch of contiguous nucleotides whereby said second stretch is at least partially identical to a target nucleic acid whereby the double-stranded structure is blunt-ended. As used in connection with the present description the term double-stranded structure is also referred to as duplex. This design of RNAi is thus clearly different from, e. g., the one of Tuschl et al. as specified in international patent application WO 01/75164 which discloses a 3'-overhang. As used herein overhang refers to a double-stranded structure whereby at least one end of one strand is longer than the corresponding end of the other strand forming the double-stranded structure, which is also referred to herein as the counter strand. Preferably, the first stretch is identical to the first strand and the second stretch is identical to the second strand.

Taken the effectiveness of blunt-ended RNAi and the advantages of an end modification of either the first or the second strand or both, respectively, of the respective ribonucleic acid it is preferred to have a combination of both design principles. In other words, it is within the present invention to have blunt-ended RNAi carrying any end modification scheme as depicted in table 1.

The fourth strategy subject to the present invention is to have an overhang at the 5'-end of the ribonucleic acid. More particularly, such overhang may in principle be present at either or both the first strand and second strand of the ribonucleic acid according to the present

invention. The length of said overhang may be as little as one nucleotide and as long as 2 to 8 nucleotides, preferably 2, 4, 6 or 8 nucleotides. It is within the present invention that the 5' overhang may be located on the first strand and/or the second strand of the ribonucleic acid according to the present application. The nucleotide(s) forming the overhang may be (a) desoxyribonucleotide(s), (a) ribonucleotide(s) or a continuation thereof.

The overhang preferably comprises at least one desoxyribonucleotide, whereby said one desoxyribonucleotide is preferably the most 5'-terminal one. It is within the present invention that the 3'-end of the respective counter-strand of the inventive ribonucleic acid does not have an overhang, more preferably not a desoxyribonucleotide overhang. Here again, any of the inventive ribonucleic acids may comprise an end modification scheme as outlined in connection with table 1 and/or end modification as outlined herein.

The fifth strategy in the design of interfering ribonucleic acids subject to the present application resides in the formation of a certain pattern of modified nucleotides on at least one of the strands and more particularly on one of the stretches of contiguous nucleotides of the ribonucleic acid(s) according to the present invention. The kind of modification of said nucleotides may be the same as discussed in connection with the other strategies for designing interfering RNA as disclosed herein and more particularly the kind of modification described herein for or as an end modification, such as, e.g., inverted abasics, methoxy, or amino and the like at the ribose moiety of at least one nucleotide forming the ribonucleotide acids according to the present application. It is to be noted that the modification of said nucleotides may be any form of modification described herein, more particularly the kind of modification as described herein as end modification with the particularity that the so-called end modification is not necessarily located at terminal nucleotides. Rather the modification is occurring at a non-terminal nucleotide. Under such conditions the modification is preferably attached to the ribose moiety of the - to be - modified nucleotide and even more preferably to the 2' position of the ribose moiety.

It is also within the present invention that any ribonucleic acid designed according to this strategy may also have the features conferred to a ribonucleic acid according to the present application by any of the other design strategies disclosed herein. Accordingly, the interfering ribonucleic acid having a pattern of modified nucleotides may have an end modification, an

end modification scheme, may be blunt ended or may have a 5' overhang or any combination of two or more of these elements or characteristics.

Apart from the aforementioned modifications which may be presented either as end modifications or as modification pattern, the ribonucleic acid backbone as such may be further modified by forming different links between the nucleotides. Such different links are, among others, described in European patent EP 0 586 520 B1 and European patent EP 0 618 925 B1. Of particular interest here are internal modification(s) of the ribonucleic acid backbone which have been shown to confer higher nuclease resistance of ribooligonucleotides. In a preferred embodiment the modification of the modified nucleotide is a methoxylation of the 2'-OH-group of the ribose moiety of the nucleotide.

In a preferred embodiment, both strands, and more particularly both the first stretch and the second stretch show this kind of modification of the nucleotides forming said strands and stretches, respectively. However, it is also within the present invention that either the first strand and first stretch, respectively, or the second strand and second stretch, respectively, show this particular pattern of modification of the nucleotides. As used herein, the term group of modified nucleotide or flanking group of nucleotide may comprise or represent as little nucleotides as one nucleotide.

Taken the stretch of contiguous nucleotides a pattern of modification of the nucleotides forming the stretch may be realised such that a single nucleotide or group of nucleotides which are covalently linked to each other via standard phosphodiester bonds or, at least partially, through phosphorothioate bonds, show such kind of modification. In case such nucleotide or group of nucleotides which is also referred to herein as group of modified nucleotides, is not forming the 5'-end or 3'-end of said stretch a nucleotide or group of nucleotides follows on both sides of the nucleotide which does not have the modification of the preceding nucleotide or group of nucleotides. It is to be noted that this kind of nucleotide or group of nucleotides, however, may have a different modification. This kind of nucleotide or group of nucleotides is also referred to herein as the flanking group of nucleotides. This sequence of modified nucleotide and group of modified nucleotides, respectively, and unmodified or differently modified nucleotide or group of unmodified or differently modified nucleotides may be repeated one or several times. Preferably, the sequence is repeated more than one time. For reason of clarity the pattern is discussed in more detail in the following,

generally referring to a group of modified nucleotides or a group of unmodified nucleotides whereby each of said group may actually comprise as little as a single nucleotide. Unmodified nucleotide as used herein means either not having any of the afore-mentioned modifications at the nucleotide forming the respective nucleotide or group of nucleotides, or having a modification which is different from the one of the modified nucleotide and group of nucleotides, respectively.

It is also within the present invention that the modification of the unmodified nucleotide(s) wherein such unmodified nucleotide(s) is/are actually modified in a way different from the modification of the modified nucleotide(s), can be the same or even different for the various nucleotides forming said unmodified nucleotides or for the various flanking groups of nucleotides.

The pattern of modified and unmodified nucleotides may be such that the 5'-terminal nucleotide of the strand or of the stretch starts with a modified group of nucleotides or starts with an unmodified group of nucleotides. However, in an alternative embodiment it is also possible that the 5'-terminal nucleotide is formed by an unmodified group of nucleotides.

This kind of pattern may be realised either on the first stretch or the second stretch of the interfering RNA or on both. It has to be noted that a 5' phosphate on the target-complementary strand of the siRNA duplex is required for siRNA function, suggesting that cells check the authenticity of siRNAs through a free 5' OH (which can be phosphorylated) and allow only such bona fide siRNAs to direct target RNA destruction (Nykanen, *et al.* (2001), Cell 107, 309-21).

Preferably, the first stretch shows a kind of pattern of modified and unmodified groups of nucleotides, i. e. of group(s) of modified nucleotides and flanking group(s) of nucleotides, whereas the second stretch does not show this kind of pattern. This may be useful insofar as the first stretch is actually the more important one for the target-specific degradation process underlying the interference phenomenon of RNA so that for specificity reasons the second stretch can be chemically modified so it is not functional in mediating RNA interference.

However, it is also within the present invention that both the first stretch and the second stretch have this kind of pattern. Preferably, the pattern of modification and non-modification is the same for both the first stretch and the second stretch.

In a preferred embodiment the group of nucleotides forming the second stretch and corresponding to the modified group of nucleotides of the first stretch are also modified whereas the unmodified group of nucleotides of or forming the second stretch correspond to the unmodified group of nucleotides of or forming the first stretch. This possibility is schematically depicted in Fig. 2A. Another alternative is that there is a phase shift of the pattern of modification of the first stretch and first strand, respectively, relative to the pattern of modification of the second stretch and second strand, respectively. Preferably, the shift is such that the modified group of nucleotides of the first strand corresponds to the unmodified group of nucleotides of the second strand and vice versa. This possibility is shown in Fig. 2B. It is also within the present invention that the phase shift of the pattern of modification is not complete but overlapping as illustrated in Fig. 2C.

In a preferred embodiment the second nucleotide at the terminus of the strand and stretch, respectively, is an unmodified nucleotide or the beginning of group of unmodified nucleotides. Preferably, this unmodified nucleotide or unmodified group of nucleotides is located at the 5'-end of the first and second strand, respectively, and even more preferably of the first strand. In a further preferred embodiment the unmodified nucleotide or unmodified group of nucleotide is located at the 5'-end of the first strand and first stretch, respectively. In a preferred embodiment the pattern consists of alternating single modified and unmodified nucleotides.

In a further preferred embodiment of this aspect of the present invention the interfering ribonucleic acid subject comprises two strands, whereby a 2'-O-methyl modified nucleotide and a non-modified nucleotide, preferably a nucleotide which is not 2'-O-methyl modified, are incorporated on both strands in an alternate manner which means that every second nucleotide is a 2'-O-methyl modified and a non-modified nucleotide, respectively. This means that on the first strand one 2'-O-methyl modified nucleotide is followed by a non-modified nucleotide which in turn is followed by 2'-O-methyl modified nucleotide and so on. The same sequence of 2'-O-methyl modification and non-modification exists on the second strand, whereby there is preferably a phase shift such that the 2'-O-methyl modified nucleotide on the

first strand base pairs with a non-modified nucleotide(s) on the second strand and vice versa. This particular arrangement, i. e. base pairing of 2'-O-methyl modified and non-modified nucleotide(s) on both strands is particularly preferred in case of short interfering ribonucleic acids, i. e. short base paired double-stranded ribonucleic acids because it is assumed, although the present inventors do not wish to be bound by that theory, that a certain repulsion exists between two base-pairing 2'-O-methyl modified nucleotides which would destabilise such duplex, preferably short duplexes. About the particular arrangement, it is preferred if the antisense strand starts with a 2'-O-methyl modified nucleotide at the 5' end whereby consequently the second nucleotide is non-modified, the third, fifth, seventh and so on nucleotides are thus again 2'-O-methyl modified whereas the second, fourth, sixth, eighth and the like nucleotides are non-modified nucleotides. Again, not wishing to be bound by any theory, it seems that a particular importance may be ascribed to the second, and optionally fourth, sixth, eighth and/or similar position(s) at the 5' terminal end of the antisense strand which should not comprise any modification, whereas the most 5' terminal nucleotide, i. e. the first 5' terminal nucleotide of the antisense strand may exhibit such modification with any uneven positions such as first, optionally third, fifth and similar position(s) at the antisense strand may be modified. In further embodiments the modification and non-modification, respectively, of the modified and non-modified nucleotide(s), respectively, may be anyone as described herein.

Although not limited thereto, the double-stranded structure of the inventive ribonucleic acid, which is also referred to as duplex, is formed by the first strand and second strand, respectively, or the first and second stretch of contiguous nucleotides. The length of the first stretch and second stretch, respectively, is typically about 15 to about 23, preferably 17 to 21, and more preferably 18 or 19 bases. In connection with this it is to be noted that a length of less than 30 nucleotides, preferably less than 21 nucleotides does not cause any biological system which is basically capable of showing RNA interference and also interferon response, to develop an interferon response. The reason for this resides in the observation that a given cell is experiencing profound physiological changes when double-stranded DNA longer than 30 base pairs binds and activates the protein kinase PKR and 2',5'-oligoadenylate synthetase. Activated PKR stalls translation via phosphorylation of eIF2a, activated 2',5'-AS causes mRNA degradation. These effects are not desired in target validation and animal models because they overrule the effect of the target specific knockdown on the phenotype.

According to a sixth strategy in the design of interfering ribonucleic acids subject to the present application the ribonucleic acid comprises a double-stranded structure whereby the double-stranded structure comprises a first strand and a second strand, whereby the first strand comprises a first stretch of contiguous nucleotides and whereby said first stretch is at least partially complementary to a target nucleic acid, and the second strand comprises a second stretch of contiguous nucleotides whereby said second stretch is at least partially identical to a target nucleic acid whereby one terminus of the first strand and one terminus of the second strand are linked by a loop structure.

In an embodiment the loop structure is comprised of a non-nucleic acid polymer. Such non-nucleic acid polymer may be polyethylene glycol or similar polymers. The non-nucleic acid polymers may in principle be chosen from the group comprising polymers which do not comprise a polynucleotide and allow that the two strands to be linked may actually hybridize to each other. To allow for such hybridization the molecule or moiety of the molecule linking the two stretches hybridizing with each other, has to have a certain molecular structure or molecular flexibility to allow the bending of the molecule so as to allow that both stretches get in close contact and in a three-dimensional orientation which permits hybridization. Such molecule or moiety factually acts as a hinge. In principle any molecule which complies with this requirement may be used in connection with the present invention. Besides polyethylene glycol amino acid based molecules may be used. Such amino acid based molecules may be either homopolymers or heteropolymers. A useful example is a homopolymer consisting of seven glycine residues which allows the generation of a hinge as required to bring the two stretches to hybridize in the close proximity as needed. This glycine based hinge is described, e. g., in Guan K. L. and Dixon J. E. (1991), *Anal Biochem.* 192, 262. In another embodiment the hinge may be formed by crown ethers known in the art.

In an alternative embodiment the loop is comprised of a nucleic acid. As used herein, LNA as described in Elayadi and Corey (2001) *Curr Opin Investig Drugs.* 2(4):558-61. Review; Orum and Wengel (2001) *Curr Opin Mol Ther.* 3(3):239-43; and PNA are regarded as nucleic acids and may also be used as loop forming polymers. Basically, the 5'-terminus of the first strand may be linked to the 3'-terminus of the second strand. As an alternative, the 3'-end of the first strand may be linked to the 5'-terminus of the second strand. The nucleotide sequence forming said loop structure is regarded as in general not being critical. However, the length of the nucleotide sequence forming such loop seems to be critical for sterical reasons.

Accordingly, a minimum length of four nucleotides seems to be appropriate to form the required loop structure. In principle, the maximum number of nucleotides forming the hinge or the link between both stretches to be hybridized is not limited. However, the longer a polynucleotide is, the more likely secondary and tertiary structures are formed and thus the required orientation of the stretches affected. Preferably, a maximum number of nucleotides forming the hinge is about 12 nucleotides. It is within the disclosure of this application that any of the designs described above may be combined with the present sixth strategy, i. e. by linking the two strands covalently in a manner that back folding (loop) can occur through a loop structure or similar structure.

The present inventors have surprisingly found that if the loop is placed 3' of the antisense strand, i. e. the first strand of the ribonucleic acid(s) according to the present invention, the activities of this kind of RNAi are higher compared to the placement of the loop 5' of the antisense strand. Accordingly, the particular arrangement of the loop relative to the antisense strand and sense strand, i. e. the first strand and the second strand, respectively, is crucial and is thus in contrast to the understanding as expressed in the prior art where the orientation is said to be of no relevance. However, this seems not true given the experimental results presented herein. The understanding as expressed in the prior art is based on the assumption that any RNAi, is subject to a processing during which non-loop linked RNAi is generated. However, if this was the case, the clearly observed increased activity of those structures having the loop placed 3' of the antisense could not be explained. Insofar a preferred arrangement in 5' → 3' direction of this kind of small interfering RNAi is second strand - loop - first strand. The respective constructs may be incorporated into suitable vector systems. Preferably the vector comprises a promoter for the expression of RNAi. Preferably the respective promoter is pol III and more preferably the promoters are the U6, H1, 7SK promoter as described in Good et al. (1997) *Gene Ther*, 4, 45-54.

Because of the general applicability of the concept of interfering RNA and thus the knockdown or knockout for a coding nucleotide such as an mRNA any gene producing such RNA may be modified in its expression by using any of the ribonucleic acid molecule according to the present invention. Because of this basic and generally applicable mechanism any application based thereon can be realised which imply the knockdown or knockout of a gene. A preferred application is the use of the inventive ribonucleic acid for target validation. As used herein, target validation shall mean a process that involves taking steps to prove that

a DNA, RNA, or protein molecule is directly involved in a biological process, preferably in a process – causally – involved in a disease or non-standard condition and is therefore a suitable target for development of a new therapeutic compound. Sequence homology studies have successfully classified genes into target families. The enormous task of deciphering which of these targets are key players in diseases and which should be used for subsequent drug development needs to be addressed in an efficient manner. Therefore, the knockdown of gene expression should be reduced by 50-100%, preferably by 90% to see significant effects on the phenotype. In other cases depending on the gene, a knockdown of as little as 20% might be sufficient to yield a phenotype. A phenotype will be defined by comparison of cells containing functional RNAi molecules with cells containing non functional RNAi molecules. This will ensure a significant readout even under conditions where the protein function is inhibited only partially. Generally there is no linear correlation between the degree of mRNA reduction and the extent of the change in phenotype. It has to be acknowledged that for some proteins a reduction of about 20 % of the protein is sufficient to create a change in the phenotype whereas in case of other genes and mRNA, respectively, as little as 5 to 10 % remaining protein is sufficient to maintain the observed phenotype.

A further use of the ribonucleic acid molecules according to the present invention is its use for the manufacture of a medicament or its use as a medicament. Such medicament could either be used for the treatment and/or prevention of diseases or conditions such as any type of cancer where a gene and its product have been linked to the onset, cause or progression of this disease. Additionally, such medicament could be used to treat diseases where the presence or overexpression of a gene product is causing a pathological phenotype. In a preferred embodiment the disease is characterised by a gain of function and may be remedied through application or administration of the corresponding, biologically active RNAi. Diseases or conditions which may be treated by the medicament comprising a ribonucleic acid as disclosed herein, may be selected from the group comprising cancer, heart diseases, metabolic diseases, dermatological diseases, inflammatory diseases, immune system disorders and autoimmune disorders. The various forms of cancer include, but are not limited to, solid tumors and tumors of the hematopoietic system, such as glioblastoma, prostate cancer, breast cancer, lung cancer, liver cancer, pancreatic cancer and leukaemia. Metabolic diseases include, but are not limited to, obesity and diabetes. Dermatological diseases include, but are not limited to, psoriasis.

In another aspect the ribonucleic acid molecules according to the present invention may be used as diagnostics, preferably for those diseases as specified in connection with the above-mentioned diseases and conditions. Such diagnosis could be based on the observation that upon applying the ribonucleic acid molecules according to the present invention to a sample which preferably contains cells, a change in the expression pattern of the sample occurs. Preferably such sample comprises cells from a subject from whom it is assumed that it may exhibit said disease or condition to be treated or a predisposition therefor.

A further application of the nucleic acids according to the present invention resides in their use in the screening of pharmaceutically active compounds and/or lead optimization. The latter is done such as to monitor or determine the effect of candidate drugs such as small molecules and compare the effect created by said candidate drugs with the effect observed upon administering specific RNAi designed on the basis of the principles disclosed herein. In doing so candidate drugs having off-target effects may be eliminated from the screening process whereas those candidate drugs which create a similar or identical phenotype are deemed as highly relevant lead compound or may even be a pharmaceutically active compound themselves. In this approach, the highly specific RNAi molecules act as gold standard against which candidate drugs are measured.

In a further aspect the invention is related to a cell, preferably a knockdown cell, which contains a ribonucleic acid as disclosed herein. Such cell is preferably a cell which is either isolated or contained in a tissue or even organ which again preferably is not contained in an organism. However, the cell may also be contained in an organism. The cell is preferably a cell which is involved in the disease or condition which is to be treated by the inventive ribonucleic acids. This kind of knock-down cells may be used to generate an expression profile based on, e. g., mRNA or protein, in order to elucidate functional relationship and to determine downstream targets.

In a further aspect the invention is related to an organism containing a ribonucleic acid as disclosed herein. Preferably such organism is a vertebrate organism and more preferably the vertebrate organism is a mammal. A mammal as used herein is, among others and not limited thereto, an ape, a dog, a cat, a goat, a sheep, a pig, a guinea pig, a rabbit, a mouse, a rat and a human being.

In a still further aspect the present invention is related to a composition containing a ribonucleic acid according to the present invention. Preferably such composition comprises negative and positive controls either in combination with the effective ribonucleic acid or separated therefrom. Such composition may further comprise a solvent, preferably a buffer.

In a further aspect the present invention is related to a pharmaceutical composition containing a ribonucleic acid according to the present invention and a pharmaceutically acceptable carrier. Pharmaceutically acceptable carriers are known to the one skilled in the art and comprise, among others, diluent, buffers and the like. The pharmaceutical composition may comprise further pharmaceutically active compounds. In case the disease or condition to be treated using the ribonucleic acid molecules according to the present invention are preferably those which are already now used in connection with the treatment of said diseases or conditions. Due to the different mode of action of the ribonucleic acid molecules according to the present invention and the medicaments used for the treatment of said diseases and conditions according to the prior art, synergistic effects will happen.

The invention is now further illustrated by reference to the figures and examples from which further features, embodiments and advantages of the present invention may be taken.

Fig. 1 shows a schematic illustration defining the terminology as used herein. The upper of the two strand is the first strand and the antisense strand of the targeted nucleic acid such as mRNA. The second strand is the one which essentially corresponds in its sequence to the targeted nucleic acid and thus forms the sense strand. Both, the first strand and second strand form a double-stranded structure, typically through Watson Crick base pairing.

Fig. 2 illustrates some embodiments of the ribonucleic acid molecules of the present invention with patterns of modified and unmodified groups of nucleotides which is also referred to herein as pattern of modification. The modified groups of nucleotides are also referred to herein as group of modified nucleotides. The unmodified nucleotides or unmodified groups of nucleotides referred to as flanking group(s) of nucleotides herein, as used herein may also have one or several of the modification(s) as disclosed herein which, however, is/are different from the modification of the nucleotides forming the group(s) of modified nucleotides. In Fig. 2A the modified and unmodified groups of nucleotides, i. e. the groups of modified nucleotides and the flanking groups of nucleotides on both the first stretch and the second

stretch are located on corresponding parts of the stretches and are thus aligned to each other (groups of modified nucleotides on the first strand aligned with groups of modified nucleotides on the second strand and flanking groups of nucleotides on the first strand aligned with flanking group of nucleotides on the second strand), whereas in Fig. 2B the pattern realised on the first strand is also realised on the second strand, however, with a phase shift such that the modified group of nucleotides of the first stretch is base pairing with an unmodified group of nucleotides of the second stretch and vice versa so that a group of modified nucleotides on the first strand aligns with a flanking group of nucleotides on the second strand. In Fig. 2C a further possibility of arranging the modified and unmodified groups of nucleotides is realised. It is also within the present invention that the pattern of the first stretch is independent from the pattern of the second stretch and that both patterns partially overlap in terms of relative position to each other in the double-stranded structure defined by base pairing. In a further embodiment the extent of this overlapping can vary over the length of the stretch(es) and strand(s), respectively.

Fig. 3 shows the result of a knockdown experiment using RNAi molecules with different end protection groups. More particularly Fig. 3A shows that the various forms of end protected RNAi molecules are functional on the knockdown of PTEN mRNA.

Fig. 3B shows the sequence of the different RNAi molecules used in the experiment the result of which is depicted in Fig. 3A. Fig. 3C shows the result of an immunoblot analysis of PTEN protein after treatment with modified RNAi molecules in comparison to PTEN specific antisense constructs.

Fig. 4 shows that the 3' overhang of RNAi molecules is not important for RNA interference. More particularly, Fig. 4A shows a dose response curve of different RNAi molecules and Fig. 4B shows the sequence of the RNAi molecules used in the experiment the result of which is shown in Fig. 4A.

Fig. 5 shows that duplex length of the RNAi molecules has to be at least 18-19 nucleotide. More particularly, Fig. 5B shows the sequence of the PTEN specific RNAi molecules used in the experiment the result of which is depicted in Fig. 5A as dose response curve.

Fig. 6 shows that four terminal mismatched nucleotides in RNAi molecules with a length of 19 nucleotides are still functional in mediating Akt1 knockdown. More particularly, Fig. 6B shows the sequence of the RNAi molecules used in the experiment the result of which is depicted in Fig. 6A.

Fig. 7 shows further results on duplex length requirements and tolerance for mutation in siRNAs. More particularly, Fig. 7A shows the various constructs used (left panel) and the respective impact on inhibition of Akt1 mRNA expression in HeLa cells relative to the expression of p110 α used in the indicated amounts of siRNA molecules (right panel). The nucleotide changes in the mismatch siRNA molecules are indicated by arrows; the 3' desoxynucleotide, if any, are indicated in capital letter. Fig. 7B shows the various PTEN specific siRNAs (left panel), the inhibition of PTEN mRNA expression in HeLa cells expressed as ratio PTEN/p110 α , at various amounts of siRNA (middle panel) and Fig. 7C a Western Blot analysis depicting the inhibition of PTEN protein expression using PTEN specific siRNA (30nM) and respective mismatch siRNA after 48 and 96 hours, respectively, with p100 α being used as loading control.

Fig. 8 shows the result of studies on the stability in serum conferred to RNAi molecules by 2'-O-methylation and that end modifications have no beneficial effects on RNAi stability. More particularly, Fig. 8A shows the result of a gel electrophoresis of the various RNAi molecules depicted in Fig. 8B being subject to incubation with fetal calf serum.

Fig. 9 shows that an amino end modification results in loss of activity. Fig. 9B shows the particular RNAi molecules used in the experiments the result of which is shown in Fig. 9A expressed as PTEN/p110 α expression level ratio. Fig. 9C shows the design principles which may be deduced from the results depicted in Fig. 9A. As used in Fig. 9C the term functional means functionally active in the particular assay system as described in the example and "not functional" means not functionally active in said system.

Fig. 10 shows that 2'-O-Alkyl (methyl) modifications stabilize RNAi molecules but also result in reduction of their activity. More particularly, Fig. 10C shows the sequence of the RNAi molecules used in the experiment the result of which is depicted as a dose response curve in Fig. 10A. Fig. 10B shows the result of a gel electrophoresis of the various RNAi molecules depicted in Fig. 10C being subject to a two hour incubation in fetal calf serum.

Fig. 11 shows the result of an experiment on the efficacy of RNAi molecules with blocks of 2'-O-methyl modifications with Fig. 11A graphically depicting the results of said experiments as a dose response curve and with Fig. 11C showing the sequences of the particular RNAi molecules used in said experiments. Fig. 11B shows the result of a gel electrophoresis of the various RNAi molecules depicted in Fig. 11C being subject to a two hour incubation in fetal calf serum.

Fig. 12 shows that alternating 2'-O-methyl modification result in activity of the modified RNAi molecules compared to unmodified forms. More particularly, Fig. 12B shows the sequence of the RNAi molecules used in this experiment the result of which is depicted in Fig. 12A. Fig. 12C shows the stability of said RNAi molecules following incubation in serum for two hours, whereas Fig. 12D shows an immunoblot for PTEN protein upon application of different RNAi molecules to HeLa cells. As may be taken therefrom RNAi molecules with alternating modifications are stabilized against endonuclease degradation and active in mediating a PTEN protein knock down.

Fig. 13 shows the result of a Western Blot analysis to determine the time course of PTEN protein knock down. Cells were continuously transfected with 2'-O-Methyl modified versus unmodified RNAi molecules using cationic lipids for 72 h. Protein lysates were prepared and analysed by immunoblot after 48 and 120 h. For the 96 h and 120 h timepoints the cells were splitted, replated and incubated in the absence of RNAi molecules for additional 24 and 48 h. .

Fig. 14 shows a Western Blot depicting the protein knock down of PTEN being persistent using alternating modified RNAi molecules versus unmodified RNAi molecules. Transfection were performed for only 5 h and new medium without transfection reagents was added. Lysates were analysed by immunoblot 72h and 96h post transfection with the indicated RNAi molecules.

Fig. 15 shows that siRNA molecules with distinct 2'-O-methyl ribonucleotides modifications show increased stability in serum and mediate protein knock-down in HeLa cells. More particularly, Fig. 15A indicates the various siRNA molecule constructs used (left panel), whereby 2'-O-methyl ribonucleotides modifications are underlined and indicated by bold letters in the sequence. Inhibition of PTEN mRNA expression in HeLa cells transfected with

the indicated amounts of modified siRNA molecules is expressed as ratio PTEN/p110 α and indicated on the right panel. Fig. 15B shows on the left panel the various siRNA constructs used and on the right panel a PAA gel electrophoresis of modified and unmodified siRNA molecules after incubation in serum; the various constructs with 2'-O-methyl ribonucleotides are indicated by underlining and bold printing. Fig. 15C shows an SDS-PAGE based immunoblot illustrating the inhibition of PTEN protein expression using various of the siRNA constructs (30 nM) as depicted in Fig. 15A and 15B, respectively. Again, p110 α is used as loading control. Finally, Fig. 15D is an immunoblot indicating a prolonged protein knock-down, i. e. the inhibition of PTEN protein expression, upon administration of siRNA molecules (30 nM) with distinct 2'-O-methylribonucleotides modifications after 48 and 128 hours. As in Fig. 15C, p 110 α is used as loading control.

Fig. 16 shows that siRNA molecules with distinct 2'-O-methylribonucleotides modifications which are specific for Akt1 and p110 β mRNA show increased stability in serum and mediate protein knock-down in HeLa cells. More particularly, Fig. 16A indicates on the left panel the various constructs used whereby again 2'-O-methylribonucleotides are underlined and printed in bold. The integrity of the indicated siRNA molecules after incubation in serum is shown in the right panel. Fig. 16B shows an immunoblot of Akt1, Akt2 and Akt phosphorylation and p110 being used as a loading control upon transfection of the cells with the indicated siRNAs (30 mM). Fig. 16C shows various p110 β specific siRNA constructs (left panel) with the 2'-O-methyl modifications being underlined and printed in bold, and the result of an immunoblot analysis (right panel) of the inhibition of the phosphorylation of the downstream kinase Akt1 by said siRNA constructs. p110 α has been used as a loading control.

Fig. 17 shows the efficacy of various RNAi molecules with hairpin structures as dose response curve while Fig. 17B shows the structure of said RNAi molecules the result of which is depicted in Fig. 17A. Synthetic siRNAs with different loops are functional in reducing the p110 β , Akt1 and Akt 2 expression. (14A) Inhibition of p110 β mRNA expression in siRNA transfected HeLa cells. Samples were analyzed in parallel for the level of p110 β mRNA expression 24h after transfection of the indicated siRNAs. The transfected bimolecular siRNAs (21mer with 3' TT overhangs, molecule 1AB) or the monomolecular siRNAs with loop structures are schematically shown. Note that the position of the loops (HIV derived pA-loop; (A)₁₂-loop) relative to the antisense sequence is reversed in 3A, 4A relative to 3B, 4B. The 2AB siRNA molecule contains 6 mismatches in the 21mer duplex and serves as a

negative control together with the untreated sample. RNA was prepared and subjected to real time RT-PCR (Taqman) analysis. p110 β mRNA levels are shown relative to the mRNA levels of p110 α , which serve as an internal reference. Each bar represents triplicate transfections (\pm standard deviation). HeLa cells were transfected at 50% confluency (2500 cells per 96 well) with siRNAs at the indicated concentrations in growth medium.

Fig. 18 shows the efficacy of various RNAi molecules with intermolecular and intramolecular loop structures as dose response curves. **(18A)** Inhibition of Akt1 mRNA expression in siRNA transfected HeLa cells. Samples were analysed in parallel for the level of Akt1 and Akt2 mRNA expression 24h after transfection of the indicated siRNAs. The different loops (A-loops; GAGA-loop and a polyethyleneglycol (PEG)-linker) and their putative secondary structure are shown schematically. The siRNA molecule 9A is specific for Akt2 and serves as a negative control. Note that 10A and 10B do not contain self-complementary sequences and are transfected in combination. Akt1 mRNA levels is shown relative to the mRNA levels of p110 β , which served as internal control. **(18B)** Inhibition of Akt2 mRNA expression in HeLa cells transfected with the indicated siRNA molecules. Akt2 mRNA levels is shown relative to the mRNA levels of p110 β . The Akt1 specific molecule 7A serves here as a negative control.

Fig. 18 C shows a Western Blot analysis on Akt protein depicting the functionality of synthetic siRNAs with different loops in specifically reducing the Akt1 and Akt 2 expression. Inhibition of Akt1 and Akt2 protein expression were analysed by immunoblot. The cells were harvested 48h after transfection of the indicated hairpin siRNAs (20nM). Cell extracts were separated by SDS-PAGE and analysed by immunoblotting using anti-p110 antibody, anti Akt 1/2. Similar results were obtained with an antibody specific for the phosphorylated form of Akt1. The positions of p110 α , another catalytic subunit of PI 3-kinase, which was used as a loading control, and of Akt1, Akt2 and phosphorylated Akt (P*-Akt) are indicated on the left.

Fig. 19 shows an NH₂ modification, also referred to herein as amino modification, which may be present at either the 3'-OH terminal nucleotide or the 5' terminal nucleotide. The amino group is attached to the phosphate which in turn is attached to the OH group of the sugar moiety, through an alkyl group comprising an alkyl chain of 1 to 8, preferably 6 C atoms, whereby the second C atom close to the phosphate group has a CH₂OH group attached thereto. As an alternative the linker may be formed by an ether whereby the ether is comprised of two alcohols whereby one alcohol is an amino alcohol and the other is a dialcohol with one

alcohol group involved in the formation of the ether group and the other one being an OH group located at either of the C atoms, preferably at the second C atom relative to the phosphate group.

Example 1: Dose response of synthetic duplex RNAi molecules

In this example the impact of NH₂ end protection groups on the activity of duplex RNAi molecules was investigated. Synthetic siRNAs were purchased from Biospring (Frankfurt, Germany). The ribo-oligonucleotides were resuspended in RNase free TE to a final concentration of 50 µM. In the case of bimolecular siRNA molecules equal aliquots (100 µM) were combined to a final concentration of 50 µM. For the formation of intramolecular duplexes the siRNAs were incubated at 50 °C for 2 min in annealing buffer (25mM NaCl; 5mM MgCl₂) and were cooled down to RT. Transfections were carried out in 96 well or 10-cm plates (at 30% to 50% confluency) by using various cationic lipids such as Oligofectamine, Lipofectamine (Life Technologies), NC388 (Ribozyme Pharmaceuticals, Inc., Boulder, CO), or FuGene 6 (Roche) according to the manufacturer's instructions. RNAi molecules were transfected by adding pre-formed 5x concentrated complex of annealed RNAi and lipid in serum-free medium to cells in complete medium. Prior to transfection 2500 HeLa cells were plated per well 15 – 18 hours before transfection for the 96 well format.

The total transfection volume was 100 µl for cells plated in 96-wells and 10 ml for cells in 10 cm plates. The final lipid concentration was 0.8 to 1.2 µg/ml depending on cell density; the RNAi concentration is indicated in each experiment.

Complex formation was allowed to take place for 30min at 37°C. Complexes were added to cells to yield a final 1x concentration of both lipid and RNAi. Depending on the analysis performed following transfection, cells were lysed using a standard cell lysis buffer for protein extraction (Klippel, A., Escobedo, J. A., Hirano, M. and Williams, L. T. (1994). *Mol Cell Biol* 14, 2675-2685) or a denaturing buffer for RNA isolation according to the RNA isolation kit (Invitex, Berlin (Germany) 24 to 48 hours post transfection for RNA analysis and 48 to 72 hours post transfection for protein analysis by Western Blot.

Determination of the relative amounts of RNA levels by Taqman analysis:

24h post transfection the RNA of cells transfected in 96-wells was isolated and purified using the Invisorb RNA HTS 96 kit (InVitek GmbH, Berlin). Inhibition of PTEN mRNA expression was detected by real time RT-PCR (Taqman) analysis using 300 nM PTEN 5' primer CACCGCCAAATTTAACTGCAGA, 300 nM PTEN 3' primer AAGGGTTTGATAAGTTCTAGCTGT and 100 nM of the PTEN Taqman probe Fam-TGCACAGTATCCTTTTGAAGACCATAACCCA-Tamra in combination with 40 nM β -actin 5' primer GTTTGAGACCTTCAACACCCCA, 40 nM β -actin 3' primer GACCAGAGGCATACAGGGACA and 100 nM of the β -actin Taqman probe Vic-CCATGTACGTAGCCATCCAGGCTGTG-Tamra. The Akt primers and probes are determined in Sternberger et al. (Sternberger, a.a.O.) and are used according to the manufacturer's instructions (Applied Biosystem; use of Amplicon Set). Also said primers and probes may be designed using the software program Primer Express (Applied Biosystem). The reaction was carried out in 50 μ l and assayed on the ABI PRISM 7700 Sequence detector (Applied Biosystems) according to the manufacturer's instructions under the following conditions: 48°C for 30 min, 95°C for 10 min, followed by 40 cycles of 15 sec at 95°C and 1 min at 60°C.

RNA knockdown is shown by real time RT-PCR analysis of HeLa cells transfected with 21 nt long siRNA duplex molecules unmodified and modified with either NH₂ or inverted Abasics groups at the 5'-end at a lipid carrier concentration of 1.0 μ g/ml. Cell density was 2000 cells/well. Modifications on the 3'-end are either RNA overhangs, RNA overhangs with amino groups or DNA overhangs.

Preparation of cell extracts and immunoblotting. Cells were washed twice with cold phosphate-buffered saline and lysed at 4°C in lysis buffer containing 20 mM Tris (pH 7.5), 137 mM NaCl, 15% (vol/vol) glycerol, 1% (vol/vol) Nonidet P-40 (NP-40), 2 mM phenylmethylsulfonyl fluoride, 10 mg aprotinin per ml, 20 mM leupeptin, 2 mM benzamidine, 1 mM sodium vanadate, 25 mM β -glycerol phosphate, 50 mM NaF and 10 mM NaPPi. Lysates were cleared by centrifugation at 14,000 x g for 5 minutes and aliquots of the cell extracts containing equal amounts of protein were analyzed for protein expression by Western-blotting: Samples were separated by SDS-PAGE and transferred to nitrocellulose-filters (Schleicher & Schuell). Filters were blocked in TBST buffer (10 mM Tris-HCl (pH

7.5), 150 mM NaCl, 0.05% (vol/vol) Tween 20, 0.5% (wt/vol) sodium azide) containing 5% (wt/vol) dried milk. The respective antibodies were added in TBST at appropriate dilutions. Bound antibody was detected using anti-mouse- or anti-rabbit-conjugated horse radish peroxidase (Transduction Laboratories) in TBST, washed, and developed using the SuperSignal West Dura (Pierce) or ECL (Amersham) chemoluminescence substrates (c.f. Sternberger et al. (2002). *Antisense. Nucl. Ac. Drug Dev. in press.*

Antibodies. The murine monoclonal anti-p110 antibody U3A and the murine monoclonal anti-p85 antibody N7B have been described (Klippel et al., 1994, aaO). Rabbit polyclonal anti-Akt and anti-phospho Akt (S473) antibodies were obtained from Cell Signaling Technology. The murine monoclonal anti-PTEN antibody was from Santa Cruz Biotechnology. The PTEN 53 specific antisense molecule, i. e. geneBloc, is described in Sternberg et al. [Sternberger, supra] having the following sequence (ucuccuuTTGTTTCTGcuaacga), whereby the nucleotide depicted in lower case are ribonucleotides whereas the nucleotide in capital letters are deoxyribonucleotides. This antisense molecule is also identical to RNAi 1A without TT.

The results are shown in Fig. 3A and the respective RNAi molecules in Fig. 3B which are directed to the mRNA of PTEN. The nucleotides written in lower case letters represent ribonucleotides whereas capital letters represent deoxyribonucleotides. The term NH₂ indicates that the 3' '-position of the ribonucleotide was modified by an amino group. The RNAi molecules used in this and other examples disclosed herein are also referred to as small interfering RNA molecules siRNA. It is to be noted that in any of the figures contained herein the upper strand is the antisense or first strand, whereas the lower strand is the sense or second strand of the interfering RNA molecule.

As can be taken from Fig. 3A amino end modifications such as amino modification and inverted abasics modification of the terminal OH group of the nucleic acid are as potent as unmodified ends when the modification is located at the 3' end of the antisense strand (see also Fig. 8A; 8B). Therefore chemical modification to stabilize or with other beneficial properties (delivery) will be tolerated without activity loss when located at the 3' OH; especially when the 3'OH is located on an overhanging nucleotide.

For the experiment shown in Fig. 3C similar conditions as outlined above were used. The first strand and the second strand of the RNAi were either modified by a NH_2 group at the 3'-position of the ribose moiety or by an inverted abasic at said positions. The first construct is designated as siRNA- NH_2 (3A3B) the second as siRNA-iB (4A4B). The sequence of both molecules is depicted in Fig. 3B. The term 3A3B indicates that the interfering ribonucleic acid consists of strand 3A as the antisense strand and strand 3B as the sense strand. For reason of comparison an antisense oligonucleotide designated GB53 (Steinberger et al., supra) was generated which was directed against the PTEN mRNA as well. The particularities of this latter experiment were as follows.

As may be taken from Fig. 3C end protected RNAi molecules depicted in Fig.3B are functional in yielding a PTEN protein knockdown.

From this example it can be taken that both end protection groups render RNAi molecules active in knocking down PTEN protein. This inhibition is as efficient as inhibition with antisense constructs but at lower concentrations used which is a clear advantage over the already very powerful antisense technology.

Example 2: Overhang requirements for RNAi duplex activity in vivo

The experimental procedures were the same as depicted in connection with example 1 except that the PTEN mRNA targeting interfering RNAi molecules were differently designed. The results are shown in Fig. 4A as dose response curves with Fig. 4B showing the particular sequence and modifications of the interfering RNAi molecules used to generate the data depicted in Fig. 4A. The nomenclature is such that, e. g., RNAi 18 is composed of strand 18A as antisense strand and strand 18B as sense strand.

Blunt ended molecules are compared to molecules with 3'-overhangs (RNAi 18) and 5'-overhangs (RNAi 30 and RNAi 31) in their activity to knockdown PTEN mRNA in HeLa cells. The activity of blunt ended molecules (RNAi 28) and molecules with 5'-overhangs is comparable to the activity of molecules with 3'-overhangs. This shows that 3'-overhangs are not essential for RNAi activity.

Example 3: Duplex length requirements of interfering RNA molecules for RNAi activity in vivo

The experimental approach was similar to the one outlined in connection with example 1 except that the interfering RNA molecules were directed against the mRNA of Akt1. The negative control to show the specificity of the RNAi molecules was again p110 mRNA. The experimental results are shown in Fig. 5A with the particularities of the interfering RNAi molecules used being represented in Fig. 5B. Similar experiments were carried out with further siRNA constructs which are depicted in Fig. 7A, left panel, whereby the arrows indicate mismatches and desoxyribonucleotides are expressed in capital letters. The inhibition of Akt1 mRNA expression in HeLa cells transfected with the indicated amounts of siRNA molecules is depicted on the right panel of Fig. 7A.

Taqman analysis on Akt RNA from HeLa cells transfected with different RNAi molecules shows that the doublestrand duplex of the siRNA molecules has to be longer than 17 base pairs to show activity whereas molecules with 17 base pair long duplexes or shorter are not functional even if sequence-specific overhangs are added. The shortest RNAi molecules successfully tested were 18 to 19 nucleotides or base pairs in length. It is to be noted that the design of the interfering RNA molecule 51A/51B referred to as RNAi 51 corresponds to the one as described in international patent application WO 01/75164. The RNAi molecule 55A/55B comprises a stretch of 17 nucleotides and has a clearly decreased activity in terms of degradation of Akt1 mRNA.

As may be taken from Fig. 7A 19 nt long duplexes are highly efficient in reducing Akt1 mRNA levels independent of the nature (desoxy- or ribonucleotides) of the 3' overhang (compare molecules 1AB, 2AB, 3AB, 4AB). The 17 nucleotide long siRNA (molecule 5AB) showed a dramatically reduced silencing activity confirming the above expressed understanding that active siRNA duplexes should be at least 18 nt or longer. Without being wished to be bound by any theory this result may be explained mechanistically by two different requirements. First, a minimum base pairing of 18 nt between the antisense of the siRNA and the target mRNA may be obligatory, or second, incorporation into the RNA-induced silencing complex (RISC) requires a minimum length of the siRNA duplex. To address this question a 19 nt long siRNA duplex molecule with one and two terminal mutations (CG and UA inversion) relative to the wildtype sequence was synthesised

(molecules 6AB and 7AB). Both molecules, even the molecule with a stretch of only 15 nt base pairing to the target mRNA were functional in inducing the Akt1 mRNA level. Therefore, it can be concluded that the duplex length itself, but not the base pairing of the antisense siRNA with the target mRNA seems to determine the minimum length of functional siRNAs. This suggests that the length of the double-stranded helix is an important determinant for the incorporation into the RISC complex. The introduced mismatches at the terminal ends of the siRNA duplexes had little effect on RNA interference.

Given the experimental results, the minimum requirement for optimum RNAi mediated interference is thus a duplex length of 18 or 19 nucleotides, independent of the further design of the RNAi molecules such as blunt end or 5'-overhang or any other form as disclosed herein, but generally applicable to RNAi molecules. However, it has to be acknowledged that the particular design of the RNAi molecules may confer further advantages to said molecules, such as, e. g., increased efficiency and increased stability, respectively.

Example 4: Target –antisense homology requirements for RNAi in vivo

The experimental set-up was similar to the one described in example 1, whereby the RNAi is specific for Akt1. In addition, a PTEN specific interfering RNA molecule was designed and used as negative control. The results are shown in Fig. 6A and Fig. 6B. Basically the same experiment was carried out using further siRNA molecules as depicted in Fig. 7B with the results being indicated in Fig. 7B (right panel) and Fig. 7C, respectively.

Having established the minimal duplex length of 18 or more than 18 nucleotides for functional siRNA molecules we have asked the question how many matching nucleotides between target mRNA and siRNA are necessary for silencing activity. As shown by Taqman analysis on Akt1 RNA a stretch of 19 to 15 nucleotides perfectly matching to the target RNA, in the present case Akt 1, is sufficient to mediate RNAi activity. A PTEN specific RNAi molecule does not reduce RNA amounts of Akt1 thus confirming the specificity of this approach. Mismatches of one or two nucleotides at any or both ends of a strand are functional suggesting that a homolog stretch of 15 nt between a target mRNA and RNAi is sufficient for gene silencing. It can be concluded from these data that unspecific gene silencing can occur by chance through unspecific binding to unrelated targets. This is based on the understanding

that a stretch of 15 to 17 matching base pairs is not specific for a single gene and will occur by chance considering the complexity and size of the genome or transcriptome of vertebrates. Apart from the afore-described experiments also the location of the mismatch was subsequently analysed. For this purpose a 19 nt long blunt siRNA directed against PTEN mRNA was used. The sequence changes in one siRNA strand were compensated by complementary changes in the other strand to avoid disrupting duplex formation. As may be taken from both Figs. 7B and C, respectively, a siRNA with only one point mutation in the centre of the molecule was severely compromised in its ability to use mRNA and protein expression levels. This result indicates that the RNA machinery is highly discriminative between perfect and imperfect base pairing between target mRNA and siRNA in the centre of the duplex. This extreme dependence on a perfect complementarity between target and siRNA has already been described for RNAi interference in the *Drosophila* system, however, not yet in connection with mammalian systems such as HeLa.

Based on this observation the present invention is reducing this off-target problem of siRNA by two approaches. First by reducing the molecule length of the siRNA molecules to the minimal requirements (18-19 nt) and thereby reducing the chance of homology to off-targets. Second, by inactivation of the sense strand to prevent a unwanted RNA silencing caused by accidental complementarity of the sense strand to a unrelated target RNA (see also Example 6).

Example 5: Stability of modified RNAi molecules in serum

Oligonucleotides were incubated in human serum for 15 min and two hours and loaded on 10% polyacrylamide gel with untreated controls. The results are shown in Fig. 8A. The various RNAi molecules used are shown and described in more detail in Fig. 8B.

From this example it can be taken that the RNAi duplex of RNA molecules with all nucleotides modified with 2'-O-methyl groups (RNAi molecules 79A79B and 28A28B) have higher stability in serum. It is also shown that a blunt duplex is more stable than the duplex molecule with overhangs. From this the conclusion may be drawn that end protection (e.g. iB or Amino) is not increasing the stability in serum.

In addition, it can also be concluded that in contrast to the understanding in the art before the filing of this application endonucleases rather than exonucleases are more important in the protection of RNAi molecules.

In view of this, in addition to the various modifications or designs of the inventive RNAi molecules as disclosed in this application a further or additional modification of the nucleotides may be the use of a phosphorothioate backbone of the RNAi molecules which may be either complete or partial in order to inhibit endonuclease function. A complete phosphorothioate backbone means that any of the nucleotides exhibits a phosphorothioate group whereas a partial phosphorothioate backbone means that not all of the nucleotides forming the RNAi molecule have a phosphorothioate modification. This modification is suitable to increase the lifetime of RNAi molecules irrespective of the further design of RNAi molecules. In this regard, a partially or completely phosphorothioate modified RNAi is subject to the present invention which may be realized in connection with the different strategies for the design of interfering RNA molecules as disclosed herein or with any of the designs known in the art.

Example 6: Inactivation of the sense strand by NH₂ end protection groups on the 5' and 3' ends

The experimental set-up was similar to the one described in connection with example 1 with the target nucleic acid sequence being PTEN mRNA. The concentration of HeLa cells was 2,000 cells per well. RNA of PTEN was analysed in Taqman assays after transfection of differently modified RNAi molecules. The different interfering RNA molecules used are depicted in Fig. 9B whereas the experimental results are shown in Fig. 9A.

As may be taken from the dose response curves of various RNAi molecules depicted in Fig. 8A RNAi molecules are functional when the sense strand, i. e. the second strand, is modified on both ends with amino groups. Particularly effective are RNAi molecules 20A26B, 18A26B, and 28A26B. The lowest activity is shown by RNAi molecule 26A26B which corresponds to end modification on all 4 ends of the duplex (Tuschl is 18AB).

However, RNAi activity is also achieved when the antisense strand, i. e. the first strand, is modified only at the 3' end leaving a free OH group at the 5' end (RNAi constructs 22A26B; 20A26B). There is no activity when the antisense strand is modified with amino groups on both the 5' and the 3' end (26A26B). This leads to the conclusion that any end of the antisense strand and more particularly the 5' end of the antisense should be kept without modifications. Additionally, it is worth stating that the NH₂ end modification can be used to inactivate the sense strand on the 5' and 3' end and therefore reduce off-target effects mediated by an otherwise functional sense strand which results in a significantly increased specificity of the RNAi molecule which is advantageous for target validation as well as for any medical use of the RNAi molecule.

The further generalisation of the results from this experiment is depicted in Fig. 9C. Functionally active RNAi are accordingly those not having an amino modification at the antisense strand or having an amino modification only at the 3' end of the antisense strand whereas an amino modification at both ends of the antisense strand is not functional, i. e. does not result in a knockdown of the target mRNA.

Example 7: Impact of 2'-O-methyl modification of RNAi molecules for endonuclease protection.

RNA knockdown is again shown using the real time RT-PCR analysis on HeLa cells transfected with RNAi duplex molecules directed against the PTEN mRNA as represented in Fig. 10A. Experimental procedures were basically the same as specified in example 1. The structure of the RNAi molecules investigated and their dose responses, which are depicted in Fig. 10A, are shown in Fig. 10C. The nucleotides printed in bold are those having a 2'-O-methyl modification.

It is illustrated by the dose response curves shown for various RNAi molecules in Fig. 10A that internal 2'-O-alkyl groups are reducing RNAi activity. Preferably such 2'-O-alkyl groups are 2'-O-methyl or 2'-O-ethyl groups. However, molecules with unmodified nucleotides in combination with 2'-O-alkyl modification show significant activity. As is also depicted in Fig. 10A there is no activity when the antisense strand is all modified with 2'-O-methyl groups and the sense strand is not modified (c. f., e. g., RNAi molecule 79A28B). Taken the

results of a stability test such as incubation of the various RNAi molecules in serum, as depicted in Fig. 10B, shows that 2'-O-alkyl modifications stabilize RNAi molecules against degradation. This clearly beneficial effect, however, is at least to a certain degree counterbalanced by the effect that 2'-O-alkyl modifications generally result in a reduced knockdown activity. Accordingly, the design of RNAi molecules has to balance stability against activity which makes it important to be aware of the various design principles as disclosed in the present application.

Example 8: Impact of blocks of internal 2'-O-methyl modifications on the stability of RNAi molecules in serum.

The experimental approach in connection with this study was actually the same as depicted in example 1. Again, PTEN RNA is analysed by real time RT-PCR on HeLa cells at a density of 2000 cells/well which were transfected with different doses of RNAi molecules. RNAi molecules were incubated in serum for two hours and analysed on a 10% polyacrylamide gel. The results of this study are illustrated in Figs. 11A to 11C, whereby Fig. 11A shows the dose response of the various RNAi molecules depicted in Fig. 11C and Fig. 11B shows the result of a stability test using some of the RNAi molecules depicted in Fig. 11C. It is to be acknowledged that the nucleotides written in bold in Fig. 11C are the ones carrying a modification which is in the present case of a 2'-O-methyl modification of the ribose moiety of the nucleotides.

There is a dose dependent inhibition by the unmodified RNAi molecules. It is also shown that the 2'-O-methyl modification of the core 9 nucleotides makes the RNAi stable in serum and allows activity of the duplex in the meaning of mediating the interference phenomenon leading to a degradation of the PTEN mRNA. Total modification of the sense strand makes the RNAi molecule stable in serum and allows certain activity.

Alternating blocks of 5 nucleotides with 2'-O-methyl modification renders the RNAi molecule stable in serum and allows activity on PTEN RNA as shown by incubating the RNAi duplex in serum for two hours and loading the samples on a 10% polyacrylamide gel. As may be taken from Fig. 11B the duplex comprising strands 80A and 80B is strongly degraded after incubation in serum for two hours. The duplex consisting of strands 82A and

82B confirms the result that the 5'-end of the first strand which comprises the antisense strand should not be modified at the 5'-terminal nucleotides (compare 82A82B with the reverse orientated 81A81B). This is also confirmed by the results obtained having the duplex consisting of the strands 86A and 86B which is both active and stabilised in serum. It is noteworthy that molecules with unmodified blocks at the terminal 5' of the antisense strand are more active whereby the 5'terminal OH group is preferably not derivatized.

Further experiments were carried out using different modification patterns of 2'-O-methyl modification of the nucleotides. The results thereof are shown in Fig. 12A to 12C and further discussed herein in example 9.

Example 9: The impact of alternating internal 2'-O-alkyl modification on serum stability of RNAi molecules.

The experimental set-up for performing this kind of study was the same as used in connection with the studies reported in example 1 and example 8, respectively, with the targeted nucleic acid being again PTEN mRNA. HeLa cells were transfected with the different RNAi molecules depicted in Fig. 12B and RNA knockdown was demonstrated using real time RT-PCR on PTEN RNA in a dose-dependent manner (Fig. 12A). The stability of the various RNAi molecules after 15 min and two hours in serum at 37°C is depicted in Fig. 12C and a Western Blot for p110 and PTEN as the target-protein of the various RNAi molecules is depicted in Fig. 12D with the RNAi molecules tested being the same in both the experiments underlying Fig. 12C and Fig. 12D.

As illustrated in Fig. 12A and Fig. 12C nucleotides modified with 2'-O-methyl groups alternating with unmodified nucleotides render RNAi molecules stable in serum while still allowing them to be active in the sense of interfering with the target mRNA. It is shown that incubation of RNAi duplex molecules for 15 min and two hours in serum will degrade the unmodified duplex and the duplex where the 10 most 5'-positioned nucleotides are unmodified.

In the RNAi molecules represented in Fig. 12B various patterns of modified and unmodified nucleotides are realised. The RNAi molecule 94A1/94B1 comprises a structure wherein a

modified nucleotide is flanked by an unmodified nucleotide with the unmodified nucleotide being located at the 5' end of the first strand. The RNAi molecule comprised of strands 94A2 and 94B2 is another example where the modified nucleotides and the unmodified nucleotides of the first and the second strand are located at opposing sites. In contrast to this the RNAi molecule comprised of strands 94A1 and 94B2 have the same pattern of modified and unmodified nucleotides. However, there is a phase shift such that the modified nucleotide forms base pairing with an unmodified nucleotide. The two RNAi molecules comprised of strands 94A1 and 94B1 and strands 94A2 and 94B2 differ from each other such that in the first case the first strand starts with an unmodified nucleotide and the corresponding first nucleotide of the second strand, i. e. the nucleotide at the 3' end of the second strand, starts with an unmodified nucleotide with the arrangement being opposite to this in the RNAi molecule comprised of 94A2 and 94B2.

Additionally, alternately modified RNAi molecules as depicted in Fig. 12B are functional in mediating a PTEN protein knock down as shown in Fig. 12D but only when the second 5' and second 3' terminal nucleotide is not modified (see 94A294B1 and 94A294B2). Taken together these data show that the most stable and most active RNAi molecules do have alternating 2' Alkyl modified and unmodified nucleotide residues. It should be noted that these molecules do show a very similar mRNA reduction when compared to unmodified siRNA molecules which being stable in serum allows increased or easier handling.

Example 10: Functional Protein Knockdown mediated by internally modified RNAi molecules

The experimental approach was similar to the one outlined in connection with example 1.

Western Blots were performed on HeLa cells harvested at various time points following transfection (48, 72, 96 and 120 hours) with alternately modified RNAi molecules as depicted in Fig. 12B. For experimental reasons it is noteworthy that at the 96 hour time point cells have been split and half the population was replated. A total of 40 nM of the various RNAi molecules were applied to the cells. Cells were continuously transfected for 72h with cationic lipids as described in example 1; then replated in the absence of transfection reagents.

Transfections were carried out in 96 well or 10-cm plates (at 30% to 50% confluency) by using various cationic lipids such as Oligofectamine, Lipofectamine (Life Technologies), NC388, L8 (Atugen, Berlin), RNAi were transfected by adding pre-formed 5x concentrated complex of siRNAs and lipid in serum-free medium to cells in complete medium. The total transfection volume was 100 μ l for cells plated in 96-wells and 10 ml for cells in 10 cm plates. The final lipid concentration was 0.8 to 1.2 μ g/ml depending on cell density; the siRNA concentration is indicated in each experiment.

The result of the Western Blot analysis is depicted in Fig. 13. As can be taken from this Figure, modified RNAi molecules of the 94A2B1 and 94A2B2 version yield a longer lasting PTEN protein knock down as unmodified molecules. The lack of protein knock down also seen in Fig. 12 with molecules of the 94A1B1 and 94A1B2 version is confirmed in this experiment. Unmodified molecules (80AB) are not as potent in supporting long lasting protein knock down when the cells are not continuously transfected.

Example 11: Persistent PTEN protein knock down with alternating 2'-O-methyl modifications of RNAi molecules

The experimental approach was similar to the one outlined in connection with example 10 with the exception that the transfection was terminated after 5 h by replacing the transfection medium with new medium. The protocol was slightly modified such that for each of the RNAi molecules a 40 nM concentration was realized using a stock solution of 1 μ g RNAi / ml cationic lipid as described in connection with example 1. 5 hours after transfection the medium was withdrawn and fresh EMEM added. The cells were split after 72, with half of the cells being lysed and the other half newly plated and lysed 24 h later (96h post transfection). The result of a Western Blot analysis using 3 different RNAi molecules (80AB, 94A1/B2, 94A2/B1) are depicted in Fig. 14. As a positive control untreated cells were used. Fig. 14 shows the expression of PTEN after 72 h and 96 h, respectively. Taken the structural particularities of the various RNAi molecules it can be taken from Fig. 14 that protein knockdown is persistent with alternating molecules of the 94A2B1 kind even over 96 h after splitting and replating cells compared to unmodified RNAi molecules (such as 80AB) and RNAi molecule 94A1B2.

A further experiment was carried out using the siRNA constructs as depicted in Fig. 15A (left panel). From the results as depicted as ratio of PTEN/p110 α mRNA degradation at the various concentrations of siRNA constructs administered to the test system, it can be taken that siRNA molecules with either one or both strands consisting of 2'-O-methyl residues were not able to induce RNA interference in the mammalian system (Fig. 15A, molecules V2, V5, V6). However, the decrease in activity was less pronounced when only parts of the strands were modified. Interestingly, a molecule having an unmodified antisense strand (which is the upper strand in the representation throughout this specification if not indicated otherwise) and a completely modified sense strand was significantly more active when compared to the reversed version (Fig. 5A, molecules V5 and V6). This result suggests that the antisense strand of the siRNA seems to be more critical and sensitive to modification. The most efficient molecules inducing PTEN mRNA had only stretches of modifications leaving the 5' end unmodified or were modified on alternating positions on both strands (Fig. 15A, molecules V10, V12).

To test the nuclease resistance the different siRNA versions were incubated in serum followed PAA gelelectrophoresis. The result is shown in Fig. 15B (right panel with the various sequences indicated on the left panel of Fig. 15B). As shown earlier, blunt ended siRNA molecules with unmodified ribonucleotides were very rapidly degraded whereas a complete substitution with 2'-O-methyl nucleotides mediated resistance against serum-derived nucleases (Fig. 15B, compare molecule AB with V1). siRNA molecules with partial 2'-O-methyl modification showed as well an increased stability when compared to unmodified siRNAs. Especially molecules with alternating modifications on both strands showed a significant improve in instability (Fig. 15B, molecules V13, V14, V15 and V12). More importantly, transfection of three of these molecules into HeLa cells resulted in a significant down regulation of PTEN protein expression as depicted in Fig. 15C, length 6, 9 and 10). In this RNA interference activity assay an unexpected preference for molecules was observed which were modified at every second nucleotide beginning with the most 5' terminal nucleotide of the antisense strand (molecules V15 and V12). Molecules which contained modifications beginning with the second nucleotide at the 5' end of the antisense strand were more stable but had a strongly reduced activity in gene silencing (molecules V13, V14). This result points towards highly specific interactions between the involved enzymes and precise nucleotides in the siRNA duplex. Taken together the data shown herein demonstrate that 2'-

O-methyl modifications at particularly selected positions in the siRNA duplex can increase nuclease resistance and do not necessarily abolish RNAi completely.

Although an increased stability of synthetic siRNA has primarily implication for *in vivo* application, it was also analysed whether the particular modification can also lead to an extended protein knock-down in cell culture systems. In accordance therewith HeLa cells were transiently transfected for six hours using different versions of PTEN specific siRNAs. The lipid siRNA complex was then washed away and the PTEN protein knock-down was analysed 48 hours and 120 hours later. Although knock-down experiments without continued transfection of siRNAs are complicated due to rapid growth of untransfected cells in this time period resulting in a very transient knock-down, the present inventors were able to demonstrate a prolonged PTEN protein knock-down with siRNA molecules stabilised by the described 2'-O-methyl modification. At 48 hours post transfection the unmodified siRNA (AB) showed the biggest reduction in PTEN protein levels, however, at 120 hours post transfection the reduction in PTEN protein expression is superior with the siRNA stabilised by alternating 2'-O-methyl modifications (Fig. 15D, compare lane 2 with lanes 4, 6 and 7).

From the results it can also be taken that preferably the starting nucleotide position of the alternating modification seems to be important. To test this preference in more detail two additional series of siRNAs were synthesised, one specific for the kinase Akt1 and the other one specific for p110 β which is one of the two catalytic subunits of PI(3-) kinase. The particular constructs are shown in Fig. 16A. As may be taken therefrom only 19 nt long siRNA duplexes either without any or with 2'-O-methyl modification on every second other nucleotide were used. Using Akt1 as a target an efficient protein knock-down as well as a dramatic reduction in phospho-Akt levels was observed with blunt, unmodified siRNAs (Fig. 16A, right panel). From the different versions of molecules with modifications on every second other nucleotide only one was efficiently mediating RNAi (Fig. 16A, molecule V5). This siRNA molecule contained an antisense strand which was modified at the most terminal 5' and 3' nucleotides. The sense strand started with the unmodified nucleotides at the most terminal position, resulting in a structure in which the modified and unmodified ribonucleotides of both strands are facing each other. As expected, this molecule was also protected against serum-derived nucleases as depicted in Fig. 16B (molecule V5).

Interestingly, a very similar 19 nt long siRNA molecule (V4) with modifications beginning at the second nucleotide of the antisense strand showed no RNA interference activation in the particular assay used. The version V6 in which the modified nucleotides of the antisense strand are facing modified nucleotides on the sense strand, was also inactive in this experiment. An identical series of 19 nt long siRNA molecules specific for p110 β confirmed these observations as depicted in Fig. 16C. Again the similarly modified siRNA molecule (V5) was the most active, as indicated by reducing Akt phosphorylation, which is indicative for a reduced P (I)-3 kinase activity due to reduced p110 β levels. The reduced activity of the molecules V6 might be explained by reduced duplex stability since the same structure was active in the PTEN knock-down experiment with 21mer siRNAs. Although it is known that 2'-O-methyl modification on both strands facing each other will reduce the stability of nucleic acid duplexes, the difference between the activity of siRNA molecules V4 and V5 (Figs. 16B and C) is probably not due to differences in duplex stability since the number of base pairing of modified and unmodified nucleotides is identical. This difference in activity might be due to specific requirements in the interacting proteins involved in the degradation of the target mRNA. Also it can be taken from these experiments that the most terminal nucleotides of the antisense strand can be modified at the 2'-OH-group with significant loss of silencing activity.

Example 12: Different loop structures are functional in mediating RNA interference

To test whether RNAi molecules, preferably synthetic RNAi molecules with self complementary structures can inhibit gene expression as efficiently as standard double stranded siRNA molecules, HeLa cells were transfected with p110 β specific synthetic siRNAs. Transfections were carried out in 96 well or 10-cm plates (at 30% to 50% confluency) by using various cationic lipids such as Oligofectamine, Lipofectamine (Life Technologies), GeneBlocs were transfected by adding pre-formed 5x concentrated complex of GB and lipid in serum-free medium to cells in complete medium. The total transfection volume was 100 μ l for cells plated in 96-wells and 10 ml for cells in 10 cm plates. The final lipid concentration was 0.8 to 1.2 μ g/ml depending on cell density; the RNAi molecule concentration is indicated in each experiment.

A dose dependent titration showed no significant difference in efficiency of mRNA knock-down achieved by the standard bimolecular double strand 21mer and the corresponding

monomolecular molecules as analysed by realtime PCR (Taqman) (Fig. 17A). Two different loop structures a (A)₁₂ loop and a HIV derived pA-loop were tested in parallel with similar results. A comparison of the relative position of the antisense sequence and the loop structure revealed an improved knock-down efficiency with the antisense sequence being located 3' to the loop (Fig. 17B; compare construct 3A, 3B and 4A, 4B).

Example 13: Studies on the efficacy of different intramolecular hairpin loops and intermolecular “bubbles”

The influence of different loop structures on inhibition of mRNA and protein expression was tested. For these experiments Akt1 and Akt2 were chosen as targets. The experimental approach was similar to the one described in example 12.

Significantly, the reduction in Akt1 mRNA as depicted in Fig. 18A, and Fig. 18B as well as Akt1 protein levels as depicted in Fig. 18C was completely independent of the loop structure tested (compare molecules 5A, 6A, 7A, 8A) (the structure of the RNAi molecule tested is always depicted underneath the bar diagram). Even a molecule containing a rather unphysiological structure such as a polyethylenglycol linker (PEG) as a loop efficiently reduced Akt1 expression indicating that size and nucleotide sequence of the loop is not crucial (Fig. 18A; molecule 8A). A synthetic siRNA molecule specific for Akt2 (9A) was used to control for specificity, and had no effect on Akt1 levels as shown in Fig. 15A. This molecule, however, efficiently silenced Akt2 expression (Fig. 18B; Fig. 18C). Self-complementary RNA molecules with loop structures have the possibility to anneal as doublestrands in monomolecular or bimolecular structures under physiological hybridization conditions (Fig. 18B, loop or bubble structure). To address the question of whether the siRNA molecules exert their function via adapting an intra-molecular loop or an inter-molecular “bubble” (schematically shown in Fig. 18B) two molecules not capable of folding back on themselves were transfected. These constructs contained Akt1- and Akt2-specific sequences within the same molecule (Fig. 18B, constructs 10A, 10B) and were designed to be restricted to form a bimolecular duplex (“bubble”). Surprisingly, this molecule efficiently mediated both Akt1 and Akt2 mRNA knock-down as well as protein knock-down when transfected after annealing of both strands.

Whether loop and bubble structures are indeed substrates for RNA processing enzymes, e.g. *Dicer*, is not clear at this point. A recent study by Paddison and coworkers suggests that hairpin containing siRNAs are more dependent on *Dicer* activity than double stranded siRNAs. However, these data demonstrating RNA interference activity using a PEG linker molecule indicate that the linker sequence is likely to be irrelevant.

The features of the present invention disclosed in the specification, the claims and/or the drawings may both separately and in any combination thereof be material for realizing the invention in various forms thereof.

Further novel forms of interfering RNA molecules

Claims

1. A ribonucleic acid comprising a double stranded structure whereby the double-stranded structure comprises a first strand and a second strand, whereby the first strand comprises a first stretch of contiguous nucleotides and whereby said first stretch is at least partially complementary to a target nucleic acid, and the second strand comprises a second stretch of contiguous nucleotides whereby said second stretch is at least partially identical to a target nucleic acid, characterised in that the double stranded structure is blunt ended.
2. A ribonucleic acid comprising a double stranded structure whereby the double-stranded structure comprises a first strand and a second strand, whereby the first strand comprises a first stretch of contiguous nucleotides and whereby said first stretch is at least partially complementary to a target nucleic acid, and the second strand comprises a second stretch of contiguous nucleotides whereby said second stretch is at least partially identical to a target nucleic acid, characterised in that the first stretch and/or the second stretch have a length of 18 or 19 nucleotides.
3. The ribonucleic acid according to claim 1, wherein the first stretch and/or the second stretch have a length of 18 or 19 nucleotides.
4. The nucleic acid according to claim 1 or 3, wherein the double stranded structure is blunt ended on both sides of the double strand.
5. The nucleic acid according to claim 1 or 3, wherein the double stranded structure is blunt ended on the double stranded structure which is defined by the 5'-end of the first strand and the 3'-end of the second strand.

6. The nucleic acid according to any of claims 1 to 5, wherein the double stranded structure is blunt ended on the double stranded structure which is defined by the 3'-end of the first strand and the 5'-end of the second strand.
7. A ribonucleic acid comprising a double stranded structure whereby the double-stranded structure comprises a first strand and a second strand, whereby the first strand comprises a first stretch of contiguous nucleotides and whereby said first stretch is at least partially complementary to a target nucleic acid, and the second strand comprises a second stretch of contiguous nucleotides and whereby said second stretch is at least partially identical to a target nucleic acid, characterised in that at least one of the two strands has an overhang of at least one nucleotide at the 5'-end.
8. The ribonucleic acid according to claim 7, wherein the overhang consists of at least one nucleotide which is selected from the group comprising ribonucleotides and desoxyribonucleotides.
9. The ribonucleic acid according to claim 8, wherein the nucleotide has a modification whereby said modification is preferably selected from the group comprising nucleotides being an inverted abasic and nucleotides having an NH₂-modification at the 2'-position.
10. The ribonucleic acid according to any of claims 7 to 9, wherein at least one of the strands has an overhang of at least one nucleotide at the 3'-end consisting of ribonucleotide or deoxyribonucleotide.
11. The ribonucleic acid according to any of claims 7 to 10, wherein the first stretch and/or the second stretch have a length of 18 or 19 nucleotides.
12. The ribonucleic acid according to any of claims 1 to 11, wherein the double-stranded structure has a length of 17 to 21 nucleotides, preferably 18 to 19 nucleotides
13. The ribonucleic acid according to any of claims 7 to 12, wherein the overhang at the 5'-end is on the second strand.

14. The ribonucleic acid according to claim 13, characterised in that the first strand comprises also an overhang, preferably at the 5'-end.
15. The ribonucleic acid according to any of claims 7 to 14, wherein the 3'-end of the first strand comprises an overhang.
16. The ribonucleic acid according to any of claims 7 to 12, wherein the overhang at the 5'-end is on the first strand.
17. The ribonucleic acid according to claim 16, characterised in that the second strand also comprise an overhang, preferably at the 5'-end.
18. The ribonucleic acid according to any of claims 7 to 12 and 16 to 17, wherein the 3'-end of the first strand comprises an overhang.
19. The ribonucleic acid according to any of claims 1 to 18, wherein at least one nucleotide of the ribonucleic acid has a modification at the 2'-position and the modification is preferably selected from the group comprising amino, fluoro, methoxy, alkoxy and alkyl.
20. A ribonucleic acid comprising a double stranded structure, whereby the double-stranded structure comprises a first strand and a second strand, whereby the first strand comprises a first stretch of contiguous nucleotides and whereby said first stretch is at least partially complementary to a target nucleic acid, and the second strand comprises a second stretch of contiguous nucleotides and whereby said second stretch is at least partially identical to a target nucleic acid,

characterised in

that said first strand and/or said second strand comprises a plurality of groups of modified nucleotides having a modification at the 2'-position whereby within the strand each group of modified nucleotides is flanked on one or both sides by a flanking group of nucleotides whereby the flanking nucleotides forming the flanking group of

nucleotides is either an unmodified nucleotide or a nucleotide having a modification different from the modification of the modified nucleotides.

21. The ribonucleic acid according to claim 20, wherein the ribonucleic acid is the ribonucleic acid according to any of claims 1 to 19.
22. The ribonucleic acid according to claims 20 or 21, wherein said first strand and/or said second strand comprise said plurality of modified nucleotides.
23. The ribonucleic acid according to claims 20 or 21, wherein said first strand comprises said plurality of groups of modified nucleotides.
24. The ribonucleic acid according to claims 20 or 21, wherein said second strand comprises said plurality of groups of modified nucleotides.
25. The ribonucleic acid according to claims 20 or 24, wherein the group of modified nucleotides and/or the group of flanking nucleotides comprises a number of nucleotides whereby the number is selected from the group comprising one nucleotide to 10 nucleotides.
26. The ribonucleic acid according to any of claims 20 to 25, wherein the pattern of modified nucleotides of said first strand is the same as the pattern of modified nucleotides of said second strand.
27. The ribonucleic acid according to any of claims 20 to 26, wherein the pattern of said first strand aligns with the pattern of said second strand.
28. The ribonucleic acid according to any of claims 20 to 26, wherein the pattern of said first strand is shifted by one or more nucleotides relative to the pattern of the second strand.
29. The ribonucleic acid according to any of claims 20 to 28, wherein the modification is selected from the group comprising amino, fluoro, methoxy, alkoxy and alkyl.

30. The ribonucleic acid according to any of claims claim 20 to 29, wherein the double stranded structure is blunt ended.
31. The ribonucleic acid according to any of claims 20 to 30, wherein the double stranded structure is blunt ended on both sides.
32. The ribonucleic acid according to any of claims 20 to 31, wherein the double stranded structure is blunt ended on the double stranded structure which is defined by the 5'-end of the first strand and the 3'-end of the second strand.
33. The nucleic acid according to any of claims 20 to 32, wherein the double stranded structure is blunt ended on the double stranded structure which is defined by at the 3'-end of the first strand and the 5'-end of the second strand.
34. The ribonucleic acid according to claim 20 or 33, wherein at least one of the two strands has an overhang of at least one nucleotide at the 5'-end.
35. The nucleic acid according to claim 34, wherein the overhang consists of at least one desoxyribonucleotide.
36. The ribonucleic acid according to claim 34 or 35, wherein at least one of the strands has an overhang of at least one nucleotide at the 3'-end.
37. The ribonucleic acid according to any of claims 1 to 36, wherein the length of the double-stranded structure has a length from about 17 to 21 and more preferably 18 or 19 bases
38. The ribonucleic acid according to any of claims 1 to 37, wherein the length of said first strand and/or the length of said second strand is independently from each other selected from the group comprising the ranges of from about 15 to about 23 bases, 17 to 21 bases and 18 or 19 bases.
39. The ribonucleic acid according to any of claims 1 to 38, wherein the complementarity between said first strand and the target nucleic acid is perfect.

40. The ribonucleic acid according to any of claims 1 to 39, wherein the duplex formed between the first strand and the target nucleic acid comprises at least 15 nucleotides wherein there is one mismatch or two mismatches between said first strand and the target nucleic acid forming said double-stranded structure.
41. The ribonucleic acid according to any of claims 20 to 40, wherein
- both the first strand and the second strand each comprise at least one group of modified nucleotides and at least one flanking group of nucleotides,
- whereby each group of modified nucleotides comprises at least one nucleotide and whereby each flanking group of nucleotides comprising at least one nucleotide;
- with each group of modified nucleotides of the first strand being aligned with a flanking group of nucleotides on the second strand, whereby
- the most terminal 5' nucleotide of the first strand is a nucleotide of the group of modified nucleotides, and
- the most terminal 3' nucleotide of the second strand is a nucleotide of the flanking group of nucleotides.
42. The ribonucleic acid according to claim 41, wherein each group of modified nucleotides consists of a single nucleotide and/or each flanking group of nucleotides consists of a single nucleotide.
43. The ribonucleic acid according to claim 42, wherein on the first strand the nucleotide forming the flanking group of nucleotides is an unmodified nucleotide which is arranged in a 3' direction relative to the nucleotide forming the group of modified nucleotides, and

wherein on the second strand the nucleotide forming the group of modified nucleotides is a modified nucleotide which is arranged in 5' direction relative to the nucleotide forming the flanking group of nucleotides.

44. The ribonucleic acid according to any of claims 41 to 44, wherein the first strand comprises eight to twelve, preferably nine to eleven, groups of modified nucleotides, and wherein the second strand comprises seven to eleven, preferably eight to ten, groups of modified nucleotides.
45. The ribonucleic acid according to any of claims 1 to 44, wherein the target gene is selected from the group comprising structural genes, housekeeping genes, transcription factors, motility factors, cell cycle factors, cell cycle inhibitors, enzymes, growth factors, cytokines and tumor suppressors.
46. The ribonucleic acid according to any of claims 1 to 45, whereby the first strand and the second strand are linked by a loop structure.
47. The ribonucleic acid according to claim 46, wherein the loop structure is comprised of a non-nucleic acid polymer.
48. The ribonucleic acid according to claim 47, characterised in that the non-nucleic acid polymer is polyethylene glycol.
49. The ribonucleic acid according to any of claims 46 to 48, wherein the loop structure is comprised of a nucleic acid.
50. The ribonucleic acid according to any of claims 46 to 49, characterised in that the 5'-terminus of the first strand is linked to the 3'-terminus of the second strand.
51. The ribonucleic acid according to any of claims 46 to 50, characterised in that the 3'-end of the first strand is linked to the 5'-terminus of the second strand.
52. Use of a ribonucleic acid according to any of claims 1 to 51, for target validation.

53. Use of a ribonucleic acid according to any of claims 1 to 51 for the manufacture of a medicament.
54. The use according to claim 53, wherein the medicament is for the treatment of a disease or of a condition which is selected from the group comprising glioblastoma, prostate cancer, breast cancer, lung cancer, liver cancer, colon cancer, pancreatic cancer and leukaemia, diabetes, obesity, cardiovascular diseases, and metabolic diseases.
55. A cell, preferably a knockdown cell, containing a ribonucleic acid according to any of claims 1 to 51.
56. An organism, preferably a knockdown organism, containing a ribonucleic acid according to any of claims 1 to 51.
57. A composition containing a ribonucleic acid according to any of claims 1 to 51.
58. A pharmaceutical composition containing a ribonucleic acid according to any of claims 1 to 51 and a pharmaceutically acceptable carrier.
59. A method for inhibiting the expression of a target gene in a cell or derivative thereof comprising introducing a ribonucleic acid according to any of claims 1 to 47 into the cell in an amount sufficient to inhibit expression of the target gene, wherein the target gene is the target gene of the a ribonucleic acid according to any of claims 1 to 51.

Further novel forms of interfering RNA molecules

Abstract

The present invention is related to a ribonucleic acid comprising a double stranded structure whereby the double- stranded structure comprises a first strand and a second strand, whereby the first strand comprises a first stretch of contiguous nucleotides and whereby said first stretch is at least partially complementary to a target nucleic acid, and the second strand comprises a second stretch of contiguous nucleotides whereby said second stretch is at least partially identical to a target nucleic acid, and whereby the double stranded structure is blunt ended.

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Fig. 1

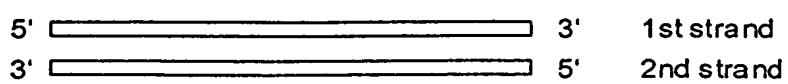


Fig. 2

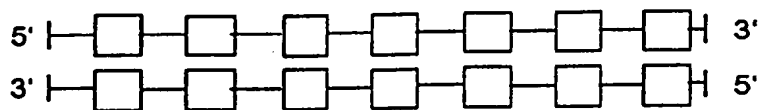


Fig. 2B

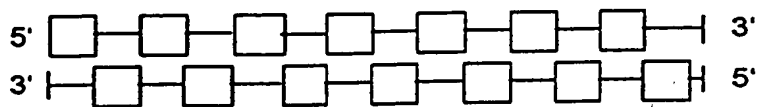


Fig. 2C

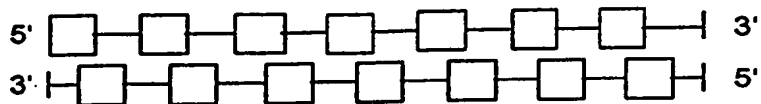
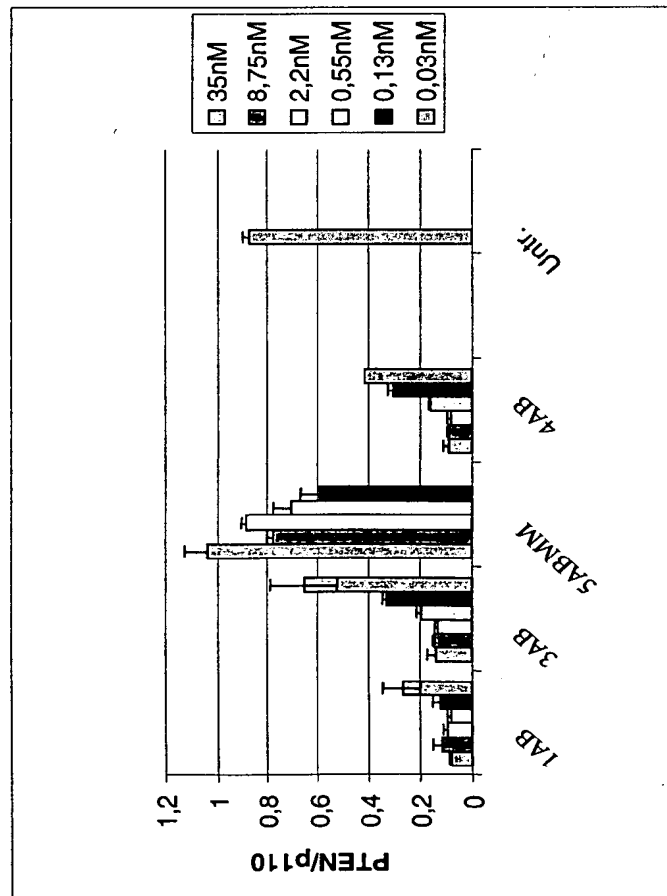


Fig.3



3A

ONLY RNA WITH 2 DEOXY AT EACH END
 1A 5'- cuccuuuuuuuugucuaacg-TT
 1B 3'-TT-gaggaacacaaagacgaugc

ONLY RNA WITH NH2 GROUPS AT EACH 3' END AND 2 DEOXY
 3A 5'- cuccuuuuuuuugucuaacg-TT-NH2
 3B 3'-NH2-TT-gaggaacacaaagacgaugc

ONLY RNA WITH INVERTED ABASIC AND 2 TT
 4. 16153-iB (iB at the 3' ends)
 4A 5'- cuccuuuuuuuugucuaacg-TT-iB
 4B 3'-iB-TT-gaggaacacaaagacgaugc

ONLY RNA WITH 2 DEOXY AT EACH END
 5A 5'- cucauuuuuuuugucucacg-TT
 5B 3'-TT-gaguaacacaaacacgagugc

3B

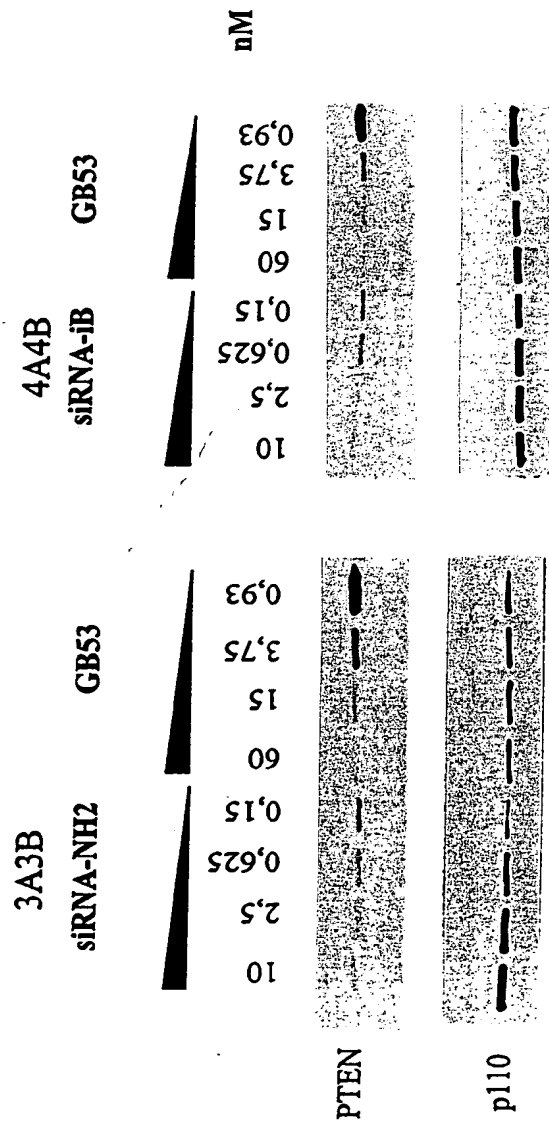
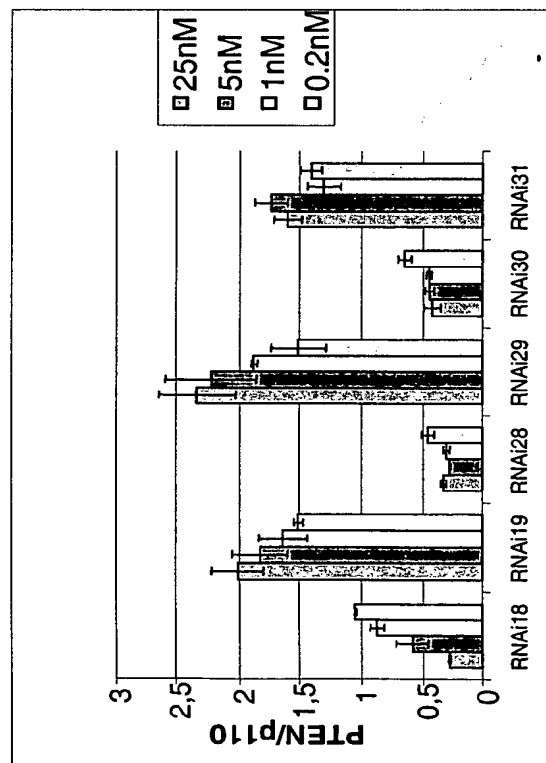


Fig. 3C

4A

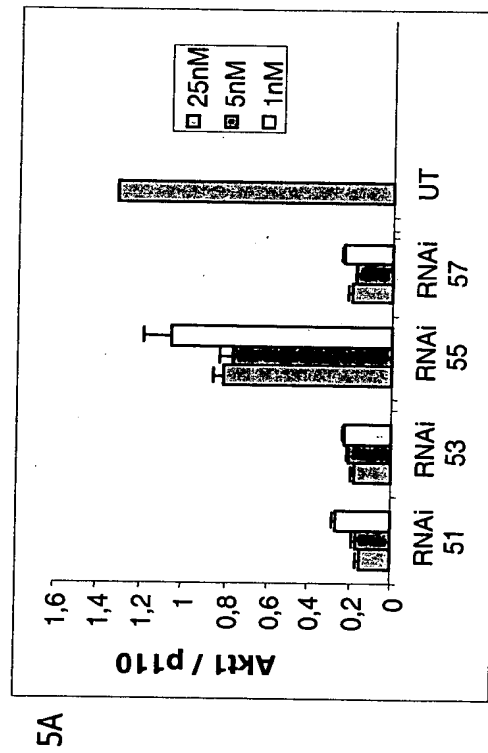


4B

18A	5'-	cuccuuuuuuuucugcuaacg-TT
18B	3'-TT-	gaggaacacaaagacgaugc
19A (MM)	5'-	cucauuuuuuuugugcucacg-TT
19B (MM)	3'-TT-	gaguaaaagaaacacgagugc
28A	5'-	cuccuuuuuuuucugcuaacg-
28B	3'-	gaggaaacacaaagacgaugc-
29A (MM)	5'-	cucauuuuuuuugugcucacg-
29B (MM)	3'-	gaguaaaagaaacacgagugc
30A	5'-TT-	cuccuuuuuuuucugcuaacg-
30B	3'-	-gaggaaacacaaagacgaugc-TT
31A (MM)	5'-TT-	cucauuuuuuuugugcucacg-
31B (MM)	3'-	-gaguaaaagaaacacgagugc-TT

Fig. 4

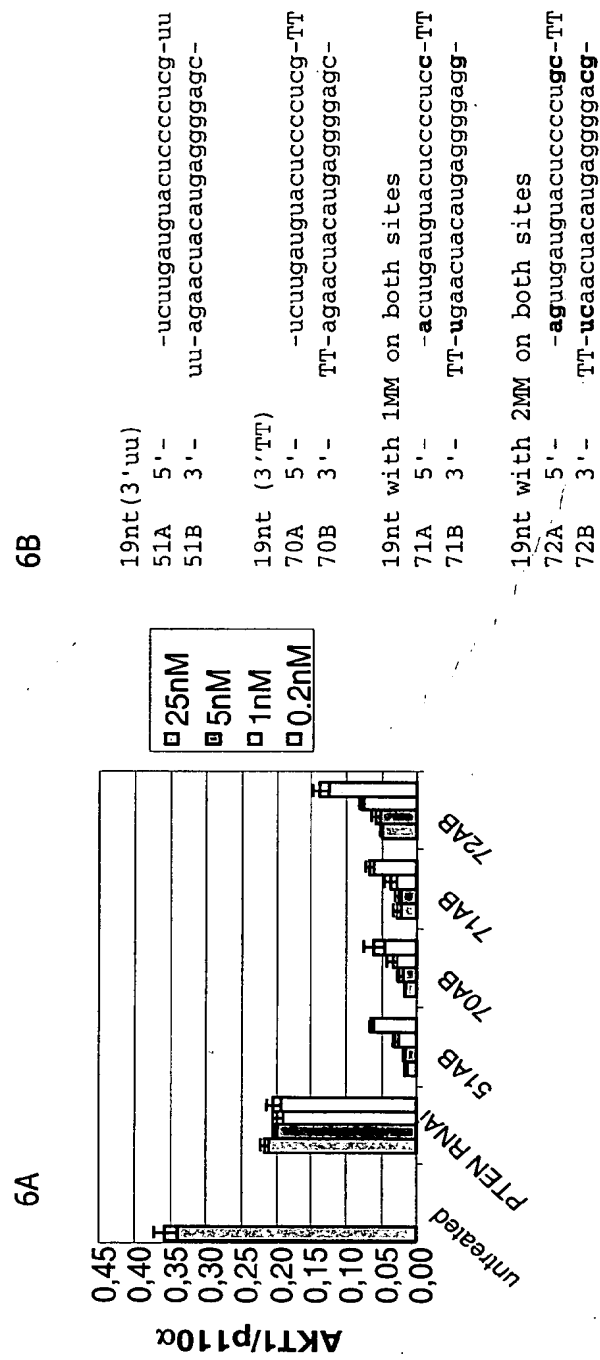
Fig. 5



5B

51A	5'-	-ucuugauguacuucccucg-uu	19nt 3'uu
51B	3'-	uu-agaacuacaugaggggagc-	
53A	5'-	-ucuugauguacucccucg-uu	19nt 3'seq.specific RNA
53B	3'-	cc-agaacuacaugaggggagc-	
55A	5'-	-cuugauguacuucccuc-gu	17nt 3'seq.specific RNA
55B	3'-	ca-gaacuacaugaggggag-	
57A	5'-	-ucuugauguacuucccucg-TT	19nt 3'seq.specific DNA
57B	3'-	CC-agaacuacaugaggggagc-	

Fig. 6



A

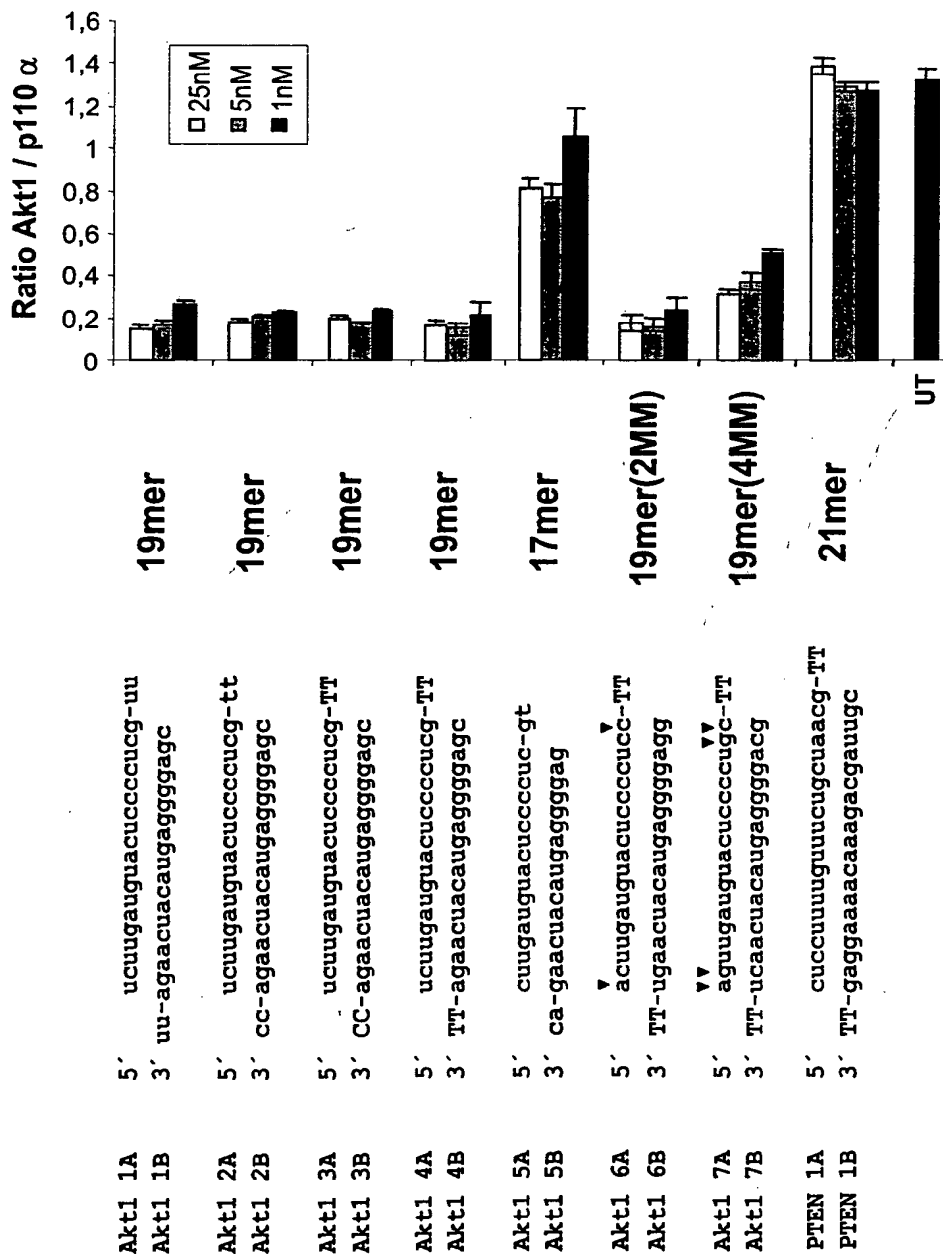


Fig. 7

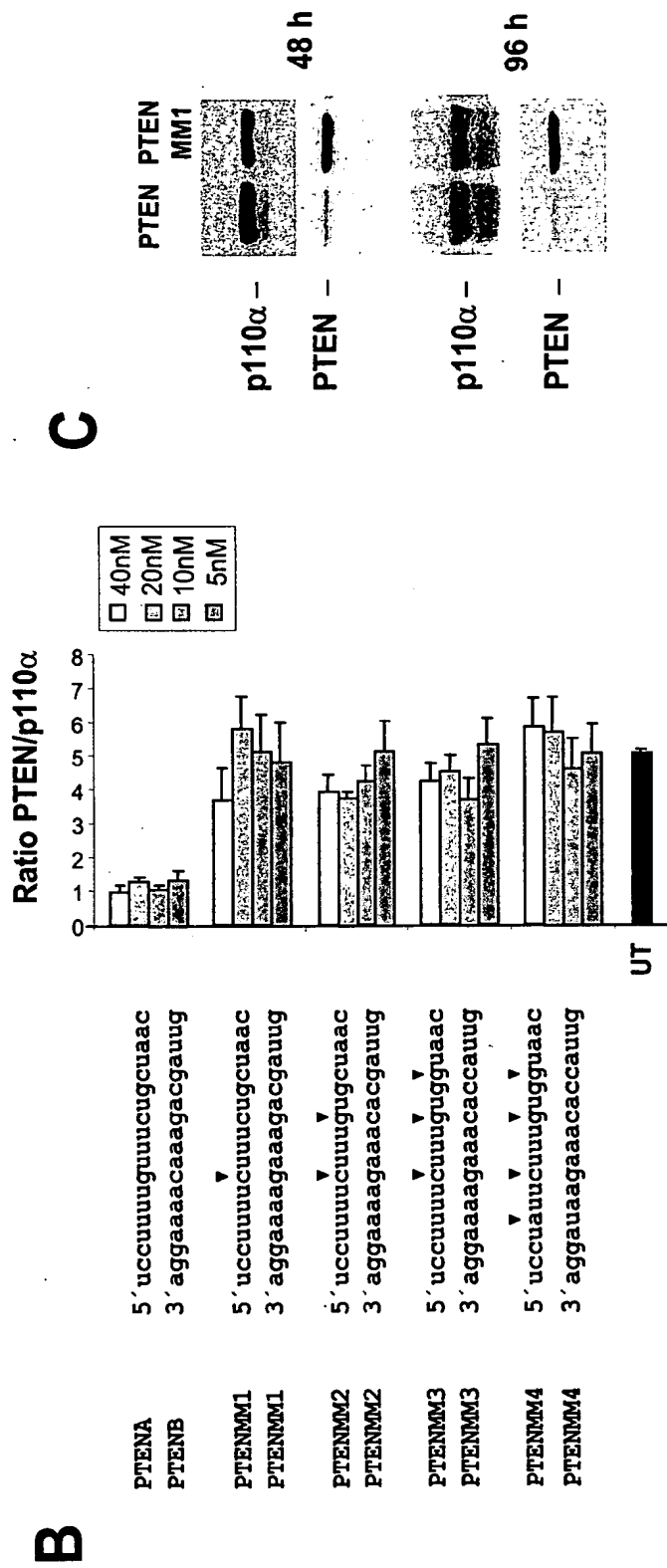


Fig. 7

Fig. 8'

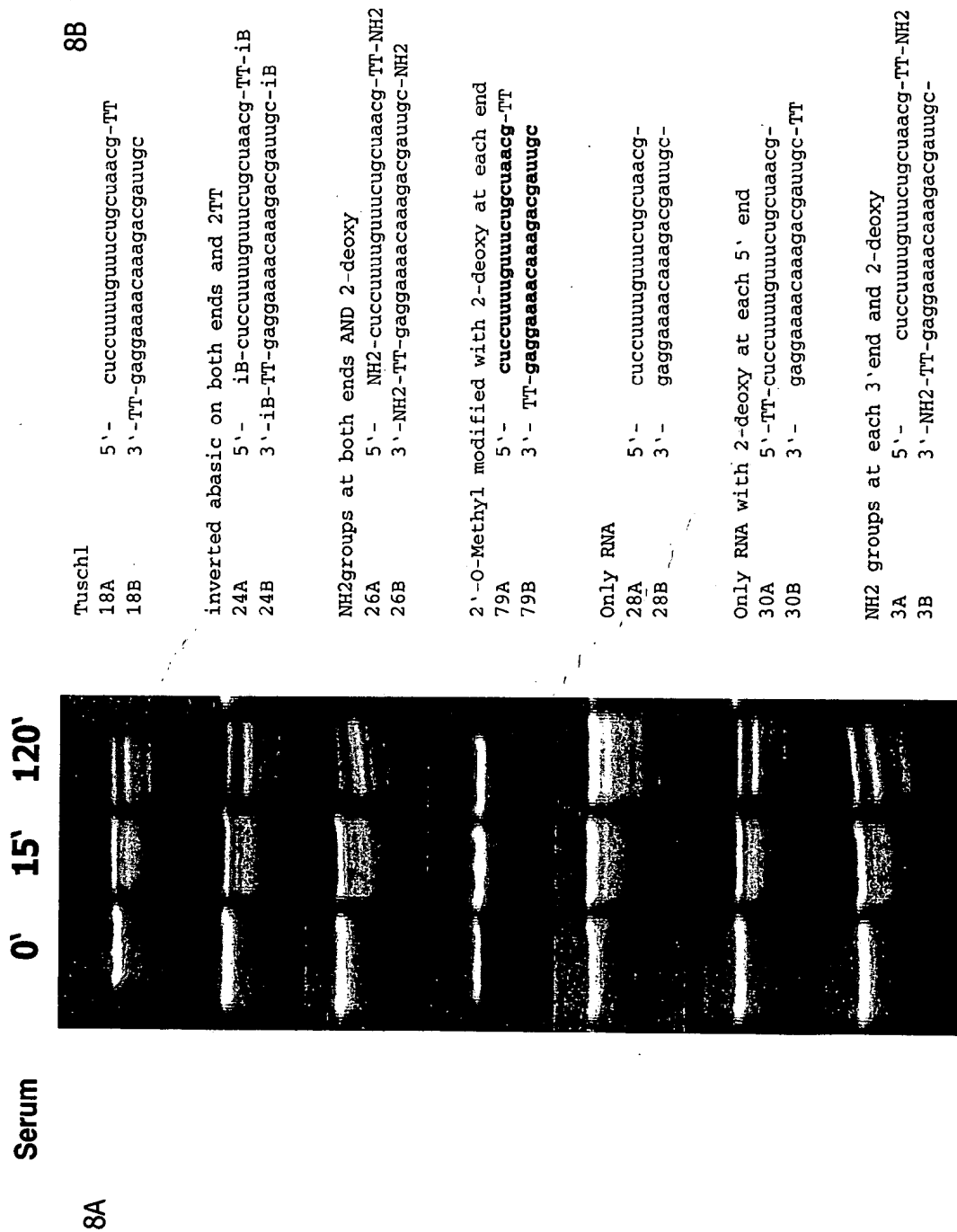
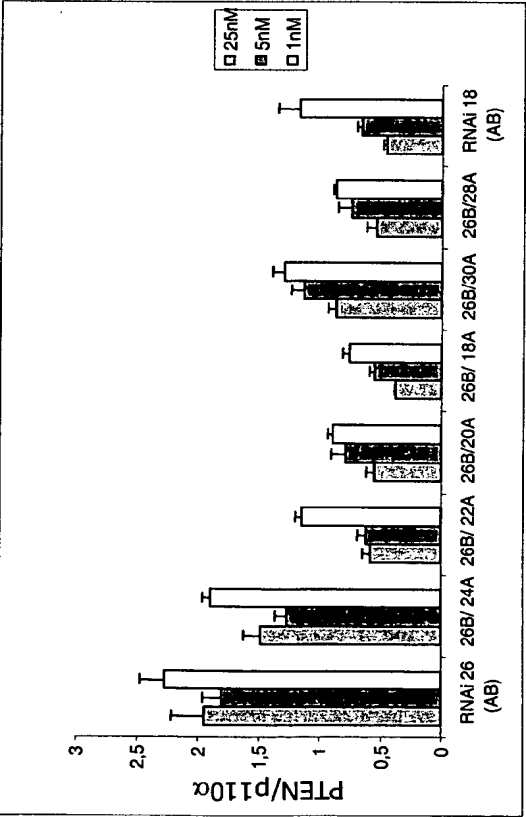


Fig. 9

9A



9B

26A	5'-	NH2-cuccuuuuuuuucugcuaacg-TT-NH2
26B	3'-	-NH2-TT-gaggaaaaaagacgauugc-NH2
24A	5'-	iB-cuccuuuuuuuucugcuaacg-TT-iB
26B	3'-	-NH2-TT-gaggaaaaaagacgauugc-NH2
22A	5'-	cuccuuuuuuuucugcuaacg-TT-iB
26B	3'-	-NH2-TT-gaggaaaaaagacgauugc-NH2
20A	5'-	cuccuuuuuuuucugcuaacg-TT-NH2
26B	3'-	-NH2-TT-gaggaaaaaagacgauugc-NH2
18A	5'-	cuccuuuuuuuucugcuaacg-TT
26B	3'-	-NH2-TT-gaggaaaaaagacgauugc-NH2
30A	5'-	TT-cuccuuuuuuuucugcuaacg-
26B	3'-	-NH2-TT-gaggaaaaaagacgauugc-NH2
28A	5'-	cuccuuuuuuuucugcuaacg-
26B	3'-	-NH2-TT-gaggaaaaaagacgauugc-NH2
18A	5'-	cuccuuuuuuuucugcuaacg-TT
18B	3'-	TT-gaggaaaaaagacgauugc

9C

-nnnnnnnnnnnnnnnn-tt		
NH2-nnnnnnnnnnnnnnn-NH2	functional	
-nnnnnnnnnnnnnnnn-tt-NH2		
NH2-nnnnnnnnnnnnnnn-NH2	functional	
NH2-nnnnnnnnnnnnnnn-tt-NH2		
tt-nnnnnnnnnnnnnnn-tt	not functional	

Fig. 10

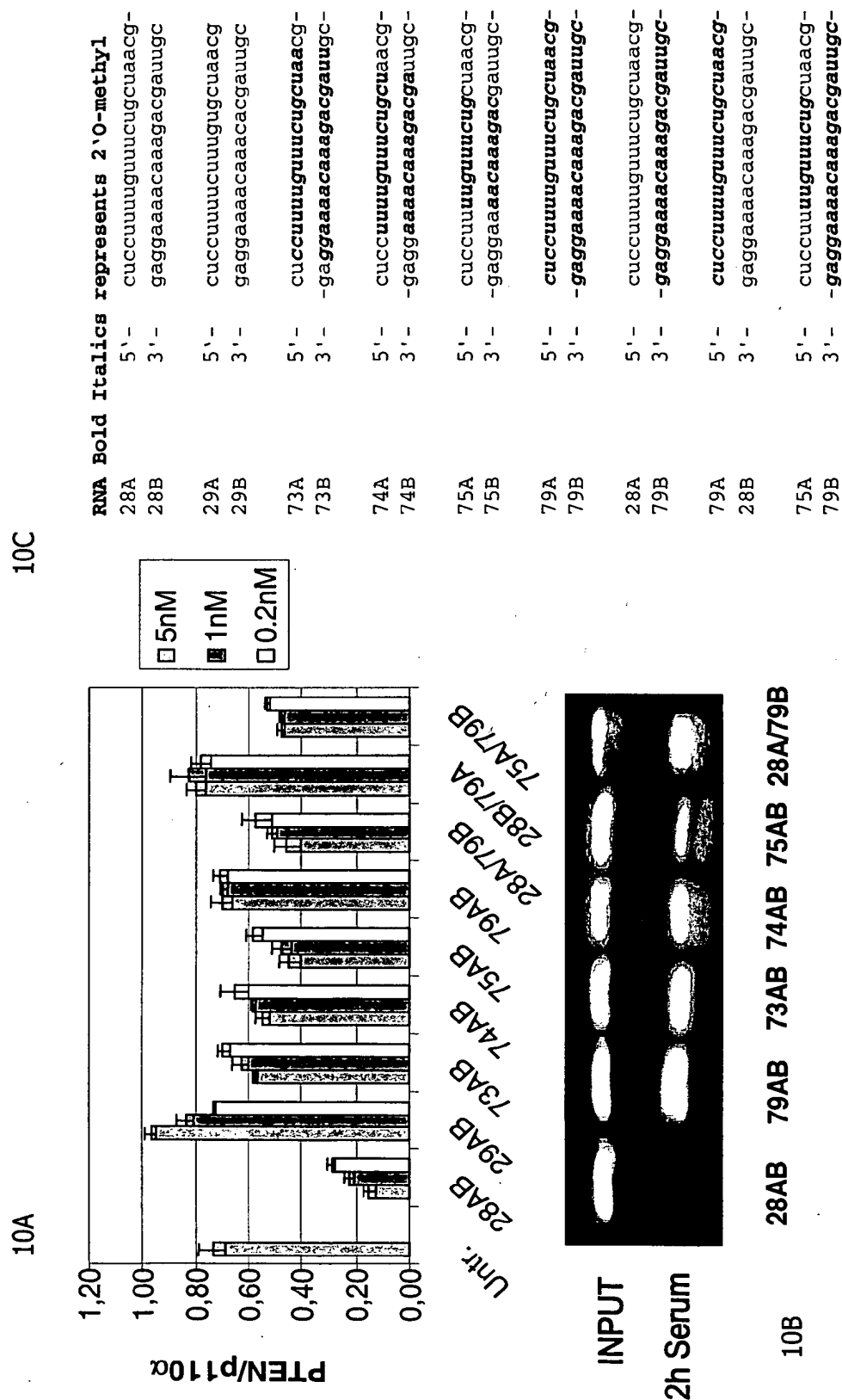


Fig. 11

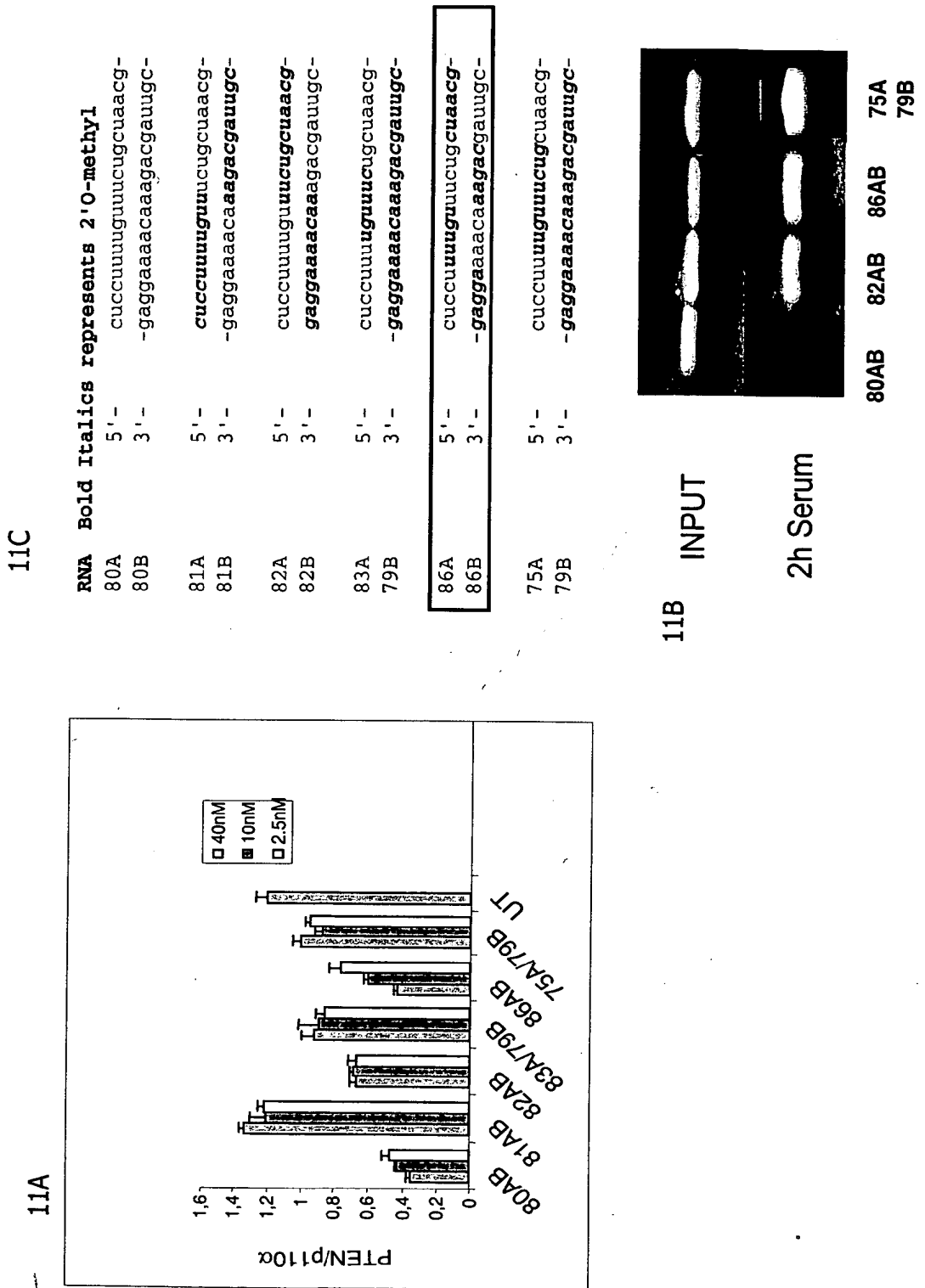


Fig. 12

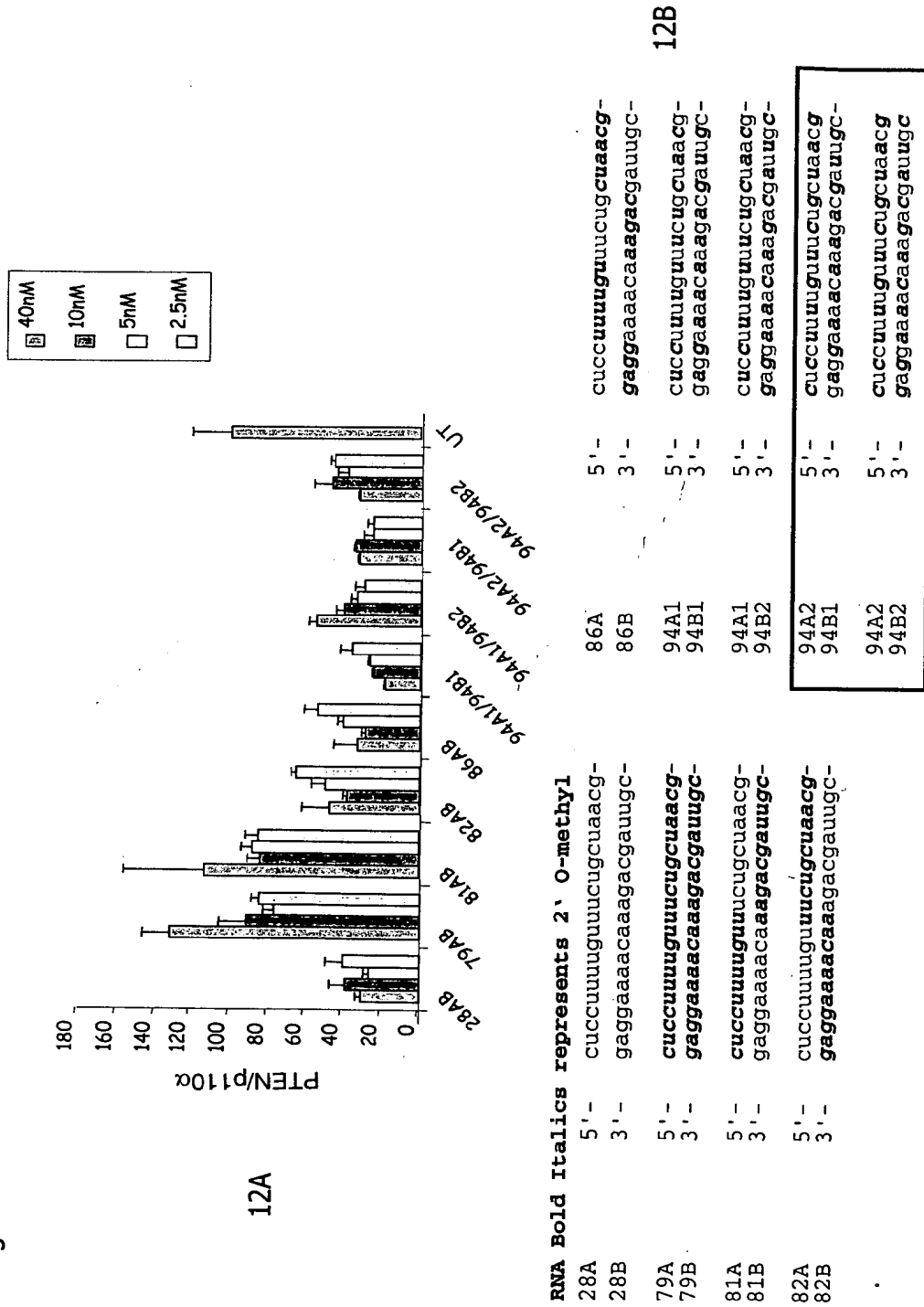


Fig. 12

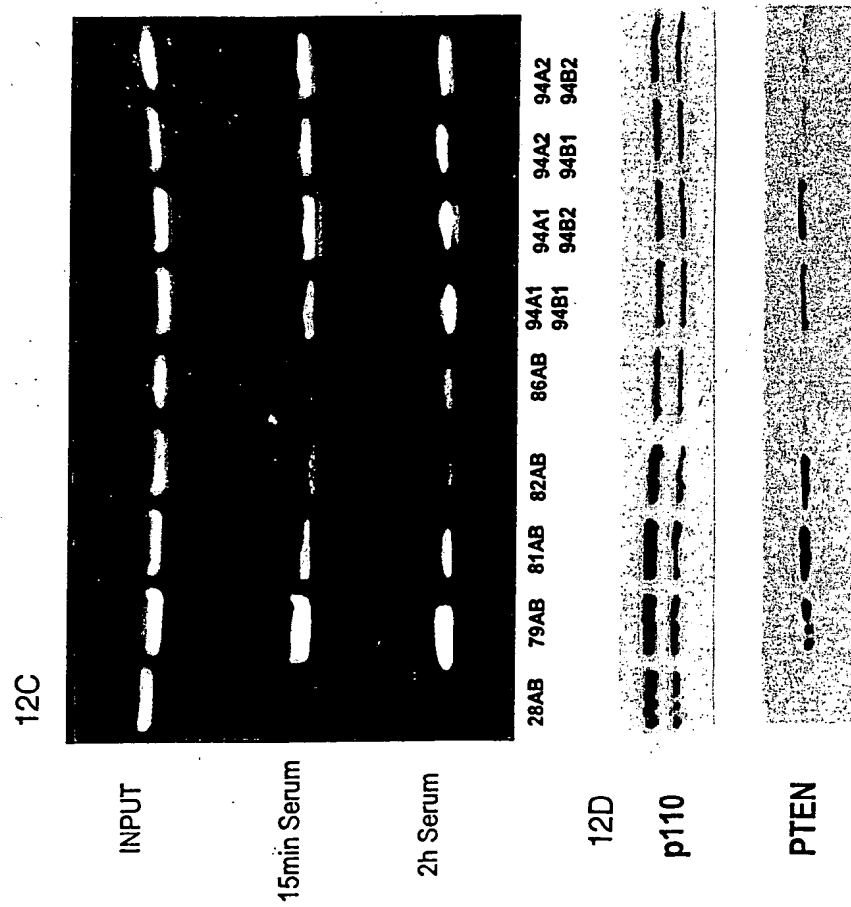
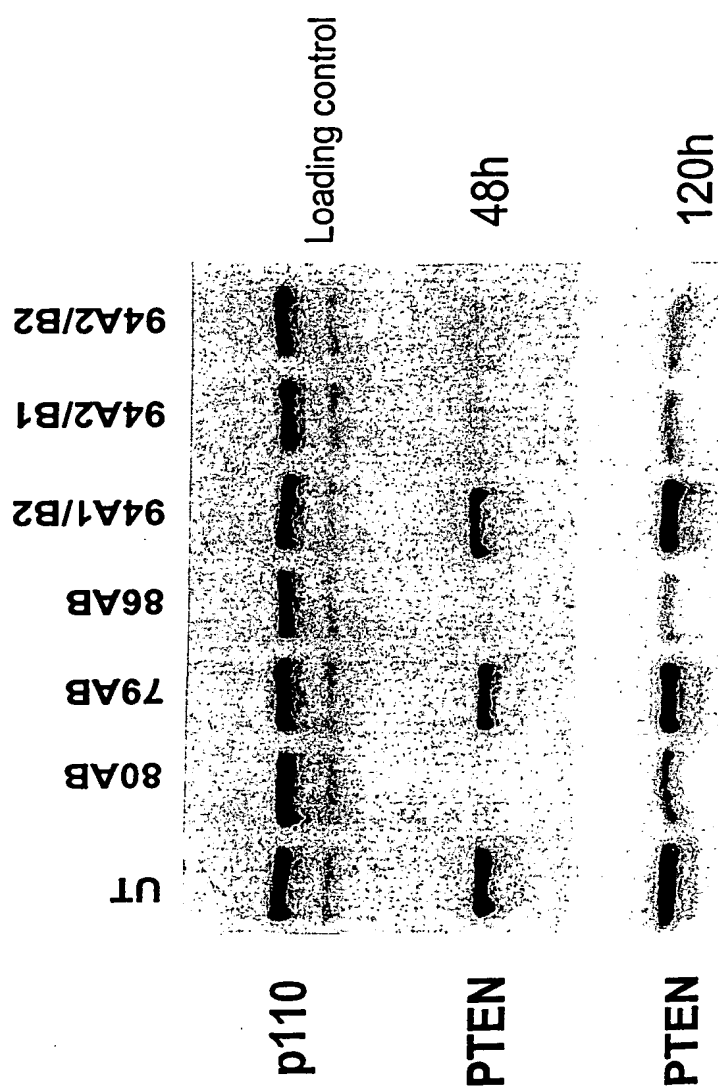


Fig. 13



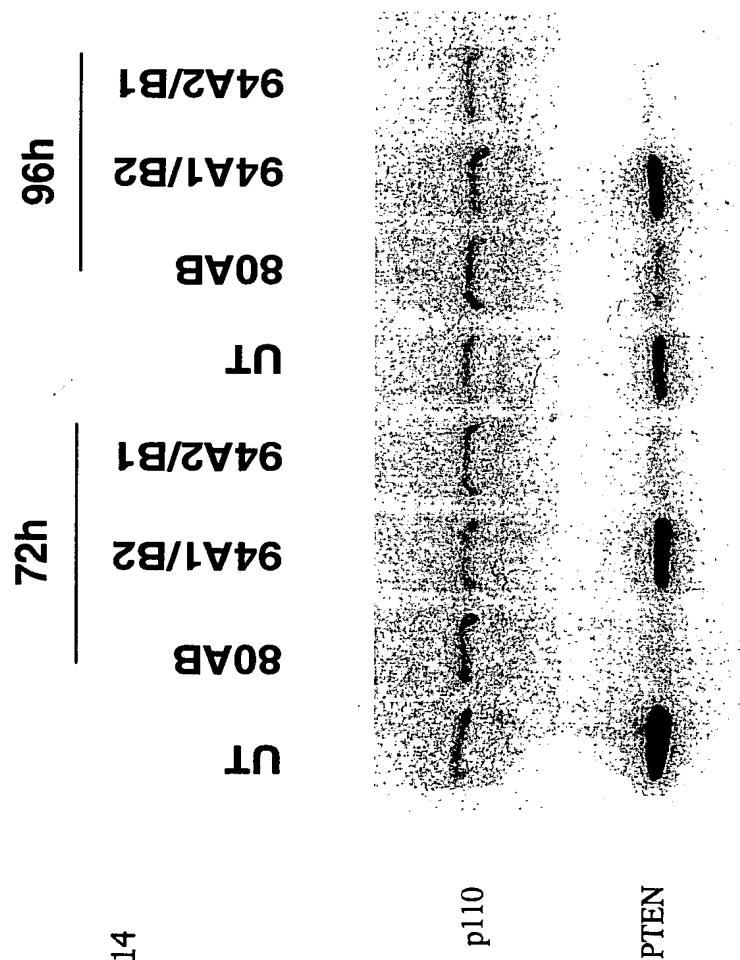


Fig. 14

A

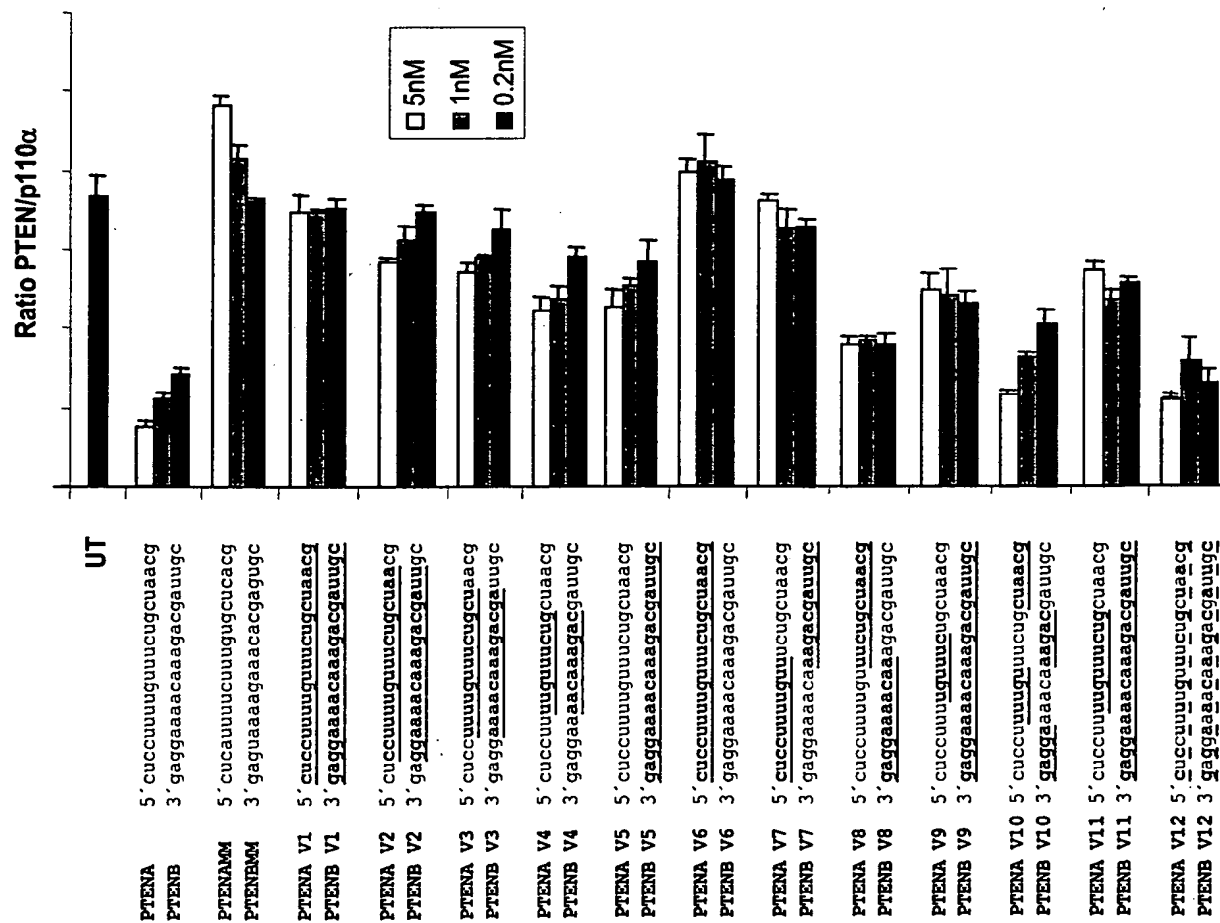


Fig. 15.

B

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PTENB 3'gaggaaaacaaagacgaungc

PTENA V1 5'cuccuuuuuuuuucugcuaacg
PTENB V1 3'gaggaaaacaaagacgaungc

PTENA V7 5'cuccuuuuuuuuucugcuaacg
PTENB V7 3'gaggaaaacaaagacgaungc

PTENA V8 5'cuccuuuuuuuuucugcuaacg
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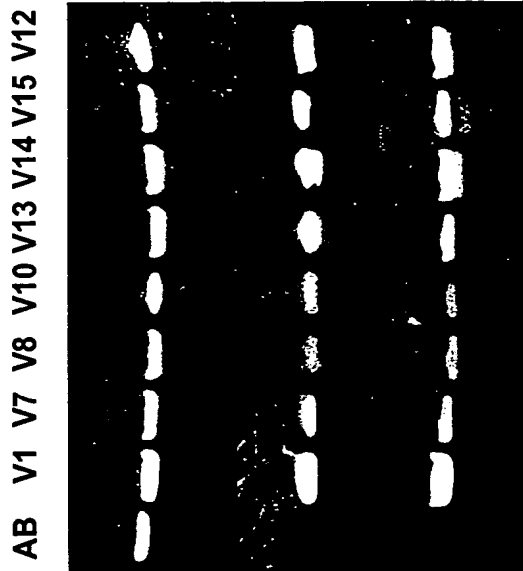
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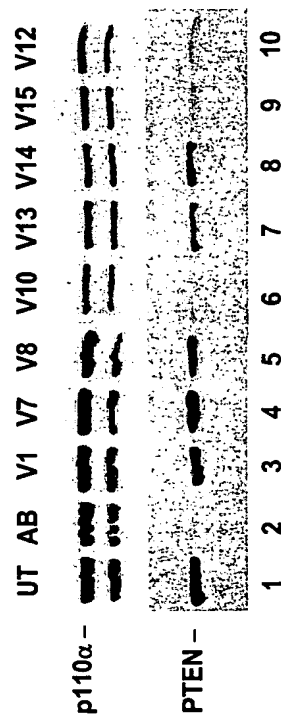
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C



D

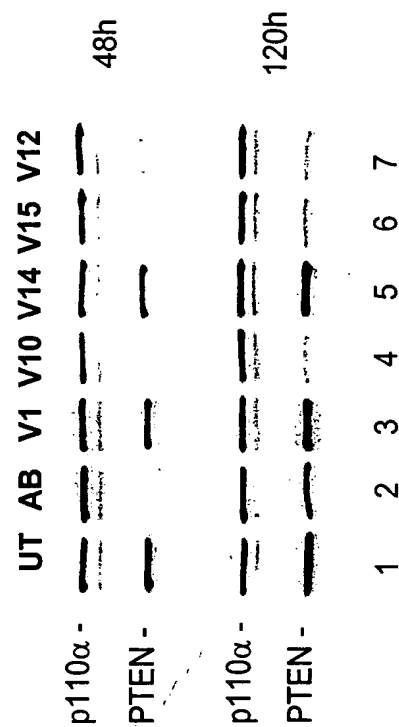
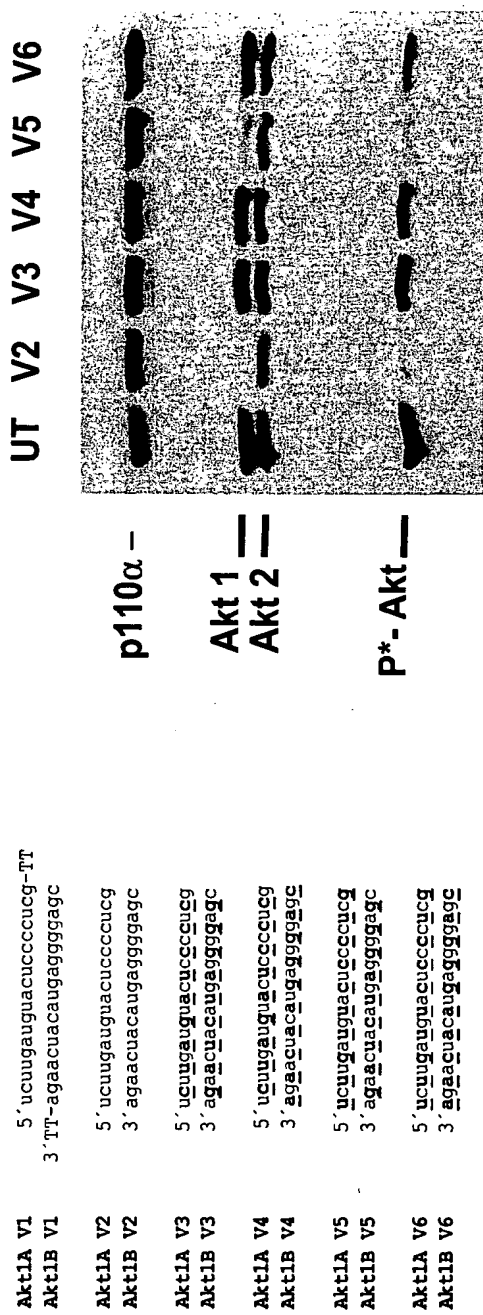


Fig. 15

A



B



Fig. 16

C

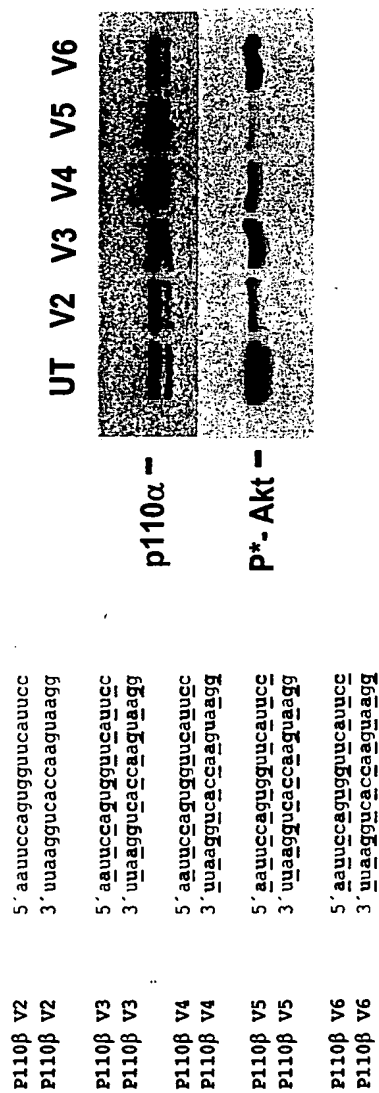


Fig. 16

Fig.17

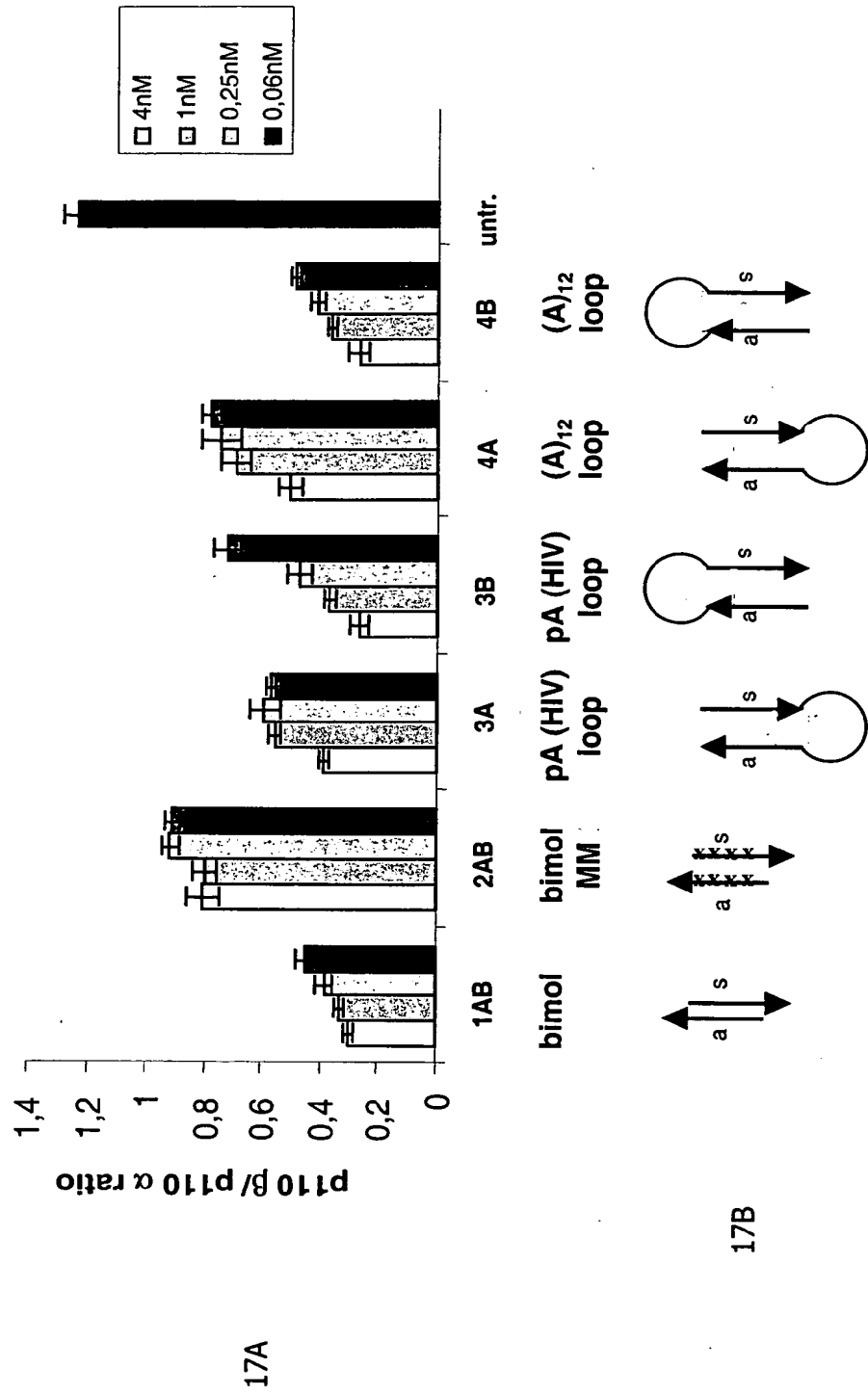
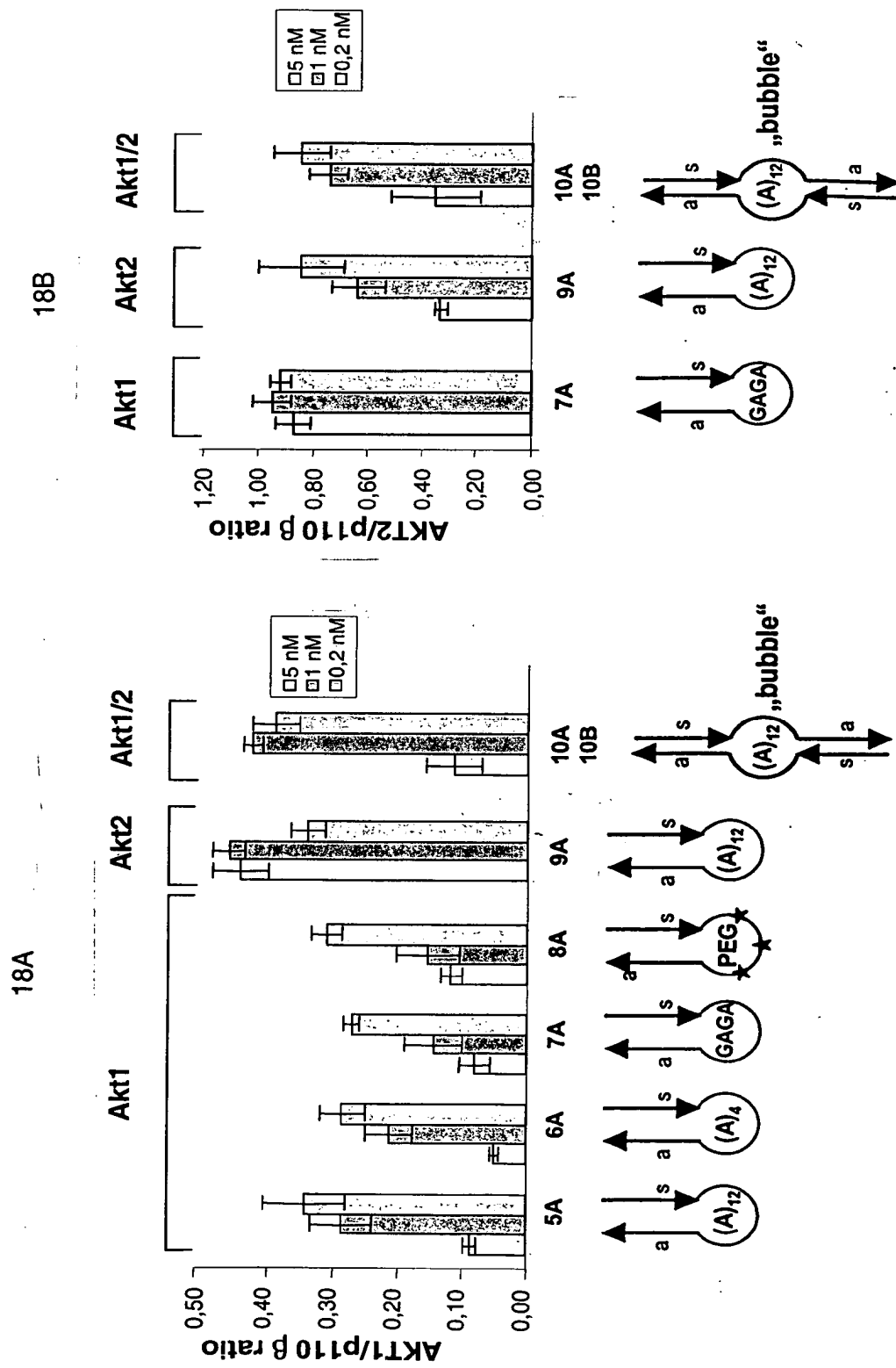
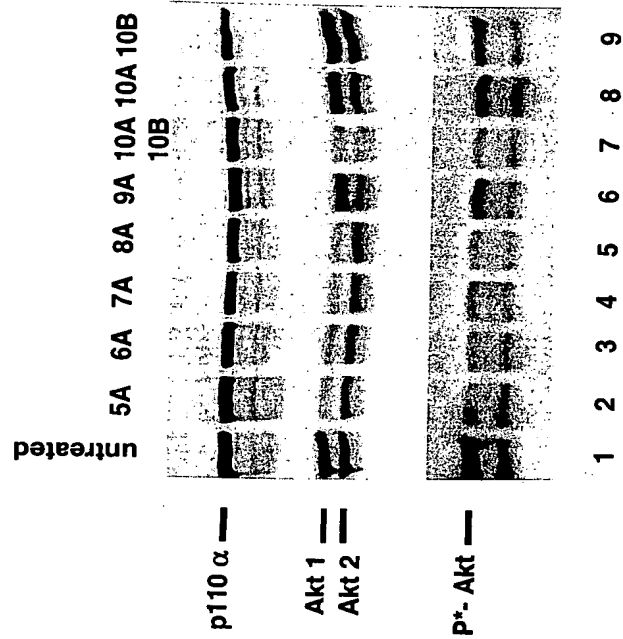


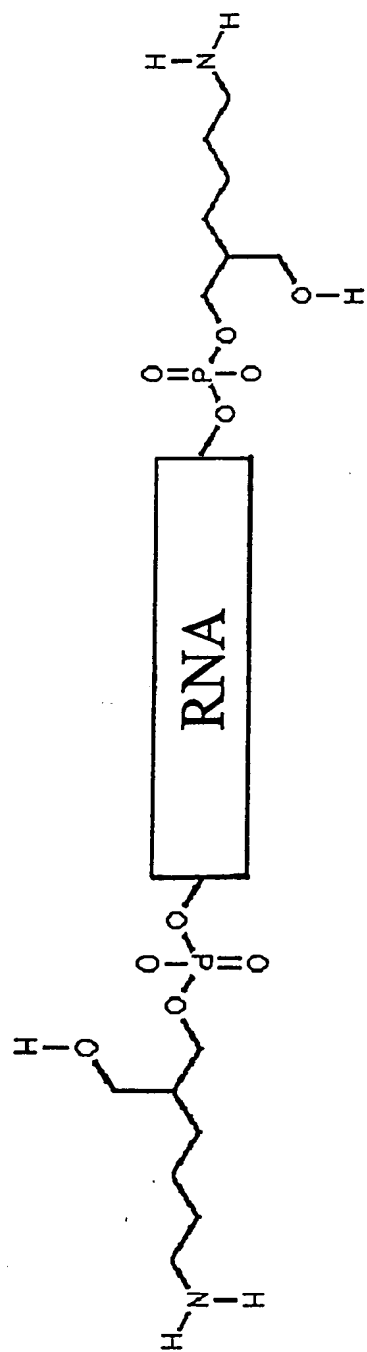
Fig. 18



18C



A



B

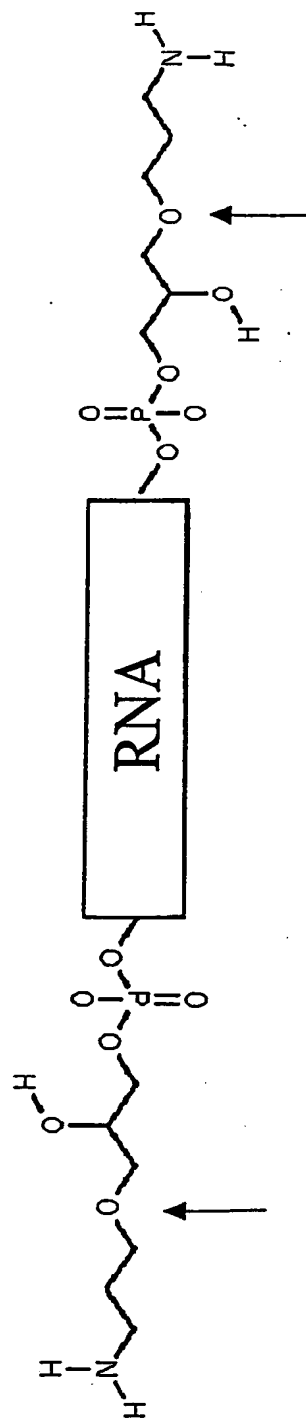


Fig. 19

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29
10. April 2003

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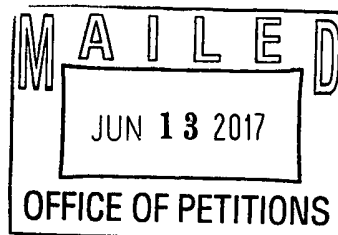
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Doc Code: TRACK1.GRANT

Decision Granting Request for Prioritized Examination (Track I or After RCE)	Application No.: 15/589,968
<p>1. THE REQUEST FILED <u>5/8/17</u> IS GRANTED.</p> <p>The above-identified application has met the requirements for prioritized examination</p> <p>A. <input checked="" type="checkbox"/> for an original nonprovisional application (Track I).</p> <p>B. <input type="checkbox"/> for an application undergoing continued examination (RCE).</p> <p>2. The above-identified application will undergo prioritized examination. The application will be accorded special status throughout its entire course of prosecution until one of the following occurs:</p> <p>A. filing a <u>petition for extension of time</u> to extend the time period for filing a reply;</p> <p>B. filing an <u>amendment to amend the application to contain more than four independent claims, more than thirty total claims</u>, or a multiple dependent claim;</p> <p>C. filing a <u>request for continued examination</u>;</p> <p>D. filing a notice of appeal;</p> <p>E. filing a request for suspension of action;</p> <p>F. mailing of a notice of allowance;</p> <p>G. mailing of a final Office action;</p> <p>H. completion of examination as defined in 37 CFR 41.102; or</p> <p>I. abandonment of the application.</p> <p>Telephone inquiries with regard to this decision should be directed to Cheryl Gibson-Baylor at (571)272-3213, Office of Petitions. In his/her absence, calls may be directed to Brian W. Brown, (571)272-5338.</p> <p>Cheryl Gibson-Baylor <u>/Cheryl Gibson-Baylor/</u> [Signature]</p> <p><u>Petitions Paralegal Specialist</u> (Title)</p>	



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APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
15/589,968	05/08/2017	Klaus GIESE	680132000106	8531

25226 7590 06/21/2017
MORRISON & FOERSTER LLP
755 PAGE MILL RD
PALO ALTO, CA 94304-1018

EXAMINER
CHONG, KIMBERLY

ART UNIT	PAPER NUMBER
1674	

NOTIFICATION DATE	DELIVERY MODE
06/21/2017	ELECTRONIC

Please find below and/or attached an Office communication concerning this application or proceeding.

The time period for reply, if any, is set in the attached communication.

Notice of the Office communication was sent electronically on above-indicated "Notification Date" to the following e-mail address(es):

EOfficePA@mofo.com
PatentDocket@mofo.com
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Office Action Summary	Application No. 15/589,968	Applicant(s) GIESE ET AL.	
	Examiner KIMBERLY CHONG	Art Unit 1674	AIA (First Inventor to File) Status No

-- The MAILING DATE of this communication appears on the cover sheet with the correspondence address --

Period for Reply

A SHORTENED STATUTORY PERIOD FOR REPLY IS SET TO EXPIRE 3 MONTHS FROM THE MAILING DATE OF THIS COMMUNICATION.

- Extensions of time may be available under the provisions of 37 CFR 1.136(a). In no event, however, may a reply be timely filed after SIX (6) MONTHS from the mailing date of this communication.
- If NO period for reply is specified above, the maximum statutory period will apply and will expire SIX (6) MONTHS from the mailing date of this communication.
- Failure to reply within the set or extended period for reply will, by statute, cause the application to become ABANDONED (35 U.S.C. § 133). Any reply received by the Office later than three months after the mailing date of this communication, even if timely filed, may reduce any earned patent term adjustment. See 37 CFR 1.704(b).

Status

- 1) ☒ Responsive to communication(s) filed on 05/08/2017.
☐ A declaration(s)/affidavit(s) under **37 CFR 1.130(b)** was/were filed on _____.
- 2a) ☐ This action is **FINAL**. 2b) ☒ This action is non-final.
- 3) ☐ An election was made by the applicant in response to a restriction requirement set forth during the interview on _____; the restriction requirement and election have been incorporated into this action.
- 4) ☐ Since this application is in condition for allowance except for formal matters, prosecution as to the merits is closed in accordance with the practice under *Ex parte Quayle*, 1935 C.D. 11, 453 O.G. 213.

Disposition of Claims*

- 5) ☒ Claim(s) 1-30 is/are pending in the application.
5a) Of the above claim(s) _____ is/are withdrawn from consideration.
- 6) ☐ Claim(s) _____ is/are allowed.
- 7) ☒ Claim(s) 1-30 is/are rejected.
- 8) ☐ Claim(s) _____ is/are objected to.
- 9) ☐ Claim(s) _____ are subject to restriction and/or election requirement.

* If any claims have been determined allowable, you may be eligible to benefit from the **Patent Prosecution Highway** program at a participating intellectual property office for the corresponding application. For more information, please see http://www.uspto.gov/patents/init_events/pph/index.jsp or send an inquiry to PPHfeedback@uspto.gov.

Application Papers

- 10) ☐ The specification is objected to by the Examiner.
- 11) ☒ The drawing(s) filed on 05/08/2017 is/are: a) ☒ accepted or b) ☐ objected to by the Examiner.
Applicant may not request that any objection to the drawing(s) be held in abeyance. See 37 CFR 1.85(a).
Replacement drawing sheet(s) including the correction is required if the drawing(s) is objected to. See 37 CFR 1.121(d).

Priority under 35 U.S.C. § 119

- 12) ☒ Acknowledgment is made of a claim for foreign priority under 35 U.S.C. § 119(a)-(d) or (f).

Certified copies:

- a) ☒ All b) ☐ Some** c) ☐ None of the:
1. ☐ Certified copies of the priority documents have been received.
 2. ☒ Certified copies of the priority documents have been received in Application No. 10/633,630.
 3. ☐ Copies of the certified copies of the priority documents have been received in this National Stage application from the International Bureau (PCT Rule 17.2(a)).

** See the attached detailed Office action for a list of the certified copies not received.

Attachment(s)

- | | |
|--|---|
| 1) <input checked="" type="checkbox"/> Notice of References Cited (PTO-892) | 3) <input type="checkbox"/> Interview Summary (PTO-413)
Paper No(s)/Mail Date. _____ |
| 2) <input checked="" type="checkbox"/> Information Disclosure Statement(s) (PTO/SB/08a and/or PTO/SB/08b)
Paper No(s)/Mail Date <u>05/08/2017</u> . | 4) <input type="checkbox"/> Other: _____ |

The present application is being examined under the pre-AIA first to invent provisions.

DETAILED ACTION

Status of the Application

Claims 1-30 are pending and are currently under examination.

Information Disclosure Statement

The submission of the Information Disclosure Statement on 05/08/2017 is in compliance with 37 CFR 1.97. The information disclosure statement has been considered by the examiner and signed copies have been placed in the file.

Claim Rejections - 35 USC § 102

The following is a quotation of the appropriate paragraphs of pre-AIA 35 U.S.C. 102 that form the basis for the rejections under this section made in this Office action:

A person shall be entitled to a patent unless –

(e) the invention was described in (1) an application for patent, published under section 122(b), by another filed in the United States before the invention by the applicant for patent or (2) a patent granted on an application for patent by another filed in the United States before the invention by the applicant for patent, except that an international application filed under the treaty defined in section 351(a) shall have the effects for purposes of this subsection of an application filed in the United States only if the international application designated the United States and was published under Article 21(2) of such treaty in the English language.

Claims 1-11, 14, 15 and 20-23 are rejected under pre-AIA 35 U.S.C. 102(e) as being anticipated by McSwiggen et al. (US 2003/0190635 of record cited on IDS filed 05/08/2017).

The instant claims are drawn to a nucleic acid molecule comprising a first and second strand comprising a first and second stretch wherein the first and second strand form a double-stranded structure comprising a first stretch and a second stretch, wherein said nucleic acid is blunt ended on a least one end or both ends, wherein the first stretch and second stretch each comprise contiguous alternating modified ribonucleotide, wherein the first strand is shifted by one nucleotide relative to the pattern of modification on the second strand and wherein the first stretch and second stretch consists of 17 and fewer than 30 ribonucleotides.

The claims recite each stretch "comprise contiguous alternating modified ribonucleotides" and this is interpreted such that each stretch does not necessarily have all nucleotides in an alternating pattern and thus a strand with a stretch of 2 nucleotides wherein one nucleotide is unmodified or differently modified with a second contiguous nucleotide would meet the claim limitation.

McSwiggen et al. discloses a ribonucleic acid comprising a double stranded structure wherein the first and second strands comprise a first stretch of contiguous nucleotides that are at least partially or perfectly complementary to target molecule (see paragraph 0017 and 0121) and wherein the double stranded structure is between 19-25 nucleotides (see paragraph 0035) and teach the double stranded nucleic acid can optionally comprise overhang regions on one or both strands (see paragraph 0019) and thus teach a structure that is blunt ended on one or both ends.

McSwiggen et al. further discloses each strand of the double stranded structure comprise contiguous alternating modified ribonucleotides wherein the alternating

modified ribonucleotides alternate with unmodified or differently modified ribonucleotides and are for example 2'-O-methyl or 2'-fluoro nucleotides (see Figure 5). McSwiggen et al. teach in Figure 5B, the pattern of modification on the first strand is shifted by one nucleotide relative to the pattern of modification on the second strand, meeting the limitation of claim 1.

Moreover, McSwiggen et al. teach, in at least Figure 5, the first stretch and second stretch each comprise contiguous alternating single 2'-O-alkyl modified nucleotides that alternate with unmodified ribonucleotides (see bolded nucleotides of SEQ ID No. 665/666 below) wherein the 2'-O-alkyl is shifted by one ribonucleotide relative to the 2'-O-alkyl modified ribonucleotide of the second strand (see grey highlighted nucleotides of SEQ ID No. 665/666 below wherein lower case is 2'-O-methyl and upper case is unmodified). The structure below illustrates the 5' ribonucleotide on the first stretch is a 2'-O-methyl ribonucleotide and alternates with an unmodified nucleotide (claims 22 and 23).

cAuGGcuGGccAuc**u**GGCGccTT

GuAccGAcGG**u****A**GAcGGG

Thus, McSwiggen et al. anticipates claims 1-11, 14, 15 and 20-23 of the instant application.

Claim Rejections - 35 USC § 103

The following is a quotation of pre-AIA 35 U.S.C. 103(a) which forms the basis for all obviousness rejections set forth in this Office action:

(a) A patent may not be obtained though the invention is not identically disclosed or described as set forth in section 102 of this title, if the differences between the subject matter sought to be patented and the prior art are such that the subject matter as a whole would have been obvious at the time the invention was made to a person having ordinary skill in the art to which said subject matter pertains. Patentability shall not be negated by the manner in which the invention was made.

Claims 1-30 are rejected under pre-AIA 35 U.S.C. 103(a) as being obvious over McSwiggen et al (US 2003/0190635 of record cited on IDS filed 05/08/2017), Arnold et al (USPN 6,262,036), Damha et al (US 2005/0142535) and McKay et al (USPN 6,133,246).

The factual inquiries set forth in *Graham v. John Deere Co.*, 383 U.S. 1, 148 USPQ 459 (1966), that are applied for establishing a background for determining obviousness under pre-AIA 35 U.S.C. 103(a) are summarized as follows:

1. Determining the scope and contents of the prior art.
2. Ascertaining the differences between the prior art and the claims at issue.
3. Resolving the level of ordinary skill in the pertinent art.
4. Considering objective evidence present in the application indicating obviousness or nonobviousness.

The instant claims are drawn to a nucleic acid molecule comprising a first and second strand comprising a first and second stretch wherein the first and second strand form a double-stranded structure comprising a first stretch and a second stretch, wherein said nucleic acid is blunt ended on a least one end or both ends, wherein the

first stretch and second stretch each comprise contiguous alternating modified ribonucleotide, wherein the first strand is shifted by one nucleotide relative to the pattern of modification on the second strand and wherein the first stretch and second stretch consists of 17 and fewer than 30 ribonucleotides.

McSwiggen et al. discloses a ribonucleic acid comprising a double stranded structure wherein the first and second strands comprise a first stretch of contiguous nucleotides that are at least partially or perfectly complementary to target molecule (see paragraph 0017 and 0121) and wherein the double stranded structure is between 19-25 nucleotides (see paragraph 0035) and teach the double stranded nucleic acid can optionally comprise overhang regions on one or both strands (see paragraph 0019) and thus teach a structure that is blunt ended on one or both ends.

McSwiggen et al. further discloses each strand of the double stranded structure comprise contiguous alternating modified ribonucleotides wherein the alternating modified ribonucleotides alternate with unmodified or differently modified ribonucleotides and are for example 2'-O-methyl or 2'-fluoro nucleotides (see Figure 5). McSwiggen et al. teach in Figure 5B, the pattern of modification on the first strand is shifted by one nucleotide relative to the pattern of modification on the second strand, meeting the limitation of claims 17 and 21. Moreover, McSwiggen et al. teach, in at least Figure 5, the first stretch and second stretch each comprise contiguous alternating single 2'-O-alkyl modified nucleotides that alternate with unmodified ribonucleotides (see bolded nucleotides of SEQ ID No. 665/666 below) wherein the 2'-O-alkyl is shifted by one ribonucleotide relative to the 2'-O-alkyl modified ribonucleotide of the second

strand (see gray highlighted nucleotides of SEQ ID No. 665/666 above wherein lower case is 2'-O-methyl and upper case is unmodified).

McSwiggen et al. do not illustrate a double stranded structure comprising alternating modified ribonucleotides wherein the 2'-O-methyl alternates with a differently modified ribonucleotide such as a 2'-O- fluoro ribonucleotide.

Arnold et al teach antisense oligonucleotides comprising alternating 2'-modified nucleosides, and the introduction of these modifications for enhancing target binding stability (see esp. example 34, col. 48-50).

Damha et al teach inhibitory oligonucleotides, including those comprising alternating 2'-modified nucleosides in antisense oligonucleotides for enhancing target binding by antisense molecules for the binding and inhibition of target gene expression.

McKay et teach the inhibition of expression of a target gene using inhibitory oligonucleotides comprising numerous motifs and combinations of modified residues within antisense oligonucleotides, including the incorporation of 2'-modified sugars which include 2'-fluoro, 2'-bromo, 2'-O-alkyl groups, modified nucleobases, modified internucleotide linkages, 2'-O-D-deoxynucleosides and combinations thereof, as well as the optimization of modifications for maximizing target binding, cellular uptake and oligonucleotide stability, as well as inhibitory function of antisense (see esp. col. 7-12; Tables 4-26, esp. Tables 11 and 12, and Table 26).

It would have been obvious to utilize siRNA oligonucleotides of the size range instant claimed for target gene inhibition, wherein the oligonucleotides incorporate alternating 2'-modifications, including fluoro and methoxy, groups, because McSwiggen

Art Unit: 1674

taught various configurations for optimizing stability and siRNA activity. Alternating motifs were well known in the art of both siRNA and antisense technology, and the ability to incorporate the instant modifications and motifs, as well as the ability to test for changes in target gene inhibition as a function of modifications were all well known in the art and involved routine experimentation.

One of ordinary skill in the art would have incorporated alternating 2'-modified nucleosides into RNAi molecules taught by McSwiggen, including the well-known modifications comprising 2'-fluoro and 2'-O-methyl modified sugars in oligonucleotides including antisense and siRNA molecules, because it was well known at the time of the invention that alternating various modifications within a motif, including alternating modified 2' residues, enhanced target binding stability of the antisense oligonucleotides for their target regions, as taught previously by Arnold and Damha.

One of ordinary skill would have expected that the incorporation of the well-known modifications comprising 2'-fluoro and 2'-O-methyl, along with the previously disclosed other various modifications, are optimized using routine experimentation because Damha, McKay and Arnold all teach optimization experiments where antisense oligonucleotides comprising an array of different combinations of these well-known modifications are tested for their ability to target and bind target genes and inhibit their expression, the ability to incorporate the modifications claimed were well known in the art, and testing different motifs was very routine at the time the instant invention was made.

One of ordinary skill in the art would have reasonably expected that the siRNA molecules comprising the well-known alternating motif as instantly claimed, would provide for enhanced stability and provide for target gene cleavage in the presence of an appropriate target gene sequence. One of ordinary skill in the art would have designed and tested such modification motifs because it was well known in the art at the time of the instant invention that incorporation of 2'-O-methoxy alky or 2'-deoxy, or 2'-fluoro modifications at many appropriate positions within the siRNA allows for enhanced oligonucleotide stability, target binding and the trigger of target gene degradation by RISC.

For these reasons, the instant invention as a whole would have been *prima facie* obvious to one of ordinary skill at the time it was made.

Double Patenting

The nonstatutory double patenting rejection is based on a judicially created doctrine grounded in public policy (a policy reflected in the statute) so as to prevent the unjustified or improper timewise extension of the "right to exclude" granted by a patent and to prevent possible harassment by multiple assignees. See *In re Goodman*, 11 F.3d 1046, 29 USPQ2d 2010 (Fed. Cir. 1993); *In re Longi*, 759 F.2d 887, 225 USPQ 645 (Fed. Cir. 1985); *In re Van Ornum*, 686 F.2d 937, 214 USPQ 761 (CCPA 1982); *In re Vogel*, 422 F.2d 438, 164 USPQ 619 (CCPA 1970); and, *In re Thorington*, 418 F.2d 528, 163 USPQ 644 (CCPA 1969).

A timely filed terminal disclaimer in compliance with 37 CFR 1.321(c) may be used to overcome an actual or provisional rejection based on a nonstatutory double patenting ground provided the conflicting application or patent is shown to be commonly owned with this application. See 37 CFR 1.130(b).

Effective January 1, 1994, a registered attorney or agent of record may sign a terminal disclaimer. A terminal disclaimer signed by the assignee must fully comply with 37 CFR 3.73(b).

Claims 1-30 are rejected under the judicially created doctrine of obviousness-type double patenting as being unpatentable over claims 1-7 of U.S. Patent No. 7,452,987. Although the conflicting claims are not identical, they are not patentably distinct from each other because the instant claims and the claims of the patent are drawn to patently indistinguishable double stranded siRNA comprising first and second strands with contiguous alternating modified ribonucleotides.

Claims 1-30 are rejected under the judicially created doctrine of obviousness-type double patenting as being unpatentable over claims 1-11 of U.S. Patent No. 7,893,245. Although the conflicting claims are not identical, they are not patentably distinct from each other because the instant claims and the claims of the patent are drawn to patently indistinguishable double stranded siRNA comprising first and second strands with contiguous alternating modified ribonucleotides.

Claims 1-30 are rejected under the judicially created doctrine of obviousness-type double patenting as being unpatentable over claims 1-7 of U.S. Patent No. 8,324,370. Although the conflicting claims are not identical, they are not patentably distinct from each other because the instant claims and the claims of the patent are drawn to patently indistinguishable double stranded siRNA comprising first and second strands with contiguous alternating modified ribonucleotides.

Claims 1-30 are rejected under the judicially created doctrine of obviousness-type double patenting as being unpatentable over claims 1-7 of U.S. Patent No. 8,933,215. Although the conflicting claims are not identical, they are not patentably distinct from each other because the instant claims and the claims of the patent are drawn to patently indistinguishable double stranded siRNA comprising first and second strands with contiguous alternating modified ribonucleotides.

Claims 1-30 are rejected under the judicially created doctrine of obviousness-type double patenting as being unpatentable over claims 1-7 of U.S. Patent No. 9,222,092. Although the conflicting claims are not identical, they are not patentably distinct from each other because the instant claims and the claims of the patent are drawn to patently indistinguishable double stranded siRNA comprising first and second strands with contiguous alternating modified ribonucleotides.

Claims 1-30 are rejected under the judicially created doctrine of obviousness-type double patenting as being unpatentable over claims 1-18 of U.S. Application No. (Patent No. pending) 20160130587. Although the conflicting claims are not identical, they are not patentably distinct from each other because the instant claims and the claims of the patent are drawn to patently indistinguishable double stranded siRNA comprising first and second strands with contiguous alternating modified ribonucleotides.

Claims 1-30 are rejected under the judicially created doctrine of obviousness-type double patenting as being unpatentable over claims 1-24 of U.S. Application No. 15/589,968. Although the conflicting claims are not identical, they are not patentably distinct from each other because the instant claims and the claims of the patent are drawn to patentably indistinguishable double stranded siRNA comprising first and second strands with contiguous alternating modified ribonucleotides.

Conclusion

Any inquiry concerning this communication or earlier communications from the examiner should be directed to **Kimberly Chong** whose telephone number is **571-272-3111**. The examiner can normally be reached Monday thru Friday between 9-5 pm.

If attempts to reach the examiner by telephone are unsuccessful please contact the SPE for 1674 Ram Shukla at 571-272-0753. The fax phone number for the organization where this application or proceeding is assigned is 571-273-8300.

Patent applicants with problems or questions regarding electronic images that can be viewed in the Patent Application Information Retrieval system (PAIR) can now contact the USPTO's Patent Electronic Business Center (Patent EBC) for assistance. Representatives are available to answer your questions daily from 6 am to midnight (EST). The toll free number is (866) 217-9197. When calling please have your application serial or patent number, the type of document you are having an image problem with, the number of pages and the specific nature of the problem. The Patent Electronic Business Center will notify applicants of the resolution of the problem within 5-7 business days. Applicants can also check PAIR to confirm that the problem has been corrected. The USPTO's Patent Electronic Business Center is a complete service center supporting all patent business on the Internet. The USPTO's PAIR system provides Internet-based access to patent application status and history information. It also enables applicants to view the scanned images of their own application file

Art Unit: 1674

folder(s) as well as general patent information available to the public. For more information about the PAIR system, see <http://pair-direct.uspto.gov>.

For all other customer support, please call the USPTO Call Center (UCC) at 800-786-9199.

/Kimberly Chong/
Primary Examiner
Art Unit 1674

Notice of References Cited	Application/Control No. 15/589,968		Applicant(s)/Patent Under Reexamination GIESE ET AL.	
	Examiner KIMBERLY CHONG		Art Unit 1674	Page 1 of 1

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*		Document Number Country Code-Number-Kind Code	Date MM-YYYY	Name	CPC Classification	US Classification
*	A	US-6,133,246 A	10-2000	McKay; Robert	C07H21/00	435/183
*	B	US-6,262,036 B1	07-2001	Arnold, Jr.; Lyle J.	C12N15/1131	435/6.14
*	C	US-2005/0142535 A1	06-2005	Damha, Masad J.	C07H21/00	435/5
	D	US-				
	E	US-				
	F	US-				
	G	US-				
	H	US-				
	I	US-				
	J	US-				
	K	US-				
	L	US-				
	M	US-				

FOREIGN PATENT DOCUMENTS

*		Document Number Country Code-Number-Kind Code	Date MM-YYYY	Country	Name	CPC Classification
	N					
	O					
	P					
	Q					
	R					
	S					
	T					

NON-PATENT DOCUMENTS

*		Include as applicable: Author, Title Date, Publisher, Edition or Volume, Pertinent Pages)
	U	
	V	
	W	
	X	

*A copy of this reference is not being furnished with this Office action. (See MPEP § 707.05(a).)
Dates in MM-YYYY format are publication dates. Classifications may be US or foreign.

Substitute for form 1449/PTO INFORMATION DISCLOSURE STATEMENT BY APPLICANT <i>(Use as many sheets as necessary)</i>				Complete if Known	
				Application Number	Not Yet Assigned
				Filing Date	Concurrently Herewith
				First Named Inventor	Klaus GIESE
				Art Unit	Not Yet Assigned
				Examiner Name	Not Yet Assigned
Sheet	1	of	12	Attorney Docket Number	680132000106

U.S. PATENT DOCUMENTS					
Examiner Initials*	Cite No. ¹	Document Number Number-Kind Code ² (if known)	Publication Date MM-DD-YYYY	Name of Patentee or Applicant of Cited Document	Pages, Columns, Lines, Where Relevant Passages or Relevant Figures Appear
	1.	US-2003/0027783-A1	02-06-2003	Zernicka-Goetz et al.	
	2.	US-2003/0108923-A1	06-12-2003	Tuschl et al.	
	3.	US-2003/0135033-A1	07-17-2003	Klippel-Giese et al.	
	4.	US-2003/0190635-A1	10-09-2003	McSwiggen, James A.	
	5.	US-2004/0053875-A1	03-18-2004	Kreutzer et al.	
	6.	US-2004/0072779-A1	04-15-2004	Kreutzer et al.	
	7.	US-2004/0102408-A1	05-27-2004	Kreutzer et al.	
	8.	US-2004/0106569-A1	06-03-2004	Klippel-Giese et al.	
	9.	US-2004/0175703-A1	09-09-2004	Kreutzer et al.	
	10.	US-2004/0180351-A1	09-16-2004	Giese et al.	
	11.	US-2004/0229266-A1	11-18-2004	Tuschl et al.	
	12.	US-2005/0043263-A1	02-24-2005	Giese et al.	
	13.	US-2005/0100907-A1	05-12-2005	Kreutzer et al.	
	14.	US-2005/0142535-A1	06-30-2005	Damha et al.	
	15.	US-2006/0094678-A1	05-04-2006	Vornlocher et al.	
	16.	US-2006/0142226-A1	06-29-2006	Polisky et al.	
	17.	US-2006/0217329-A1	09-28-2006	Feinstein, Elena	
	18.	US-2006/0240022-A1	10-26-2006	Klippel-Giese et al.	
	19.	US-2007/0027097-A1	02-01-2007	Lewis, D. L.	
	20.	US-2007/0093445-A1	04-26-2007	Tuschl et al.	
	21.	US-2007/0135372-A1	06-14-2007	MacLachlan et al.	
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	24.	US- 4,217,294	08-12-1980	Petty, Herbert E.	
	25.	US- 5,466,786	11-14-1995	Buhr et al.	
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	28.	US-5,670,633	09-23-1997	Cook et al.	
	29.	US-5,792,847	08-11-1998	Buhr et al.	
	30.	US-5,872,232	02-16-1999	Cook et al.	
	31.	US-5,898,031	04-27-1999	Crooke, Stanley T.	
	32.	US-5,914,396	06-22-1999	Cook et al.	
	33.	US-5,955,589	09-21-1999	Cook et al.	
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	35.	US-6,107,094	08-22-2000	Crooke, Stanley T.	
	36.	US-6,129,912	10-10-2000	Hortin et al.	
	37.	US-6,153,737	11-28-2000	Manoharan et al.	
	38.	US-6,165,772	12-26-2000	Bertling, Wolf	
	39.	US-6,239,272-B1	05-29-2001	Beigelman et al.	
	40.	US-6,284,267-B1	09-04-2001	Aneja, Rajindra	
	41.	US- 60/352,873	02-01-2002	Damha et al.	
	42.	US-60/358,580	02-20-2002	Beigelman et al.	
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	45.	US-6,476,205-B1	11-05-2002	Buhr et al.	
	46.	US-6,506,559-B1	01-14-2003	Fire et al.	
	47.	US-6,531,584-B1	03-11-2003	Cook et al.	
	48.	US-6,617,438-B1	09-09-2003	Beigelman et al.	

Substitute for form 1449/PTO INFORMATION DISCLOSURE STATEMENT BY APPLICANT <i>(Use as many sheets as necessary)</i>				Complete if Known	
				Application Number	Not Yet Assigned
				Filing Date	Concurrently Herewith
				First Named Inventor	Klaus GIESE
				Art Unit	Not Yet Assigned
				Examiner Name	Not Yet Assigned
Sheet	2	of	12	Attorney Docket Number	680132000106

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Examiner Signature	/KIMBERLY CHONG/	Date Considered	06/14/2017
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*EXAMINER: Initial if reference considered, whether or not citation is in conformance with MPEP 609. Draw line through citation if not in conformance and not considered. Include copy of this form with next communication to applicant.

¹Applicant's unique citation designation number (optional). ²Applicant is to place a check mark here if English language Translation is attached.

Search Notes 	Application/Control No. 15589968	Applicant(s)/Patent Under Reexamination GIESE ET AL.
	Examiner KIMBERLY CHONG	Art Unit 1674

CPC- SEARCHED		
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C07H 2104	6/14/2017	KC

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US CLASSIFICATION SEARCHED			
Class	Subclass	Date	Examiner

SEARCH NOTES		
Search Notes	Date	Examiner
EAST	6/14/2017	KC
PALM inventor name search	6/14/2017	KC
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APPLICANTS Silence Therapeutics GMBH, Berlin, GERMANY;				
INVENTORS Klaus GIESE, Berlin, GERMANY; Jörg KAUFMANN, Berlin, GERMANY; Anke KLIPPEL-GIESE, Berchtesgaden, GERMANY;				
** CONTINUING DATA ***** This application is a CON of 14/977,710 12/22/2015 PAT 9695423 which is a CON of 14/578,636 12/22/2014 PAT 9222092 which is a CON of 13/692,178 12/03/2012 PAT 8933215 which is a CON of 12/986,389 01/07/2011 PAT 8324370 which is a CON of 12/200,296 08/28/2008 PAT 7893245 which is a CON of 10/633,630 08/05/2003 PAT 7452987 which claims benefit of 60/402,541 08/12/2002				
** FOREIGN APPLICATIONS ***** EUROPEAN PATENT OFFICE (EPO) 03008383.6 04/10/2003 EUROPEAN PATENT OFFICE (EPO) 02017601.2 08/05/2002				
** IF REQUIRED, FOREIGN FILING LICENSE GRANTED ** 05/19/2017				
Foreign Priority claimed <input checked="" type="checkbox"/> Yes <input type="checkbox"/> No 35 USC 119(a-d) conditions met <input checked="" type="checkbox"/> Yes <input type="checkbox"/> No Verified and Acknowledged <u>/KIMBERLY CHONG/</u> Examiner's Signature	<input type="checkbox"/> Met after Allowance Initials	STATE OR COUNTRY GERMANY	SHEETS DRAWINGS 29	TOTAL CLAIMS 30
INDEPENDENT CLAIMS 1				
ADDRESS MORRISON & FOERSTER LLP 755 PAGE MILL RD PALO ALTO, CA 94304-1018 UNITED STATES				
TITLE INTERFERING RNA MOLECULES				
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Dated: July 13, 2017

Electronic Signature for Conchita Picazo-Mejia: /Conchita Picazo-Mejia/

Docket No.: 680132000106
(PATENT)

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Patent Application of:
Klaus GIESE et al.

Application No.: 15/589,968

Confirmation No.: 8531

Filed: May 8, 2017

Art Unit: 1674

For: INTERFERING RNA MOLECULES

Examiner: K. Chong

RESPONSE TO NON-FINAL OFFICE ACTION UNDER 37 C.F.R. 1.111

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Dear Sir:

INTRODUCTORY COMMENTS

This is in response to the non-final Office Action dated June 21, 2017 (Paper No. 20170614), for which a response is due on September 21, 2017. Accordingly, this response is timely filed. Reconsideration and allowance of the pending claims in light of the remarks presented herein are respectfully requested.

Remarks/Arguments begin on page 2 of this paper.

A declaration under 37 C.F.R. § 1.131 (**Exhibit A**) and Exhibits 1-2 referred to therein are also submitted herewith.

REMARKS

Claims 1-30 are pending in the present application.

Claim Rejections - 35 USC § 102

Claims 1-11, 14, 15 and 20-23 are rejected under pre-AIA 35 U.S.C. §102(e) as allegedly being anticipated by McSwiggen et al. (US 2003/0190635, “McSwiggen”). Applicant respectfully traverses this rejection.

McSwiggen was published on October 9, 2003, and was filed as a U.S. patent application on July 25, 2002. McSwiggen claims priority to U.S. provisional applications 60/386,782 filed on June 6, 2002, 60/363,124 filed on March 11, 2002, and 60/358,580 filed on February 20, 2002. Therefore, the earliest § 102(e) reference date of McSwiggen is February 20, 2002.

Without acquiescing to the rejection and solely in an effort to expedite prosecution, Applicant herewith submits a 37 C.F.R. §1.131 declaration (enclosed herein as Exhibit A, “131 Declaration”) by inventors of the present application, which provides evidence of prior invention. Specifically, the 131 Declaration provides evidence that prior to the reference date of McSwiggen (*i.e.* February 20, 2002), inventors have synthesized an exemplary siRNA molecule as recited in claim 1, and demonstrated inhibition of PTEN expression by the siRNA molecule. Thus, the 131 Declaration provides clear evidence of actual reduction to practice prior to the reference date of McSwiggen and overcomes McSwiggen. Withdrawal of the §102(e) rejection is respectfully requested.

Claim Rejections – 35 USC § 103

Claims 1-30 are rejected under pre-AIA 35 U.S.C. §103(a) as being obvious over McSwiggen et al (US 2003/0190635, “McSwiggen”), Arnold et al (USPN 6,262,036, “Arnold”), Damha et al (US 2005/0142535, “Damha”), and McKay et al (USPN 6,133,246, “McKay”). Applicant respectfully traverses this rejection.

In particular, the Examiner is of the opinion that one of skill in the art would have combined the teachings of McSwiggen with the teachings of Arnold and Damha, which allegedly disclose that “alternating various modifications within a motif, including alternating modified 2’

residues, enhanced target binding stability of the antisense oligonucleotides for their target regions,” to arrive at the presently claimed invention. Additionally, the Examiner is of the opinion that one of skill in the art would have optimized the claimed RNAi molecule using routine experimentation because Damha, McKay and Arnold allegedly disclose “optimization experiments where antisense oligonucleotides comprising an array of different combinations of these well-known modifications are tested for their ability to target and bind target genes and inhibit their expression.” *See*, page 8 of the Non-final Office Action. Applicant respectfully disagrees.

As discussed above, McSwiggen is antedated by the 131 Declaration and thus does not qualify as a §103(a) art against claims 1-30 of the present application. Thus, an obviousness rejection relying on McSwiggen should be withdrawn.

Furthermore, none of Arnold, Damha, and McKay, either singly or in combination, teaches or suggests a double-stranded siRNA molecule against a target nucleic acid having a first strand comprising a first stretch and a second strand comprising a second stretch, wherein the first stretch and the second stretch **each** “comprises contiguous alternating 2’-O-alkyl ribonucleotides, wherein the 2’-O-alkyl ribonucleotides alternate with unmodified or differently modified ribonucleotides, wherein the 2’-O-alkyl ribonucleotides of the first strand are shifted by one ribonucleotide relative to the 2’-O-alkyl ribonucleotides of the second strand” as recited in claim 1. Thus, claims 1-30 are not obvious in view of Arnold, Damha and McKay.

Therefore, withdrawal of the §103(a) rejection is respectfully requested.

Double Patenting

Claims 1-30 are rejected under the judicially created doctrine of obviousness-type double patenting as allegedly being unpatentable over claims 1-7 of U.S. Patent No. 7,452,987. Applicant respectfully disagrees.

Without acquiescing to this obviousness-type double patenting rejection and solely in an effort to expedite prosecution, Applicant herewith files a terminal disclaimer in the present application to disclaim any term beyond the term of U.S. Patent No. 7,452,987.

Claims 1-30 are rejected under the judicially created doctrine of obviousness-type double patenting as allegedly being unpatentable over claims 1-11 of U.S. Patent No. 7,893,245. Applicant respectfully disagrees.

Without acquiescing to this obviousness-type double patenting rejection and solely in an effort to expedite prosecution, Applicant herewith files a terminal disclaimer in the present application to disclaim any term beyond the term of U.S. Patent No. 7,893,245.

Claims 1-30 are rejected under the judicially created doctrine of obviousness-type double patenting as allegedly being unpatentable over claims 1-7 of U.S. Patent No. 8,324,370. Applicant respectfully disagrees.

Without acquiescing to this obviousness-type double patenting rejection and solely in an effort to expedite prosecution, Applicant herewith files a terminal disclaimer in the present application to disclaim any term beyond the term of U.S. Patent No. 8,324,370.

Claims 1-30 are rejected under the judicially created doctrine of obviousness-type double patenting as allegedly being unpatentable over claims 1-7 of U.S. Patent No. 8,933,215. Applicant respectfully disagrees.

Without acquiescing to this obviousness-type double patenting rejection and solely in an effort to expedite prosecution, Applicant herewith files a terminal disclaimer in the present application to disclaim any term beyond the term of U.S. Patent No. 8,933,215.

Claims 1-30 are rejected under the judicially created doctrine of obviousness-type double patenting as allegedly being unpatentable over claims 1-7 of U.S. Patent No. 9,222,092. Applicant respectfully disagrees.

Without acquiescing to this obviousness-type double patenting rejection and solely in an effort to expedite prosecution, Applicant herewith files a terminal disclaimer in the present application to disclaim any term beyond the term of U.S. Patent No. 9,222,092.

Claims 1-30 are rejected under the judicially created doctrine of obviousness-type double patenting as allegedly being unpatentable over claims 1-18 of U.S. Application No. 14/977,710 (U.S. Application Publication No. 20160130587, Patent No. 9,695,423)¹. Applicant respectfully disagrees.

Without acquiescing to this obviousness-type double patenting rejection and solely in an effort to expedite prosecution, Applicant herewith files a terminal disclaimer in the present application to disclaim any term beyond the term of U.S. Application No. 14/977,710 (U.S. Application Publication No. 20160130587, Patent No. 9,695,423).

Claims 1-30 are rejected under the judicially created doctrine of obviousness-type double patenting as being unpatentable over Claims 1-24 of U.S. Application No. 15/589,968. Applicant respectfully submits that U.S. Application No. 15/589,968 is the present application. Thus, the obviousness-type double patenting rejection should be withdrawn.

In view of the foregoing, Applicant respectfully requests withdrawal of the obviousness-type double patenting rejections of Claims 1-30 of the present application.

¹ In the non-final Office Action, the Examiner cites “U.S. Application No. 20160130587” for the obviousness-type double patenting rejection. However, 20160130587 is the U.S. Application Publication No. corresponding to U.S. Application No. 14/977,710. The corresponding U.S. Patent No. 9,695,423 was issued on July 4, 2017.

CONCLUSION

In view of the above, each of the presently pending claims in this application is believed to be in immediate condition for allowance. Accordingly, the Examiner is respectfully requested to withdraw the outstanding rejection of the claims and to pass this application to issue. If it is determined that a telephone conference would expedite the prosecution of this application, the Examiner is invited to telephone the undersigned at the number given below.

In the event the U.S. Patent and Trademark Office determines that an extension and/or other relief is required, Applicant petitions for any required relief including extensions of time and authorizes the Commissioner to charge the cost of such petitions and/or other fees due in connection with the filing of this document to **Deposit Account No. 03-1952** referencing docket no. **680132000106**. However, the Commissioner is not authorized to charge the cost of the issue fee to the Deposit Account.

Dated: July 13, 2017

Respectfully submitted,

Electronic signature: /Janet Xiao /
Jian Xiao

Registration No.: 55,748
MORRISON & FOERSTER LLP
755 Page Mill Road
Palo Alto, California 94304-1018
(650) 813-5736

EXHIBIT A

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Attorney Docket No: 14677-003

Applicant(s): Klaus Giese *et al.*

Confirmation No.: 6369

Serial No.: 10/633,630

Examiner: Kimberly Chong

Filing Date: August 5, 2003

Group Art Unit: 1635

Title: INTERFERING RNA MOLECULES

DECLARATION UNDER 37 C.F.R § 1.131

We, Klaus Giese, Anke Klippel-Giese and Jörg Kaufmann, do hereby declare as follows:

1. We understand that the claims in the captioned application have been rejected over U.S. patent application 2003/0190635, which lists February 20, 2002 as the earliest priority date, and U.S. patent application 2005/0142535, which lists February 1, 2002 as the earliest priority date.

2. We submit this declaration, based on our personal knowledge to demonstrate that the invention claimed in the captioned application was completed prior to February 2002 and therefore prior to the earliest prior art date of either U.S. patent application 2003/0190635, or 2005/0142535. All dates on the attached Exhibits have been masked out.

3. The delivery receipt attached as EXHIBIT 1 shows that two complementary oligoribonucleotide molecules, 94A2 and 94B1 were synthesized prior to February 1, 2002. 94A2 is the antisense strand and 94B1 is the sense strand. These molecules are described in the specification of the captioned application in Figure 15 where they are shown as PTENA V15 and PTENB V15.

4. In the delivery receipt, the lower case letters a, u, c, and g indicate the conventional unmodified ribonucleotides adenosine, uracil, cytosine, and guanosine, respectively. The number 5 indicates 2'O-methyl-ribo U, number 6 indicates 2'O-methyl-ribo A, number 7 indicates 2'O-

1/6

methy-ribo C, and number 8 indicates 2'-O-methyl-ribo G. Accordingly, the sequences of the two oligoribonucleotides are:

94A2: 5'-CuCcUuUuGuUuCuGcUaAcG-3' and

94B1: 5'-cGuUaGcAgAaAcAaAaGgAg-3'.

In these sequences a lower case letter indicates the unmodified nucleotide and the upper case indicates the 2'-O-methyl nucleotide. The nucleosides are linked by phosphodiester bonds.

5. Comparison of the sequences of 94A2 and 94B1 shows that they are both 21 nucleotides long, are complementary and have contiguous alternating 2'-O-methyl modified and single unmodified ribonucleotides, where a modified ribonucleotide on one strand is base paired with an unmodified ribonucleotide on the second strand and vice versa. 94A2 is complementary to a part of the PTEN gene. When 94A2 and 94B1 are combined, the resulting double stranded molecule is blunt ended.

6. Prior to February 1, 94A2 and 94B1 were combined to form a double stranded molecule designated 94A2/94B1 and demonstrated to inhibit expression of PTEN as shown in EXHIBIT 2. The results shown in EXHIBIT 2 were recorded prior to February 1, 2002.

7. EXHIBIT 2 shows the results of an experiment on the ability of RNAi molecules to inhibit PTEN expression in HeLaB cells. PTEN mRNA was measured using quantitative real time PCR analysis and was normalized to p110a mRNA as control. The bar graph in EXHIBIT 2 shows the results obtained with various double stranded RNA molecules. The 80AB molecule was an unmodified blunt siRNA molecule, and represented a positive control. The 79AB molecule was a completely 2'-O-methyl modified siRNA molecule and served as a negative control. All molecules were tested at four different concentrations: 40 nM, 10 nM, 5 nM, 2.5 nM.

8. As can be seen from the data, molecule 94A2/94B1 shows activity that is very similar to the positive control 80AB, demonstrating that the molecule was effective at reducing PTEN mRNA expression in cells.

9. We hereby declare that all statements made herein of our own knowledge are true, and that all statements made on information and belief are believed to be true; and further, that these statements are made with the knowledge that willful false statements, and the like so made, are punishable by fine or imprisonment, or both, under Section 1001, Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

Feb 10, 2008

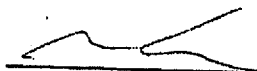
Date

Feb 10, 2008

Date

Feb. 08/2008

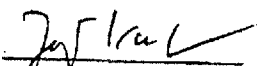
Date



Klaus Giese



Anke Klippel-Giese



Jörg Kaufmann

EXHIBIT 1 of
EXHIBIT A

BioSpring
 Gesellschaft für Biotechnologie mbH
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Jörg Kaufmann

atugen AG / Haus 80

Robert-Rössle-Str.10

D-13125 Berlin

Ihre Bestnr.

Lieferscheinr.3925

Kundennummer 10003

Bestellung vom

1

94A1

5'-c5c 7u5 u5g 5u5 c5g 7u6 a7g-3'

IDO	16893	OD	12,9 bei	260 nm	Länge	21-mer
Scale	0,2 µmol	Menge		35361 pmol	Molekular Gew.	5348,20
Reinigung	Modifikation			35 nmol	GC Gehalt	29 %
DNA-Typ	DNA			189 µg	Tm (GC)	39 °C
					ε (mM)	364,810

Zusammensetzung

A=1,0

C=3,0

G=3,0

T=4,0

Modi=10,0

Mod. 5'	Mod. 5 2'OMe-rU	Mod. 7 2'OMe-rC
Mod. 3'	Mod. 6 2'OMe-rA	Mod. 8

2

94A2

5'-7u7 c5u 5u8 u5u 7u8 c5a 6c8-3'

IDO	16894	OD	33,4 bei	260 nm	Länge	21-mer
Scale	0,2 µmol	Menge		88514 pmol	Molekular Gew.	4799,86
Reinigung	Modifikation			89 nmol	GC Gehalt	14 %
DNA-Typ	DNA			425 µg	Tm (GC)	34 °C
					ε (mM)	377,340

Zusammensetzung

A=1,0

C=3,0

G=0,0

T=6,0

Modi=11,0

Mod. 5'	Mod. 5 2'OMe-rU	Mod. 7 2'OMe-rC
Mod. 3'	Mod. 6 2'OMe-rA	Mod. 8 2'OMe-rG

3

94B1

5'-c8u 5a8 c6g 6a6 c6a 6a8 g6g-3'

IDO	16910	OD	18,9 bei	260 nm	Länge	21-mer
Scale	0,2 µmol	Menge		56445 pmol	Molekular Gew.	6523,12
Reinigung	Modifikation			56 nmol	GC Gehalt	29 %
DNA-Typ	DNA			368 µg	Tm (GC)	39 °C
					ε (mM)	334,840

Zusammensetzung

A=4,0

C=3,0

G=3,0

T=1,0

Modi=10,0

Mod. 5'	Mod. 5 2'OMe-rU	Mod. 7
Mod. 3'	Mod. 6 2'OMe-rA	Mod. 8 2'OMe-rG

BioSpring
 Gesellschaft für Biotechnologie mbH

Geschäftsführer:
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Bank: Frankfurter Volksbank
 Konto: 134295 BLZ: 50190000
 Amtsgericht: Frankfurt am Main
 Handelsregisternummer: HRB 43255
 Ust-IdNr. DE187592327

4		94B2				
5'-7g5 u6g 7a8 a6a 7a6 a6g 8a8-3'						
IDO	16911	OD	32,7 bei 260 nm	Länge	21-mer	
Scale	0,2 µmol	Menge	96665 pmol	Molekular Gew.	6555,12	
Reinigung	Modifikation		97 nmol	GC Gehalt	14 %	
DNA-Typ	DNA		634 µg	Tm (GC)	34 °C	
				ε (mM)	338,280	
Zusammensetzung		A=6,0	C=0,0	G=3,0	T=1,0	Modi=11,0
Mod. 5'		Mod. 5 2'OMe-rU		Mod. 7 2'OMe-rC		
Mod. 3'		Mod. 6 2'OMe-rA		Mod. 8 2'OMe-rG		

EXHIBIT 2 of
EXHIBIT A

► 96 well PTEN RNAi Transfection

[illegible]

A	80 AB
B	19 AB
C	81 AB
D	6 AB
E	61 AB
F	
G	
H	
A	9TA/9B
B	9TA/9B
C	9TA/9B
D	9TA/9B
E	
F	
G	
H	

40mH / 60mH / 2.5mH

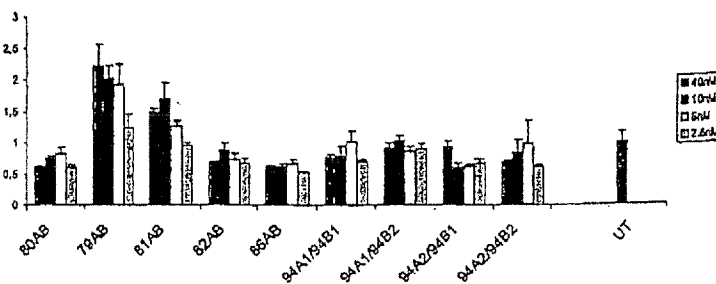
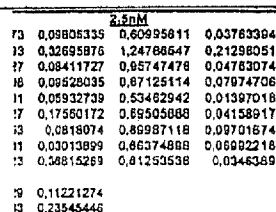
* Tagihan 2x RBN / p Mo e

	1	2	3	4	5	6	7	8	9	10	11	12	
A	SECRET												A
B	1948												B
C	M. A. B.												C
D	1948												D
E	C. A. B.												E
F	400												F
G	400 400 400 400 400 400 400 400 400 400 400 400												G
H	-9												H

	1	2	3	4	5	6	7	8	9	10	11	12
A	MAY 1968											
B	MAY 1968											
C	MAY 1968											
D	MAY 1968											
E	MAY 1968											
F	MAY 1968											
G	MAY 1968											
H	MAY 1968											
I	MAY 1968											
J	MAY 1968											
K	MAY 1968											
L	MAY 1968											
M	MAY 1968											
N	MAY 1968											
O	MAY 1968											
P	MAY 1968											
Q	MAY 1968											
R	MAY 1968											
S	MAY 1968											
T	MAY 1968											
U	MAY 1968											
V	MAY 1968											
W	MAY 1968											
X	MAY 1968											
Y	MAY 1968											
Z	MAY 1968											

! Wiederholung d. Transfection protokolles: da beim RNA-Trap was schief gelaufen ist

Pten RNAi's in HeLaB
(2500c/w NC 388 1µg/ml)



Continued on Page

Read and Understood By

Ch. Technu

Signed

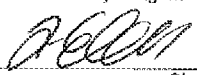
Date _____

Melanie Baker

Signed

Date _____

Under the Paperwork Reduction Act of 1995, no persons are required to respond to a collection of information unless it displays a valid OMB control number.

TERMINAL DISCLAIMER TO OBVIATE A DOUBLE PATENTING REJECTION OVER A "PRIOR" PATENT	Docket Number (Optional) 680132000106
<p>In re Application of: Klaus GIESE et al.</p> <p>Application No.: 15/589,968</p> <p>Filed: May 8, 2017</p> <p>For: INTERFERING RNA MOLECULES</p> <p>The applicant, <u>Silence Therapeutics GMBH</u>, owner of <u>100</u> percent interest in the instant application hereby disclaims, except as provided below, the terminal part of the statutory term of any patent granted on the instant application which would extend beyond the expiration date of the full statutory term of prior patent No. <u>(see attached)</u> as the term of said prior patent is presently shortened by any terminal disclaimer. The applicant hereby agrees that any patent so granted on the instant application shall be enforceable only for and during such period that it and the prior patent are commonly owned. This agreement runs with any patent granted on the instant application and is binding upon the grantee, its successors or assigns.</p> <p>In making the above disclaimer, the applicant does not disclaim the terminal part of the term of any patent granted on the instant application that would extend to the expiration date of the full statutory term of the prior patent, "as the term of said prior patent is presently shortened by any terminal disclaimer," in the event that said prior patent later:</p> <ul style="list-style-type: none"> expires for failure to pay a maintenance fee; is held unenforceable; is found invalid by a court of competent jurisdiction; is statutorily disclaimed in whole or terminally disclaimed under 37 CFR 1.321; has all claims canceled by a reexamination certificate; is reissued; or is in any manner terminated prior to the expiration of its full statutory term as presently shortened by any terminal disclaimer. <p>Check either box 1 or 2 below, if appropriate.</p> <p>1. <input checked="" type="checkbox"/> The undersigned is the applicant. If the applicant is an assignee, the undersigned is authorized to act on behalf of the assignee.</p> <p>I hereby acknowledge that any willful false statements made are punishable under 18 U.S.C. 1001 by fine or imprisonment of not more than five (5) years, or both.</p> <p>2. <input type="checkbox"/> The undersigned is an attorney or agent of record. Reg. No. _____</p> <div style="display: flex; justify-content: space-between; margin-top: 20px;"> <div style="text-align: center;">  _____ Signature </div> <div style="text-align: center;"> 6 JULY 2017 _____ Date </div> </div> <div style="display: flex; justify-content: space-between; margin-top: 10px;"> <div style="text-align: center;"> DAVID ELLAM _____ Typed or printed name </div> <div style="text-align: center;"> 020 345 769 00 _____ Telephone Number </div> </div> <div style="display: flex; justify-content: space-between; margin-top: 10px;"> <div style="text-align: center;"> CFO _____ Title </div> </div> <p><input checked="" type="checkbox"/> Terminal disclaimer fee under 37 CFR 1.20(d) included.</p> <p style="text-align: center;">WARNING: Information on this form may become public. Credit card information should not be included on this form. Provide credit card information and authorization on PTO-2038.</p>	

SUPPLEMENTAL SHEET FOR TERMINAL DISCLAIMER TO OBVIATE A DOUBLE PATENTING REJECTION OVER A "PRIOR" PATENT	Docket Number (Optional) 680132000106
---	---

Prior patent Nos. applicable to this terminal disclaimer (referenced on first page):

7,452,987, 7,893,245, 8,324,370, 8,933,215, 9,222,092, 9,695,423.

STATEMENT UNDER 37 CFR 3.73(c)

Applicant/Patent Owner: Silence Therapeutics GMBH

Application No./Patent No.: 15/589,968 Filed/Issue Date: May 8, 2017

Titled: INTERFERING RNA MOLECULES

Silence Therapeutics GMBH, a corporation
(Name of Assignee) (Type of Assignee, e.g., corporation, partnership, university, government agency, etc.)

states that, for the patent application/patent identified above, it is (choose **one** of options 1, 2, 3 or 4 below):

1. ☒ The assignee of the entire right, title, and interest.
2. ☐ An assignee of less than the entire right, title, and interest (check applicable box):
- ☐ The extent (by percentage) of its ownership interest is _____ %. Additional Statement(s) by the owners holding the balance of the interest must be submitted to account for 100% of the ownership interest.
- ☐ There are unspecified percentages of ownership. The other parties, including inventors, who together own the entire right, title and interest are:

Additional Statement(s) by the owner(s) holding the balance of the interest must be submitted to account for the entire right, title, and interest.

3. ☐ The assignee of an undivided interest in the entirety (a complete assignment from one of the joint inventors was made). The other parties, including inventors, who together own the entire right, title, and interest are:

Additional Statement(s) by the owner(s) holding the balance of the interest must be submitted to account for the entire right, title, and interest.

4. ☐ The recipient, via a court proceeding or the like (e.g., bankruptcy, probate), of an undivided interest in the entirety (a complete transfer of ownership interest was made). The certified document(s) showing the transfer is attached.

The interest identified in option 1, 2 or 3 above (not option 4) is evidenced by either (choose **one** of options A or B below):

- A. ☐ An assignment from the inventor(s) of the patent application/patent identified above. The assignment was recorded in the United States Patent and Trademark Office at Reel _____, Frame _____, or for which a copy thereof is attached.
- B. ☒ A chain of title from the inventor(s), of the patent application/patent identified above, to the current assignee as follows:

1. From: Klaus GIESE, Joerg KAUFMANN and Anke KLIPPEL-GIESE To: ATUGEN AG
The document was recorded in the United States Patent and Trademark Office at
Reel 014958, Frame 0009, or for which a copy thereof is attached.
2. From: ATUGEN AG (AKTIENGESELLSCHAFT) To: SILENCE THERAPEUTICS AKTIENGESELLSCHAFT (AG)
The document was recorded in the United States Patent and Trademark Office at
Reel 020700, Frame 0750, or for which a copy thereof is attached.

Attorney Docket No. 680132000106

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STATEMENT UNDER 37 CFR 3.73(c)3. From: SILENCE THERAPEUTICS AG To: SILENCE THERAPEUTICS GMBH

The document was recorded in the United States Patent and Trademark Office at

Reel 034767 , Frame 0324 , or for which a copy thereof is attached.

4. From: _____ To: _____

The document was recorded in the United States Patent and Trademark Office at

Reel _____ , Frame _____ , or for which a copy thereof is attached.

5. From: _____ To: _____

The document was recorded in the United States Patent and Trademark Office at

Reel _____ , Frame _____ , or for which a copy thereof is attached.

6. From: _____ To: _____

The document was recorded in the United States Patent and Trademark Office at

Reel _____ , Frame _____ , or for which a copy thereof is attached.

☐ Additional documents in the chain of title are listed on a supplemental sheet(s).☒ As required by 37 CFR 3.73(c)(1)(i), the documentary evidence of the chain of title from the original owner to the assignee was, or concurrently is being, submitted for recordation pursuant to 37 CFR 3.11.

[NOTE: A separate copy (i.e., a true copy of the original assignment document(s)) must be submitted to Assignment Division in accordance with 37 CFR Part 3, to record the assignment in the records of the USPTO. See MPEP 302.08]

The undersigned (whose title is supplied below) is authorized to act on behalf of the assignee.

Signature /Janet Xiao /_____
Date July 13, 2017_____
Printed or Typed Name Jian Xiao, Reg. No. 55,748_____
Title or Registration Number Attorney for Assignee

Attorney Docket No. 680132000106

[Page 2 of 2]

I hereby certify that this paper is being transmitted via the Office electronic filing system in accordance with 37 CFR § 1.6(a)(4).

Dated: June 13, 2017

Electronic Signature for Conchita Picazo-Mejia: /Conchita Picazo-Mejia/

Electronic Patent Application Fee Transmittal				
Application Number:		15589968		
Filing Date:		08-May-2017		
Title of Invention:		INTERFERING RNA MOLECULES		
First Named Inventor/Applicant Name:		Klaus GIESE		
Filer:		Jian Xiao/Conchita Picazo-Mejia		
Attorney Docket Number:		680132000106		
Filed as Large Entity				
Filing Fees for Utility under 35 USC 111(a)				
Description	Fee Code	Quantity	Amount	Sub-Total in USD(\$)
Basic Filing:				
Pages:				
Claims:				
Miscellaneous-Filing:				
Petition:				
Patent-Appeals-and-Interference:				
Post-Allowance-and-Post-Issuance:				
Extension-of-Time:				

Description	Fee Code	Quantity	Amount	Sub-Total in USD(\$)
Miscellaneous:				
STATUTORY OR TERMINAL DISCLAIMER	1814	1	160	160
Total in USD (\$)				160

Electronic Acknowledgement Receipt

EFS ID:	29776906
Application Number:	15589968
International Application Number:	
Confirmation Number:	8531
Title of Invention:	INTERFERING RNA MOLECULES
First Named Inventor/Applicant Name:	Klaus GIESE
Customer Number:	25226
Filer:	Jian Xiao/Conchita Picazo-Mejia
Filer Authorized By:	Jian Xiao
Attorney Docket Number:	680132000106
Receipt Date:	13-JUL-2017
Filing Date:	08-MAY-2017
Time Stamp:	19:08:57
Application Type:	Utility under 35 USC 111(a)

Payment information:

Submitted with Payment	yes
Payment Type	DA
Payment was successfully received in RAM	\$ 160
RAM confirmation Number	071417INTEFSW00005563031952
Deposit Account	031952
Authorized User	Conchita Picazo-Mejia

The Director of the USPTO is hereby authorized to charge indicated fees and credit any overpayment as follows:

37 CFR 1.21 (Miscellaneous fees and charges)

File Listing:					
Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)
1		NFOA_TRACK_One_Response_68013_2000106.pdf	42156	yes	6
			99649e9b0c8836f658e4d6c8585a8b9d1635d8b4		
	Multipart Description/PDF files in .zip description				
	Document Description		Start	End	
	Amendment/Req. Reconsideration-After Non-Final Reject		1	1	
	Applicant Arguments/Remarks Made in an Amendment		2	6	
Warnings:					
Information:					
2	Miscellaneous Incoming Letter	ExhibitA_131_declaration.pdf	267358	no	9
			ab13a0e34e303de1210b9ee058786b91e09be3a0		
Warnings:					
Information:					
3	Terminal Disclaimer Filed	EXE_TD_patents_68013_2000106.pdf	127973	no	2
			717cb2c7cad81a0d47037f3ab67c184aef0c1840		
Warnings:					
Information:					
4	Assignee showing of ownership per 37 CFR 3.73	Statement_Under_37_CFR_68013_2000106.pdf	28690	no	2
			0a22d698bb620d5cc673acb378f9caedf8d3213e		
Warnings:					
Information:					
5	Fee Worksheet (SB06)	fee-info.pdf	30495	no	2
			36e67fa0b340a600f9c852912a4cf7f9dfdd884d		
Warnings:					
Information:					
424					

This Acknowledgement Receipt evidences receipt on the noted date by the USPTO of the indicated documents, characterized by the applicant, and including page counts, where applicable. It serves as evidence of receipt similar to a Post Card, as described in MPEP 503.

New Applications Under 35 U.S.C. 111


If a new application is being filed and the application includes the necessary components for a filing date (see 37 CFR 1.53(b)-(d) and MPEP 506), a Filing Receipt (37 CFR 1.54) will be issued in due course and the date shown on this Acknowledgement Receipt will establish the filing date of the application.

National Stage of an International Application under 35 U.S.C. 371

If a timely submission to enter the national stage of an international application is compliant with the conditions of 35 U.S.C. 371 and other applicable requirements a Form PCT/DO/EO/903 indicating acceptance of the application as a national stage submission under 35 U.S.C. 371 will be issued in addition to the Filing Receipt, in due course.

New International Application Filed with the USPTO as a Receiving Office

If a new international application is being filed and the international application includes the necessary components for an international filing date (see PCT Article 11 and MPEP 1810), a Notification of the International Application Number and of the International Filing Date (Form PCT/RO/105) will be issued in due course, subject to prescriptions concerning national security, and the date shown on this Acknowledgement Receipt will establish the international filing date of the application.


Application Number 	Application/Control No. 15/589,968	Applicant(s)/Patent under Reexamination GIESE ET AL.	

Document Code - DISQ	Internal Document – DO NOT MAIL
-----------------------------	--

TERMINAL DISCLAIMER	<input checked="" type="checkbox"/> APPROVED	<input type="checkbox"/> DISAPPROVED
Date Filed : 13 July, 2017	This patent is subject to a Terminal Disclaimer	

Approved/Disapproved by: <u>/CRYSTAL QUEEN/</u> Technology Center: <u>PLRC</u> Telephone: _____

U.S. Patent and Trademark Office

Application Number 	Application/Control No. 15/589,968	Applicant(s)/Patent under Reexamination GIESE ET AL.	

Document Code - DISQ	Internal Document – DO NOT MAIL
-----------------------------	--

TERMINAL DISCLAIMER	<input checked="" type="checkbox"/> APPROVED	<input type="checkbox"/> DISAPPROVED
Date Filed : 7/13/17	This patent is subject to a Terminal Disclaimer	

Approved/Disapproved by:
jean proctor

U.S. Patent and Trademark Office



UNITED STATES PATENT AND TRADEMARK OFFICE

UNITED STATES DEPARTMENT OF COMMERCE
United States Patent and Trademark Office
Address: COMMISSIONER FOR PATENTS
P.O. Box 1450
Alexandria, Virginia 22313-1450
www.uspto.gov

NOTICE OF ALLOWANCE AND FEE(S) DUE

25226 7590 08/10/2017
MORRISON & FOERSTER LLP
755 PAGE MILL RD
PALO ALTO, CA 94304-1018

EXAMINER

CHONG, KIMBERLY

ART UNIT

PAPER NUMBER

1674

DATE MAILED: 08/10/2017

APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
-----------------	-------------	----------------------	---------------------	------------------

15/589,968

05/08/2017

Klaus GIESE

680132000106

8531

TITLE OF INVENTION: INTERFERING RNA MOLECULES

APPLN. TYPE	ENTITY STATUS	ISSUE FEE DUE	PUBLICATION FEE DUE	PREV. PAID ISSUE FEE	TOTAL FEE(S) DUE	DATE DUE
nonprovisional	UNDISCOUNTED	\$960	\$0	\$0	\$960	11/13/2017

THE APPLICATION IDENTIFIED ABOVE HAS BEEN EXAMINED AND IS ALLOWED FOR ISSUANCE AS A PATENT. PROSECUTION ON THE MERITS IS CLOSED. THIS NOTICE OF ALLOWANCE IS NOT A GRANT OF PATENT RIGHTS. THIS APPLICATION IS SUBJECT TO WITHDRAWAL FROM ISSUE AT THE INITIATIVE OF THE OFFICE OR UPON PETITION BY THE APPLICANT. SEE 37 CFR 1.313 AND MPEP 1308.

THE ISSUE FEE AND PUBLICATION FEE (IF REQUIRED) MUST BE PAID WITHIN THREE MONTHS FROM THE MAILING DATE OF THIS NOTICE OR THIS APPLICATION SHALL BE REGARDED AS ABANDONED. THIS STATUTORY PERIOD CANNOT BE EXTENDED. SEE 35 U.S.C. 151. THE ISSUE FEE DUE INDICATED ABOVE DOES NOT REFLECT A CREDIT FOR ANY PREVIOUSLY PAID ISSUE FEE IN THIS APPLICATION. IF AN ISSUE FEE HAS PREVIOUSLY BEEN PAID IN THIS APPLICATION (AS SHOWN ABOVE), THE RETURN OF PART B OF THIS FORM WILL BE CONSIDERED A REQUEST TO REAPPLY THE PREVIOUSLY PAID ISSUE FEE TOWARD THE ISSUE FEE NOW DUE.

HOW TO REPLY TO THIS NOTICE:

I. Review the ENTITY STATUS shown above. If the ENTITY STATUS is shown as SMALL or MICRO, verify whether entitlement to that entity status still applies.

If the ENTITY STATUS is the same as shown above, pay the TOTAL FEE(S) DUE shown above.

If the ENTITY STATUS is changed from that shown above, on PART B - FEE(S) TRANSMITTAL, complete section number 5 titled "Change in Entity Status (from status indicated above)".

For purposes of this notice, small entity fees are 1/2 the amount of undiscounted fees, and micro entity fees are 1/2 the amount of small entity fees.

II. PART B - FEE(S) TRANSMITTAL, or its equivalent, must be completed and returned to the United States Patent and Trademark Office (USPTO) with your ISSUE FEE and PUBLICATION FEE (if required). If you are charging the fee(s) to your deposit account, section "4b" of Part B - Fee(s) Transmittal should be completed and an extra copy of the form should be submitted. If an equivalent of Part B is filed, a request to reapply a previously paid issue fee must be clearly made, and delays in processing may occur due to the difficulty in recognizing the paper as an equivalent of Part B.

III. All communications regarding this application must give the application number. Please direct all communications prior to issuance to Mail Stop ISSUE FEE unless advised to the contrary.

IMPORTANT REMINDER: Utility patents issuing on applications filed on or after Dec. 12, 1980 may require payment of maintenance fees. It is patentee's responsibility to ensure timely payment of maintenance fees when due.

PART B - FEE(S) TRANSMITTAL

Complete and send this form, together with applicable fee(s), to: **Mail** **Mail Stop ISSUE FEE**
Commissioner for Patents
P.O. Box 1450
Alexandria, Virginia 22313-1450
or Fax (571)-273-2885

INSTRUCTIONS: This form should be used for transmitting the ISSUE FEE and PUBLICATION FEE (if required). Blocks 1 through 5 should be completed where appropriate. All further correspondence including the Patent, advance orders and notification of maintenance fees will be mailed to the current correspondence address as indicated unless corrected below or directed otherwise in Block 1, by (a) specifying a new correspondence address; and/or (b) indicating a separate "FEE ADDRESS" for maintenance fee notifications.

CURRENT CORRESPONDENCE ADDRESS (Note: Use Block 1 for any change of address)

Note: A certificate of mailing can only be used for domestic mailings of the Fee(s) Transmittal. This certificate cannot be used for any other accompanying papers. Each additional paper, such as an assignment or formal drawing, must have its own certificate of mailing or transmission.

25226 7590 08/10/2017
MORRISON & FOERSTER LLP
755 PAGE MILL RD
PALO ALTO, CA 94304-1018

Certificate of Mailing or Transmission

I hereby certify that this Fee(s) Transmittal is being deposited with the United States Postal Service with sufficient postage for first class mail in an envelope addressed to the Mail Stop ISSUE FEE address above, or being facsimile transmitted to the USPTO (571) 273-2885, on the date indicated below.

(Depositor's name)
(Signature)
(Date)

APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
15/589,968	05/08/2017	Klaus GIESE	680132000106	8531

TITLE OF INVENTION: INTERFERING RNA MOLECULES

APPLN. TYPE	ENTITY STATUS	ISSUE FEE DUE	PUBLICATION FEE DUE	PREV. PAID ISSUE FEE	TOTAL FEE(S) DUE	DATE DUE
nonprovisional	UNDISCOUNTED	\$960	\$0	\$0	\$960	11/13/2017

EXAMINER	ART UNIT	CLASS-SUBCLASS
CHONG, KIMBERLY	1674	536-024500

1. Change of correspondence address or indication of "Fee Address" (37 CFR 1.363).

- ☐ Change of correspondence address (or Change of Correspondence Address form PTO/SB/122) attached.
- ☐ "Fee Address" indication (or "Fee Address" Indication form PTO/SB/47; Rev 03-02 or more recent) attached. **Use of a Customer Number is required.**

2. For printing on the patent front page, list

- (1) The names of up to 3 registered patent attorneys or agents OR, alternatively, 1 _____
- (2) The name of a single firm (having as a member a registered attorney or agent) and the names of up to 2 registered patent attorneys or agents. If no name is listed, no name will be printed. 2 _____
- 3 _____

3. ASSIGNEE NAME AND RESIDENCE DATA TO BE PRINTED ON THE PATENT (print or type)

PLEASE NOTE: Unless an assignee is identified below, no assignee data will appear on the patent. If an assignee is identified below, the document has been filed for recordation as set forth in 37 CFR 3.11. Completion of this form is NOT a substitute for filing an assignment.

(A) NAME OF ASSIGNEE

(B) RESIDENCE: (CITY and STATE OR COUNTRY)

Please check the appropriate assignee category or categories (will not be printed on the patent): ☐ Individual ☐ Corporation or other private group entity ☐ Government

4a. The following fee(s) are submitted:

- ☐ Issue Fee
- ☐ Publication Fee (No small entity discount permitted)
- ☐ Advance Order - # of Copies _____

4b. Payment of Fee(s): (Please first reapply any previously paid issue fee shown above)

- ☐ A check is enclosed.
- ☐ Payment by credit card. Form PTO-2038 is attached.
- ☐ The director is hereby authorized to charge the required fee(s), any deficiency, or credits any overpayment, to Deposit Account Number _____ (enclose an extra copy of this form).

5. Change in Entity Status (from status indicated above)

- ☐ Applicant certifying micro entity status. See 37 CFR 1.29
- ☐ Applicant asserting small entity status. See 37 CFR 1.27
- ☐ Applicant changing to regular undiscounted fee status.

NOTE: Absent a valid certification of Micro Entity Status (see forms PTO/SB/15A and 15B), issue fee payment in the micro entity amount will not be accepted at the risk of application abandonment.

NOTE: If the application was previously under micro entity status, checking this box will be taken to be a notification of loss of entitlement to micro entity status.

NOTE: Checking this box will be taken to be a notification of loss of entitlement to small or micro entity status, as applicable.

NOTE: This form must be signed in accordance with 37 CFR 1.31 and 1.33. See 37 CFR 1.4 for signature requirements and certifications.

Authorized Signature _____

Date _____

Typed or printed name _____

Registration No. _____



UNITED STATES PATENT AND TRADEMARK OFFICE

UNITED STATES DEPARTMENT OF COMMERCE
United States Patent and Trademark Office
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Alexandria, Virginia 22313-1450
www.uspto.gov

APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
15/589,968	05/08/2017	Klaus GIESE	680132000106	8531
25226	7590	08/10/2017	EXAMINER	
MORRISON & FOERSTER LLP			CHONG, KIMBERLY	
755 PAGE MILL RD			ART UNIT	
PALO ALTO, CA 94304-1018			PAPER NUMBER	
			1674	
DATE MAILED: 08/10/2017				

Determination of Patent Term Adjustment under 35 U.S.C. 154 (b) (Applications filed on or after May 29, 2000)

The Office has discontinued providing a Patent Term Adjustment (PTA) calculation with the Notice of Allowance.

Section 1(h)(2) of the AIA Technical Corrections Act amended 35 U.S.C. 154(b)(3)(B)(i) to eliminate the requirement that the Office provide a patent term adjustment determination with the notice of allowance. See Revisions to Patent Term Adjustment, 78 Fed. Reg. 19416, 19417 (Apr. 1, 2013). Therefore, the Office is no longer providing an initial patent term adjustment determination with the notice of allowance. The Office will continue to provide a patent term adjustment determination with the Issue Notification Letter that is mailed to applicant approximately three weeks prior to the issue date of the patent, and will include the patent term adjustment on the patent. Any request for reconsideration of the patent term adjustment determination (or reinstatement of patent term adjustment) should follow the process outlined in 37 CFR 1.705.

Any questions regarding the Patent Term Extension or Adjustment determination should be directed to the Office of Patent Legal Administration at (571)-272-7702. Questions relating to issue and publication fee payments should be directed to the Customer Service Center of the Office of Patent Publication at 1-(888)-786-0101 or (571)-272-4200.

OMB Clearance and PRA Burden Statement for PTOL-85 Part B

The Paperwork Reduction Act (PRA) of 1995 requires Federal agencies to obtain Office of Management and Budget approval before requesting most types of information from the public. When OMB approves an agency request to collect information from the public, OMB (i) provides a valid OMB Control Number and expiration date for the agency to display on the instrument that will be used to collect the information and (ii) requires the agency to inform the public about the OMB Control Number's legal significance in accordance with 5 CFR 1320.5(b).

The information collected by PTOL-85 Part B is required by 37 CFR 1.311. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.14. This collection is estimated to take 12 minutes to complete, including gathering, preparing, and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, Virginia 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. SEND TO: Commissioner for Patents, P.O. Box 1450, Alexandria, Virginia 22313-1450. Under the Paperwork Reduction Act of 1995, no persons are required to respond to a collection of information unless it displays a valid OMB control number.

Privacy Act Statement

The Privacy Act of 1974 (P.L. 93-579) requires that you be given certain information in connection with your submission of the attached form related to a patent application or patent. Accordingly, pursuant to the requirements of the Act, please be advised that: (1) the general authority for the collection of this information is 35 U.S.C. 2(b)(2); (2) furnishing of the information solicited is voluntary; and (3) the principal purpose for which the information is used by the U.S. Patent and Trademark Office is to process and/or examine your submission related to a patent application or patent. If you do not furnish the requested information, the U.S. Patent and Trademark Office may not be able to process and/or examine your submission, which may result in termination of proceedings or abandonment of the application or expiration of the patent.

The information provided by you in this form will be subject to the following routine uses:

1. The information on this form will be treated confidentially to the extent allowed under the Freedom of Information Act (5 U.S.C. 552) and the Privacy Act (5 U.S.C. 552a). Records from this system of records may be disclosed to the Department of Justice to determine whether disclosure of these records is required by the Freedom of Information Act.
2. A record from this system of records may be disclosed, as a routine use, in the course of presenting evidence to a court, magistrate, or administrative tribunal, including disclosures to opposing counsel in the course of settlement negotiations.
3. A record in this system of records may be disclosed, as a routine use, to a Member of Congress submitting a request involving an individual, to whom the record pertains, when the individual has requested assistance from the Member with respect to the subject matter of the record.
4. A record in this system of records may be disclosed, as a routine use, to a contractor of the Agency having need for the information in order to perform a contract. Recipients of information shall be required to comply with the requirements of the Privacy Act of 1974, as amended, pursuant to 5 U.S.C. 552a(m).
5. A record related to an International Application filed under the Patent Cooperation Treaty in this system of records may be disclosed, as a routine use, to the International Bureau of the World Intellectual Property Organization, pursuant to the Patent Cooperation Treaty.
6. A record in this system of records may be disclosed, as a routine use, to another federal agency for purposes of National Security review (35 U.S.C. 181) and for review pursuant to the Atomic Energy Act (42 U.S.C. 218(c)).
7. A record from this system of records may be disclosed, as a routine use, to the Administrator, General Services, or his/her designee, during an inspection of records conducted by GSA as part of that agency's responsibility to recommend improvements in records management practices and programs, under authority of 44 U.S.C. 2904 and 2906. Such disclosure shall be made in accordance with the GSA regulations governing inspection of records for this purpose, and any other relevant (i.e., GSA or Commerce) directive. Such disclosure shall not be used to make determinations about individuals.
8. A record from this system of records may be disclosed, as a routine use, to the public after either publication of the application pursuant to 35 U.S.C. 122(b) or issuance of a patent pursuant to 35 U.S.C. 151. Further, a record may be disclosed, subject to the limitations of 37 CFR 1.14, as a routine use, to the public if the record was filed in an application which became abandoned or in which the proceedings were terminated and which application is referenced by either a published application, an application open to public inspection or an issued patent.
9. A record from this system of records may be disclosed, as a routine use, to a Federal, State, or local law enforcement agency, if the USPTO becomes aware of a violation or potential violation of law or regulation.

Notice of Allowability	Application No. 15/589,968	Applicant(s) GIESE ET AL.	
	Examiner KIMBERLY CHONG	Art Unit 1674	AIA (First Inventor to File) Status No

-- The MAILING DATE of this communication appears on the cover sheet with the correspondence address--

All claims being allowable, PROSECUTION ON THE MERITS IS (OR REMAINS) CLOSED in this application. If not included herewith (or previously mailed), a Notice of Allowance (PTOL-85) or other appropriate communication will be mailed in due course. **THIS NOTICE OF ALLOWABILITY IS NOT A GRANT OF PATENT RIGHTS.** This application is subject to withdrawal from issue at the initiative of the Office or upon petition by the applicant. See 37 CFR 1.313 and MPEP 1308.

1. ☒ This communication is responsive to 07/21/2017.
☒ A declaration(s)/affidavit(s) under **37 CFR 1.130(b)** was/were filed on 07/13/2017.
2. ☐ An election was made by the applicant in response to a restriction requirement set forth during the interview on ____; the restriction requirement and election have been incorporated into this action.
3. ☒ The allowed claim(s) is/are 1-30. As a result of the allowed claim(s), you may be eligible to benefit from the **Patent Prosecution Highway** program at a participating intellectual property office for the corresponding application. For more information, please see http://www.uspto.gov/patents/init_events/pph/index.jsp or send an inquiry to PPHfeedback@uspto.gov.
4. ☐ Acknowledgment is made of a claim for foreign priority under 35 U.S.C. § 119(a)-(d) or (f).

Certified copies:

- a) ☐ All b) ☐ Some *c) ☐ None of the:
1. ☐ Certified copies of the priority documents have been received.
 2. ☐ Certified copies of the priority documents have been received in Application No. ____.
 3. ☐ Copies of the certified copies of the priority documents have been received in this national stage application from the International Bureau (PCT Rule 17.2(a)).

* Certified copies not received: ____.

Applicant has THREE MONTHS FROM THE "MAILING DATE" of this communication to file a reply complying with the requirements noted below. Failure to timely comply will result in ABANDONMENT of this application.

THIS THREE-MONTH PERIOD IS NOT EXTENDABLE.

5. ☐ CORRECTED DRAWINGS (as "replacement sheets") must be submitted.
☐ including changes required by the attached Examiner's Amendment / Comment or in the Office action of Paper No./Mail Date ____.
Identifying indicia such as the application number (see 37 CFR 1.84(c)) should be written on the drawings in the front (not the back) of each sheet. Replacement sheet(s) should be labeled as such in the header according to 37 CFR 1.121(d).
6. ☐ DEPOSIT OF and/or INFORMATION about the deposit of BIOLOGICAL MATERIAL must be submitted. Note the attached Examiner's comment regarding REQUIREMENT FOR THE DEPOSIT OF BIOLOGICAL MATERIAL.

Attachment(s)

- | | |
|--|--|
| 1. <input type="checkbox"/> Notice of References Cited (PTO-892) | 5. <input type="checkbox"/> Examiner's Amendment/Comment |
| 2. <input type="checkbox"/> Information Disclosure Statements (PTO/SB/08),
Paper No./Mail Date ____ | 6. <input checked="" type="checkbox"/> Examiner's Statement of Reasons for Allowance |
| 3. <input type="checkbox"/> Examiner's Comment Regarding Requirement for Deposit
of Biological Material | 7. <input type="checkbox"/> Other ____. |
| 4. <input type="checkbox"/> Interview Summary (PTO-413),
Paper No./Mail Date ____. | |

/KIMBERLY CHONG/
Primary Examiner, Art Unit 1674

The present application is being examined under the pre-AIA first to invent provisions.

The following is an examiner's statement of reasons for allowance: the Declaration under 37 C.F.R. 1.131 is sufficient to overcome the McSwiggen et al. reference US 20030190635 and therefore the 102 and 103 rejections are moot.

The claims are in condition for allowance.

Any comments considered necessary by applicant must be submitted no later than the payment of the issue fee and, to avoid processing delays, should preferably accompany the issue fee. Such submissions should be clearly labeled "Comments on Statement of Reasons for Allowance."

Conclusion

Any inquiry concerning this communication or earlier communications from the examiner should be directed to **Kimberly Chong** whose telephone number is **571-272-3111**. The examiner can normally be reached Monday thru Friday between 9-5 pm.

If attempts to reach the examiner by telephone are unsuccessful please contact the SPE for 1674 Ram Shukla at 571-272-0753. The fax phone number for the organization where this application or proceeding is assigned is 571-273-8300.


Patent applicants with problems or questions regarding electronic images that can be viewed in the Patent Application Information Retrieval system (PAIR) can now contact the USPTO's Patent Electronic Business Center (Patent EBC) for assistance. Representatives are available to answer your questions daily from 6 am to midnight

Art Unit: 1674

(EST). The toll free number is (866) 217-9197. When calling please have your application serial or patent number, the type of document you are having an image problem with, the number of pages and the specific nature of the problem. The Patent Electronic Business Center will notify applicants of the resolution of the problem within 5-7 business days. Applicants can also check PAIR to confirm that the problem has been corrected. The USPTO's Patent Electronic Business Center is a complete service center supporting all patent business on the Internet. The USPTO's PAIR system provides Internet-based access to patent application status and history information. It also enables applicants to view the scanned images of their own application file folder(s) as well as general patent information available to the public. For more information about the PAIR system, see <http://pair-direct.uspto.gov>.

For all other customer support, please call the USPTO Call Center (UCC) at 800-786-9199.


/Kimberly Chong/
Primary Examiner
Art Unit 1674

Issue Classification 	Application/Control No. 15589968	Applicant(s)/Patent Under Reexamination GIESE ET AL.	
	Examiner KIMBERLY CHONG	Art Unit 1674	

CPC						
Symbol					Type	Version
C12N		15		113	F	2013-01-01
C12N		15		1137	I	2013-01-01
C12Y		207		11001	I	2013-01-01
C12N		2310		14	A	2013-01-01
C12N		2310		321	A	2013-01-01
C12N		2310		322	A	2013-01-01
C12N		2310		531	A	2013-01-01
C12N		2320		51	A	2013-01-01

CPC Combination Sets				
Symbol	Type	Set	Ranking	Version

NONE (Assistant Examiner) _____ (Date) _____		Total Claims Allowed: 30	
/KIMBERLY CHONG/ Primary Examiner.Art Unit 1674 (Primary Examiner) _____ (Date) _____		O.G. Print Claim(s) 1	O.G. Print Figure none

Search Notes 	Application/Control No. 15589968	Applicant(s)/Patent Under Reexamination GIESE ET AL.
	Examiner KIMBERLY CHONG	Art Unit 1674

CPC- SEARCHED		
Symbol	Date	Examiner
C07H 2104	6/14/2017	KC

CPC COMBINATION SETS - SEARCHED		
Symbol	Date	Examiner

US CLASSIFICATION SEARCHED			
Class	Subclass	Date	Examiner

* See search history printout included with this form or the SEARCH NOTES box below to determine the scope of the search.

SEARCH NOTES		
Search Notes	Date	Examiner
EAST	6/14/2017	KC
PALM inventor name search	6/14/2017	KC
Google Scholar text search	6/14/2017	KC
updated	8/5/2017	KC

INTERFERENCE SEARCH			
US Class/ CPC Symbol	US Subclass / CPC Group	Date	Examiner
C07H	2104	8/5/2017	KC

--	--

PART B - FEE(S) TRANSMITTAL

Complete and send this form, together with applicable fee(s), to: **Mail** Mail Stop ISSUE FEE
Commissioner for Patents
P.O. Box 1450
Alexandria, Virginia 22313-1450
or Fax (571)-273-2885

INSTRUCTIONS: This form should be used for transmitting the ISSUE FEE and PUBLICATION FEE (if required). Blocks 1 through 5 should be completed where appropriate. All further correspondence including the Patent, advance orders and notification of maintenance fees will be mailed to the current correspondence address as indicated unless corrected below or directed otherwise in Block 1, by (a) specifying a new correspondence address; and/or (b) indicating a separate "TEE ADDRESS" for maintenance fee notifications.

CURRENT CORRESPONDENCE ADDRESS (Note: Use Block 1 for any change of address)

Note: A certificate of mailing can only be used for domestic mailings of the Fee(s) Transmittal. This certificate cannot be used for any other accompanying papers. Each additional paper, such as an assignment or formal drawing, must have its own certificate of mailing or transmission.

25226 7590 08/10/2017
MORRISON & FOERSTER LLP
755 PAGE MILL RD
PALO ALTO, CA 94304-1018

Certificate of Mailing or Transmission

I hereby certify that this Fee(s) Transmittal is being deposited with the United States Postal Service with sufficient postage for first class mail in an envelope addressed to the Mail Stop ISSUE FEE address above, or being facsimile transmitted to the USPTO (571) 273-2885, on the date indicated below.

(Depositor's name)
(Signature)
(Date)

APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
15/589,968	05/08/2017	Klaus GIESE	680132000106	8531

TITLE OF INVENTION: INTERFERING RNA MOLECULES

APPLN. TYPE	ENTITY STATUS	ISSUE FEE DUE	PUBLICATION FEE DUE	PREV. PAID ISSUE FEE	TOTAL FEE(S) DUE	DATE DUE
nonprovisional	UNDISCOUNTED	\$960	\$0	\$0	\$960	11/13/2017

EXAMINER	ART UNIT	CLASS-SUBCLASS
CHONG, KIMBERLY	1674	536-024500

1. Change of correspondence address or indication of "Fee Address" (37 CFR 1.363).

- ☐ Change of correspondence address (or Change of Correspondence Address form PTO/SB/122) attached.
- ☐ "Fee Address" indication (or "Fee Address" Indication form PTO/SB/47; Rev 03-02 or more recent) attached. **Use of a Customer Number is required.**

2. For printing on the patent front page, list

- (1) The names of up to 3 registered patent attorneys or agents OR, alternatively,
- (2) The name of a single firm (having as a member a registered attorney or agent) and the names of up to 2 registered patent attorneys or agents. If no name is listed, no name will be printed.

1 **Morrison & Foerster LLP**

2 _____

3 _____

3. ASSIGNEE NAME AND RESIDENCE DATA TO BE PRINTED ON THE PATENT (print or type)

PLEASE NOTE: Unless an assignee is identified below, no assignee data will appear on the patent. If an assignee is identified below, the document has been filed for recordation as set forth in 37 CFR 3.11. Completion of this form is NOT a substitute for filing an assignment.

(A) NAME OF ASSIGNEE

(B) RESIDENCE: (CITY and STATE OR COUNTRY)

Silence Therapeutics GMBH

Berlin, Germany

Please check the appropriate assignee category or categories (will not be printed on the patent): ☐ Individual ☒ Corporation or other private group entity ☐ Government

4a. The following fee(s) are submitted:

- ☒ Issue Fee
- ☒ Publication Fee (No small entity discount permitted)
- ☐ Advance Order - # of Copies _____

4b. Payment of Fee(s): (Please first reapply any previously paid issue fee shown above)

- ☐ A check is enclosed.
- ☐ Payment by credit card. Form PTO-2038 is attached.
- ☒ The director is hereby authorized to charge the required fee(s), any deficiency, or credits any overpayment, to Deposit Account Number **03-1952** (enclose an extra copy of this form).

5. Change in Entity Status (from status indicated above)

- ☐ Applicant certifying micro entity status. See 37 CFR 1.29
- ☐ Applicant asserting small entity status. See 37 CFR 1.27
- ☐ Applicant changing to regular undiscounted fee status.

NOTE: Absent a valid certification of Micro Entity Status (see forms PTO/SB/15A and 15B), issue fee payment in the micro entity amount will not be accepted at the risk of application abandonment.

NOTE: If the application was previously under micro entity status, checking this box will be taken to be a notification of loss of entitlement to micro entity status.

NOTE: Checking this box will be taken to be a notification of loss of entitlement to small or micro entity status, as applicable.

NOTE: This form must be signed in accordance with 37 CFR 1.31 and 1.33. See 37 CFR 1.4 for signature requirements and certifications.

Authorized Signature /Jian Xiao/

Date August 14, 2017

Typed or printed name Jian Xiao

Registration No. 55,748

Under the Paperwork Reduction Act of 1995, no persons are required to respond to a collection of information unless it displays a valid OMB control number.

TRANSMITTAL FOR POWER OF ATTORNEY TO ONE OR MORE REGISTERED PRACTITIONERS

NOTE: This form is to be submitted with the Power of Attorney by Applicant form (PTO/AIA/82B) to identify the application to which the Power of Attorney is directed, in accordance with 37 CFR 1.5, unless the application number and filing date are identified in the Power of Attorney by Applicant form. If neither form PTO/AIA/82A nor form PTO/AIA/82B identifies the application to which the Power of Attorney is directed, the Power of Attorney will not be recognized in the application.

Application Number	15/589,968		
Filing Date	May 8, 2017		
First Named Inventor	Klaus GIESE		
Title	INTERFERING RNA MOLECULES		
Art Unit	1674		
Examiner Name	K. Chong		
Attorney Docket Number	680132000106		
SIGNATURE of Applicant or Patent Practitioner			
Signature	/Jian Xiao/	Date (Optional)	August 14, 2017
Name	Jian Xiao	Registration Number	55,748
Title (if Applicant is a juristic entity)	Attorney of Record		
Applicant Name (if Applicant is a juristic entity)	Silence Therapeutics GMBH		
NOTE: This form must be signed in accordance with 37 CFR 1.33. See 37 CFR 1.4(d) for signature requirements and certifications. If more than one applicant, use multiple forms.			
<input type="checkbox"/> *Total of _____ forms are submitted.			

I hereby certify that this paper is being transmitted via the Office electronic filing system in accordance with 37 CFR § 1.6(a)(4).

Dated: August 14, 2017

Electronic Signature: /Doreen Rovetti/ (Doreen Rovetti)

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POWER OF ATTORNEY BY APPLICANT

I hereby revoke all previous powers of attorney given in the application identified in either the attached transmittal letter or the boxes below.

Application Number	Filing Date

(Note: The boxes above may be left blank if information is provided on form PTO/AIA/82A.)

- ☒ I hereby appoint the Patent Practitioner(s) associated with the following Customer Number as my/our attorney(s) or agent(s), and to transact all business in the United States Patent and Trademark Office connected therewith for the application referenced in the attached transmittal letter (form PTO/AIA/82A) or identified above: 25226
- OR
- ☐ I hereby appoint Practitioner(s) named in the attached list (form PTO/AIA/82C) as my/our attorney(s) or agent(s), and to transact all business in the United States Patent and Trademark Office connected therewith for the patent application referenced in the attached transmittal letter (form PTO/AIA/82A) or identified above. (Note: Complete form PTO/AIA/82C.)

Please recognize or change the correspondence address for the application identified in the attached transmittal letter or the boxes above to:

- ☒ The address associated with the above-mentioned Customer Number

OR

- ☐ The address associated with Customer Number:

OR

Firm or Individual Name			
Address			
City	State	Zip	
Country			
Telephone	Email		

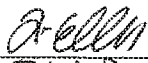
I am the Applicant (if the Applicant is a juristic entity, list the Applicant name in the box):

Silence Therapeutics GMBH

- ☐ Inventor or Joint Inventor (title not required below)
- ☐ Legal Representative of a Deceased or Legally Incapacitated Inventor (title not required below)
- ☒ Assignee or Person to Whom the Inventor is Under an Obligation to Assign (provide signer's title if applicant is a juristic entity)
- ☐ Person Who Otherwise Shows Sufficient Proprietary Interest (e.g., a petition under 37 CFR 1.46(b)(2) was granted in the application or is concurrently being filed with this document) (provide signer's title if applicant is a juristic entity)

SIGNATURE of Applicant for Patent

The undersigned (whose title is supplied below) is authorized to act on behalf of the applicant (e.g., where the applicant is a juristic entity).

Signature		Date (Optional)	14 Aug 2017
Name	DAVID ELLAM		
Title	CEO, Silence Therapeutics GMBH		

NOTE: Signature - This form must be signed by the applicant in accordance with 37 CFR 1.33. See 37 CFR 1.4 for signature requirements and certifications. If more than one applicant, use multiple forms.

- ☐ Total of _____ forms are submitted.

Attorney Docket No.: 680132800000

Electronic Patent Application Fee Transmittal				
Application Number:		15589968		
Filing Date:		08-May-2017		
Title of Invention:		INTERFERING RNA MOLECULES		
First Named Inventor/Applicant Name:		Klaus GIESE		
Filer:		Jian Xiao		
Attorney Docket Number:		680132000106		
Filed as Large Entity				
Filing Fees for Utility under 35 USC 111(a)				
Description	Fee Code	Quantity	Amount	Sub-Total in USD(\$)
Basic Filing:				
Pages:				
Claims:				
Miscellaneous-Filing:				
Petition:				
Patent-Appeals-and-Interference:				
Post-Allowance-and-Post-Issuance:				

Description	Fee Code	Quantity	Amount	Sub-Total in USD(\$)
UTILITY APPL ISSUE FEE	1501	1	960	960
STATUTORY OR TERMINAL DISCLAIMER	1814	1	160	160
Extension-of-Time:				
Miscellaneous:				
Total in USD (\$)				1120

Electronic Acknowledgement Receipt

EFS ID:	30068323
Application Number:	15589968
International Application Number:	
Confirmation Number:	8531
Title of Invention:	INTERFERING RNA MOLECULES
First Named Inventor/Applicant Name:	Klaus GIESE
Customer Number:	25226
Filer:	Jian Xiao
Filer Authorized By:	
Attorney Docket Number:	680132000106
Receipt Date:	14-AUG-2017
Filing Date:	08-MAY-2017
Time Stamp:	18:45:32
Application Type:	Utility under 35 USC 111(a)

Payment information:

Submitted with Payment	yes
Payment Type	DA
Payment was successfully received in RAM	\$ 1120
RAM confirmation Number	081517INTEFSW00006016031952
Deposit Account	
Authorized User	

The Director of the USPTO is hereby authorized to charge indicated fees and credit any overpayment as follows:

File Listing:					
Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)
1	Terminal Disclaimer Filed	Terminal_Disclaimer.pdf	115964	no	1
			595fa92622e5e8abd284678c5c8c49c9d076403a		
Warnings:					
Information:					
2	Issue Fee Payment (PTO-85B)	PTOL85_Part_B.pdf	195940	no	1
			82f35b6f5bb1a44aa770685bf0580952d5c58837		
Warnings:					
Information:					
3	Power of Attorney	Power_Of_Attorney.pdf	149294	no	2
			fc82d0844642ebc569d2fb606c4a043818b9dd2e		
Warnings:					
Information:					
4	Fee Worksheet (SB06)	fee-info.pdf	31682	no	2
			27dc394740f298f38aec49e6bcd44825b1092239		
Warnings:					
Information:					
Total Files Size (in bytes):			492880		

This Acknowledgement Receipt evidences receipt on the noted date by the USPTO of the indicated documents, characterized by the applicant, and including page counts, where applicable. It serves as evidence of receipt similar to a Post Card, as described in MPEP 503.

New Applications Under 35 U.S.C. 111

If a new application is being filed and the application includes the necessary components for a filing date (see 37 CFR 1.53(b)-(d) and MPEP 506), a Filing Receipt (37 CFR 1.54) will be issued in due course and the date shown on this Acknowledgement Receipt will establish the filing date of the application.

National Stage of an International Application under 35 U.S.C. 371

If a timely submission to enter the national stage of an international application is compliant with the conditions of 35 U.S.C. 371 and other applicable requirements a Form PCT/DO/EO/903 indicating acceptance of the application as a national stage submission under 35 U.S.C. 371 will be issued in addition to the Filing Receipt, in due course.

New International Application Filed with the USPTO as a Receiving Office

If a new international application is being filed and the international application includes the necessary components for an international filing date (see PCT Article 11 and MPEP 1810), a Notification of the International Application Number and of the International Filing Date (Form PCT/RO/105) will be issued in due course, subject to prescriptions concerning national security, and the date shown on this Acknowledgement Receipt will establish the international filing date of the application.

TERMINAL DISCLAIMER TO OBVIATE A PROVISIONAL DOUBLE PATENTING REJECTION OVER A PENDING "REFERENCE" APPLICATION	Docket Number (Optional) 680132000106
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In re Application of: Klaus GIESE et al.

Application No.: 15/589,968

Filed: May 8, 2017

For:

INTERFERING RNA MOLECULES

The applicant, Silence Therapeutics GMBH, owner of 100 percent interest in the instant application hereby disclaims, except as provided below, the terminal part of the statutory term of any patent granted on the instant application which would extend beyond the expiration date of the full statutory term of any patent granted on pending reference Application Numbers

15/589,971	May 08, 2017
15/594,349	May 12, 2017
15/594,438	May 12, 2017

filed,

as the term of any patent granted on said reference application may be shortened by any terminal disclaimer filed prior to the grant of any patent on the pending reference application. The applicant hereby agrees that any patent so granted on the instant application shall be enforceable only for and during such period that it and any patent granted on the reference application are commonly owned. This agreement runs with any patent granted on the instant application and is binding upon the grantee, its successors or assigns.

In making the above disclaimer, the applicant does not disclaim the terminal part of any patent granted on the instant application that would extend to the expiration date of the full statutory term of any patent granted on said reference application, "as the term of any patent granted on said reference application may be shortened by any terminal disclaimer filed prior to the grant of any patent on the pending reference application," in the event that: any such patent granted on the pending reference application expires for failure to pay a maintenance fee, is held unenforceable, is found invalid by a court of competent jurisdiction, is statutorily disclaimed in whole or terminally disclaimed under 37 CFR 1.321, has all claims canceled by a reexamination certificate, is reissued, or is in any manner terminated prior to the expiration of its full statutory term as shortened by any terminal disclaimer filed prior to its grant.

Check either box 1 or 2 below, if appropriate.

1. ☒ The undersigned is the applicant. If the applicant is an assignee, the undersigned is authorized to act on behalf of the assignee.

I hereby acknowledge that any willful false statements made are punishable under 18 U.S.C. 1001 by fine or imprisonment of not more than five (5) years, or both.

2. ☐ The undersigned is an attorney or agent of record. Reg. No. _____


Signature

14 Aug 2017
Date


DAVID ELLAM
Typed or printed name

CFO
Title

020 3457 6900
Telephone Number

- ☒ Terminal disclaimer fee under 37 CFR 1.20(d) is included.

WARNING: Information on this form may become public. Credit card information should not be included on this form. Provide credit card information and authorization on PTO-2038.

Application Number 	Application/Control No. 15/589,968	Applicant(s)/Patent under Reexamination GIESE ET AL.	
Document Code - DISQ		Internal Document – DO NOT MAIL	

TERMINAL DISCLAIMER	<input checked="" type="checkbox"/> APPROVED	<input type="checkbox"/> DISAPPROVED
Date Filed : 14 August, 2017	This patent is subject to a Terminal Disclaimer	

Approved/Disapproved by:
<u>/CRYSTAL QUEEN/</u> Technology Center: <u>PLRC</u> Telephone: _____

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Alexandria, Virginia 22313-1450
www.uspto.gov

APPLICATION NUMBER	FILING OR 371(C) DATE	FIRST NAMED APPLICANT	ATTY. DOCKET NO./TITLE
15/589,968	05/08/2017	Klaus GIESE	680132000106

25226
MORRISON & FOERSTER LLP
755 PAGE MILL RD
PALO ALTO, CA 94304-1018

CONFIRMATION NO. 8531
POA ACCEPTANCE LETTER



Date Mailed: 08/17/2017

NOTICE OF ACCEPTANCE OF POWER OF ATTORNEY

This is in response to the Power of Attorney filed 08/14/2017.

The Power of Attorney in this application is accepted. Correspondence in this application will be mailed to the above address as provided by 37 CFR 1.33.

Questions about the contents of this notice and the requirements it sets forth should be directed to the Office of Data Management, Application Assistance Unit, at (571) 272-4000 or (571) 272-4200 or 1-888-786-0101.

/kxaysana/



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Alexandria, Virginia 22313-1450
www.uspto.gov

APPLICATION NUMBER	FILING OR 371(C) DATE	FIRST NAMED APPLICANT	ATTY. DOCKET NO./TITLE
15/589,968	05/08/2017	Klaus GIESE	680132000106

CONFIRMATION NO. 8531

PUBLICATION NOTICE



OC000000093800119

25226
MORRISON & FOERSTER LLP
755 PAGE MILL RD
PALO ALTO, CA 94304-1018

Title:INTERFERING RNA MOLECULES

Publication No.US-2017-0247697-A1

Publication Date:08/31/2017

NOTICE OF PUBLICATION OF APPLICATION

The above-identified application will be electronically published as a patent application publication pursuant to 37 CFR 1.211, et seq. The patent application publication number and publication date are set forth above.

The publication may be accessed through the USPTO's publically available Searchable Databases via the Internet at www.uspto.gov. The direct link to access the publication is currently <http://www.uspto.gov/patft/>.

The publication process established by the Office does not provide for mailing a copy of the publication to applicant. A copy of the publication may be obtained from the Office upon payment of the appropriate fee set forth in 37 CFR 1.19(a)(1). Orders for copies of patent application publications are handled by the USPTO's Public Records Division. The Public Records Division can be reached by telephone at (571) 272-3150 or (800) 972-6382, by facsimile at (571) 273-3250, by mail addressed to the United States Patent and Trademark Office, Public Records Division, Alexandria, VA 22313-1450 or via the Internet.

In addition, information on the status of the application, including the mailing date of Office actions and the dates of receipt of correspondence filed in the Office, may also be accessed via the Internet through the Patent Electronic Business Center at www.uspto.gov using the public side of the Patent Application Information and Retrieval (PAIR) system. The direct link to access this status information is currently <https://portal.uspto.gov/pair/PublicPair>. Prior to publication, such status information is confidential and may only be obtained by applicant using the private side of PAIR.

Further assistance in electronically accessing the publication, or about PAIR, is available by calling the Patent Electronic Business Center at 1-866-217-9197.

Office of Data Management, Application Assistance Unit (571) 272-4000, or (571) 272-4200, or 1-888-786-0101



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APPLICATION NO.	ISSUE DATE	PATENT NO.	ATTORNEY DOCKET NO.	CONFIRMATION NO.
15/589,968	10/17/2017	9790501	680132000106	8531

25226 7590 09/27/2017
MORRISON & FOERSTER LLP
755 PAGE MILL RD
PALO ALTO, CA 94304-1018

ISSUE NOTIFICATION

The projected patent number and issue date are specified above.

Determination of Patent Term Adjustment under 35 U.S.C. 154 (b) (application filed on or after May 29, 2000)

The Patent Term Adjustment is 0 day(s). Any patent to issue from the above-identified application will include an indication of the adjustment on the front page.

If a Continued Prosecution Application (CPA) was filed in the above-identified application, the filing date that determines Patent Term Adjustment is the filing date of the most recent CPA.

Applicant will be able to obtain more detailed information by accessing the Patent Application Information Retrieval (PAIR) WEB site (<http://pair.uspto.gov>).

Any questions regarding the Patent Term Extension or Adjustment determination should be directed to the Office of Patent Legal Administration at (571)-272-7702. Questions relating to issue and publication fee payments should be directed to the Application Assistance Unit (AAU) of the Office of Data Management (ODM) at (571)-272-4200.

APPLICANT(s) (Please see PAIR WEB site <http://pair.uspto.gov> for additional applicants):

Silence Therapeutics GMBH, Berlin, GERMANY;
Klaus GIESE, Berlin, GERMANY;
Jörg KAUFMANN, Berlin, GERMANY;
Anke KLIPPEL-GIESE, Berchtesgaden, GERMANY;

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