

# Chemical and Structural Diversity of siRNA Molecules

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**Abstract:** Short interfering RNAs (siRNAs) are 21-23 nt long double-stranded oligoribonucleotides which in mammalian cells exhibit a potency for sequence-specific gene silencing via an RNA interference (RNAi) pathway. It has been already proven that exogenous, chemically synthesized siRNA molecules are effective inhibitors of gene expression and are widely applied for analysis of protein function and proteomics-based target identification. Moreover, since their discovery siRNA molecules have been implemented as potential candidates for therapeutic applications. Various modified siRNA molecules containing sugar modifications (2'-OMe, -F, -O-allyl, -amino, orthoesters and LNA analogues), internucleotide phosphodiester bond modifications (phosphorothioates, boranophosphates), base modifications ( $s^2U$ ) as well as 3'-terminal cholesterol-conjugated constructs were investigated as potential candidates for effective inhibition of gene expression. This chapter reviews an impact of chemical and structural modifications of siRNA molecules on their serum and thermal stability, cellular and *in vivo* activity, cellular uptake, biodistribution and cytotoxicity. Functional analysis of chemically modified siRNA molecules allows for better understanding of the mechanism of the RNA interference process as well as demonstrates immense efforts in optimizing *in vivo* potency of siRNA molecules for RNAi-based drug design.

**Keywords:** RNA interference, small interfering RNA, gene silencing, siRNA, modified siRNA, chemical modification, RNA structure.

## INTRODUCTION

An initial enthusiasm paid to antisense strategy developed in 80-ties of past century using DNA oligonucleotides and their analogues has not fulfilled early expectations to date [1, 2]. Only one antisense oligo(nucleoside phosphorothioate) has been approved till now as a drug against cytomegaloviral (CMV) infection in HIV-1 infected patients (Fomivirsen, Vitravene<sup>®</sup>, Isis Pharmaceuticals, 1998) [3]. Another oligonucleotide, active as an aptamer of vascular endothelial growth factor (VEGF) was approved by FDA in 2005 (Macugen<sup>®</sup>, Pfizer) for age-related macular degeneration (AMD) therapy [4]. New hopes for sequence-specific control of gene expression emerged with the discovery of RNA interference (RNAi) mechanism. Shortly after Fire's first report on RNAi in *Caenorhabditis elegans* [5], some of its features were found common to other mechanisms of post-transcriptional gene silencing in many different species like plants [6], fungi [7] and insects [8]. In these mechanisms common is the function of short ribonucleic acids, so far much underestimated as less stable members of nucleic acids family.

The post-transcriptional gene silencing is based on degradation of mRNA complementary to exogenous double stranded RNA. Three years after discovery of the phenomenon of RNA interference, the process was intentionally induced in mammalian cells [9]. That was possible due to the use of synthetic short interfering RNA (siRNA) duplexes which do not induce antiviral defense system in eukaryotic

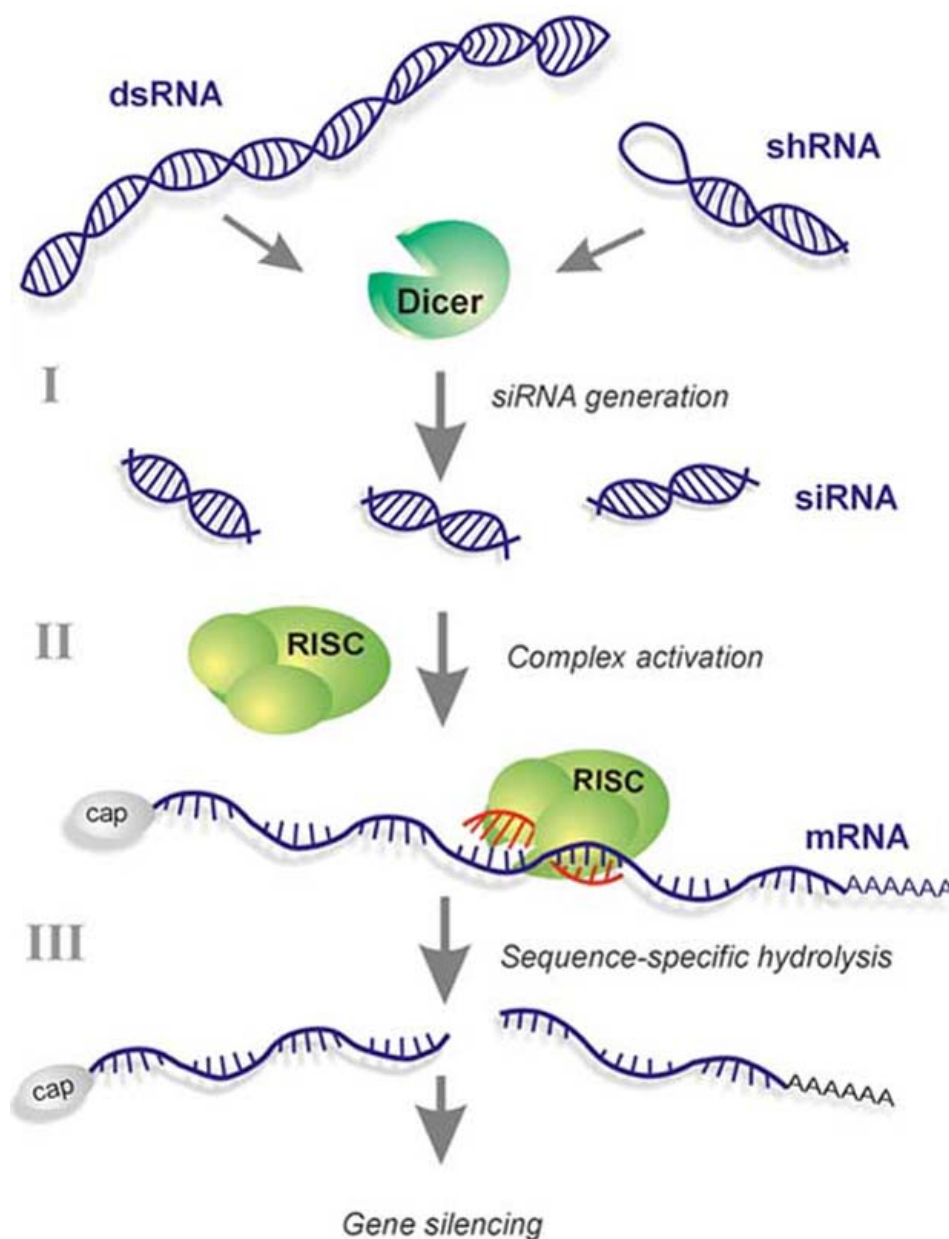
cells. In spite of the fact that antiviral defense system recognizes long double stranded RNA (> 30 bp) as a signal for apoptosis and induces interferon response [10], scientists soon started to consider siRNA molecules as the most promising tool for gene silencing in mammalian cells as well as in higher organisms.

Although siRNA molecules suffer from their low stability *in vivo*, insufficient cellular uptake and induced off-target effects, they seem to be more useful than antisense oligonucleotides for analysis of function of newly discovered genes, and in silencing of genes to combat certain diseases. Nonetheless, significant progress was made to overcome these difficulties, which capitalized on knowledge gained during attempts at chemical modification of antisense oligonucleotides. These achievements made the siRNA molecules more potent and more useful, albeit yet much unpredictable. Thus, application of the siRNA molecules requires further studies, also in respect to new chemical modifications of these tools improving their in-serum and thermal stability, intracellular and *in vivo* activity, cellular uptake, biodistribution and low cytotoxicity.

## RNAi IN MAMMALIAN CELLS

In general, an RNAi phenomenon involves three major steps [see Fig. (1)]. Within the first step, the Dicer nuclease specifically hydrolyzes long double stranded RNA into siRNAs. The one strand of the resulting siRNA duplex is incorporated into a protein complex, called RISC (RNA Induced Silencing Complex). Activated in this way RISC hydrolyzes phosphodiester bond in the target mRNA in a region complementary to the guide strand of siRNA [11, 12]. The steps of Dicer-promoted formation of siRNA and unwinding of siRNA duplex just before incorporation of one strand into the RISC [13], both depend on ATP. However,

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**Fig. (1).** Mechanism of gene silencing by induction of RNA interference. Step I: long double stranded RNA is recognized by nuclease Dicer and cleaved into 21-23 nt short interfering RNAs (siRNAs). Step II: nuclease-helicase complex RISC (RNA Induced Silencing Complex) is activated by incorporation of the antisense strand of siRNA duplex. Step III: activated RISC guided by the antisense strand of siRNA recognizes complementary strand of mRNA and catalyzes cleavage of the target sequence. The cleaved mRNA is further degraded by cellular nucleases. Degradation of mRNA leads to silencing of the coded gene.

neither the exact mechanism nor the proteins involved in these processes are so far well established. The RISC-promoted hydrolysis of mRNA does not consume ATP and takes place only in the presence of  $Mg^{2+}$  cations to yield the 5'-end phosphorylated product [14, 15]. The cleaved mRNA, deprived of the natural protection at the cleavage site, is immediately recognized by the cellular nucleases and promptly degraded. It is assumed that the activated RISC may hydrolyze several molecules of the target mRNA [13, 16, 17], while bound siRNA chain acts as the guide strand

recognizing the target sequence within a coding region of mRNA [15].

Another mechanism resulting in steric blocking of protein biosynthesis is switched on when the RNA strand present in RISC complex is not fully complementary to the 3'-untranslated region of mRNA (3'-UTR) [18-20]. In this case, the RNA strand incorporated into RISC comes from so called micro RNA family of short RNA molecules possessing a hairpin structure. The principal difference between RNAi and micro RNA mechanisms is the origin of

precursors, which for miRNA are encoded in the genome. Short RNAs may also regulate gene expression during the transcription as they are involved in condensation of heterochromatin [21]. In all these processes similar proteins (belonging to the Argonaute family) are involved. However, natural mechanisms of control of the gene expression, in which short nucleic acids play a main role, are not fully understood yet and it is difficult to assess without doubts possible relations and similarities.

The process of RNA interference in mammalian cells can be induced with the omitting of the first step [Fig. (1)]. Sequence specific gene silencing in mammalian cells was achieved by delivery to the cells of either ready to use siRNAs [9, 12] or their precursors, short hairpin RNAs (shRNAs), which are the substrates for the Dicer nuclease [22, 23]. The siRNA and shRNAs molecules can be generated endogenously or synthesized by chemical either enzymatic approach. Among different vectors encoding shRNA under the control of the RNA polymerase III (Pol III) promoters the viral vectors including lentiviral and adeno-associated vectors seem to be the most promising [24, 25]. Unfortunately, these strategies suffer from possible severe side effects like restoration of the viral virulence. Another approach was explored based on the synthesis of the total pool of so called esiRNAs, which could be generated *in vitro* by RNase III-assisted cleavage of long double stranded RNA complementary to the target gene [26]. The effectiveness of prepared on such way oligomers is usually high, but selection of sufficiently specific target sequence is difficult. Historically first and still widely used method of preparation of siRNA or shRNA is the chemical synthesis, which offers full control of the structure of the product in respect to the sequence and purposely introduced chemical modifications. Moreover, synthetic siRNA molecules, possessing chemical modifications in defined regions of the duplex, enabled to gain information on the mechanism of binding of the siRNA strand into RISC [27, 28].

The aim of this account is to present the state of the art concerning the knowledge on synthetic siRNAs, their desired features, selection of active target sequences, and new options coming from chemical modifications in duplexes.

## STRUCTURAL DIVERSITY OF siRNA DUPLEXES

siRNA molecules were identified in cellular extracts from *Drosophila melanogaster* embryos transfected with dsRNA [12]. The majority of isolated short RNAs were 20-23-bp duplexes. Further experiments demonstrated that the most potent siRNA molecules were 19-bp RNA duplexes with 2-nt overhangs at their 3'-ends [29] [Fig. (2A)]. These two overhanging nucleotides could be replaced with DNA units, preferably thymidines [29]. Subsequent studies showed that such modified duplexes were less active at lower concentrations [30]. The family of siRNA duplexes has been extended recently with blunt-ended constructs [31, 32]. Also, increased activity was found for fork siRNA duplexes possessing not fully complementary (two mismatches) blunt ends [33]. The presence of the two unpaired nucleotides might be beneficial for the RNA-protein interaction due to the dinucleotide-binding pocket identified at PAZ domain of Argonaute protein [34-36]. However, since the pattern of interactions within RISC is not

well understood, above experimental observations of high activity of blunt-ended siRNAs must be interpreted with caution.

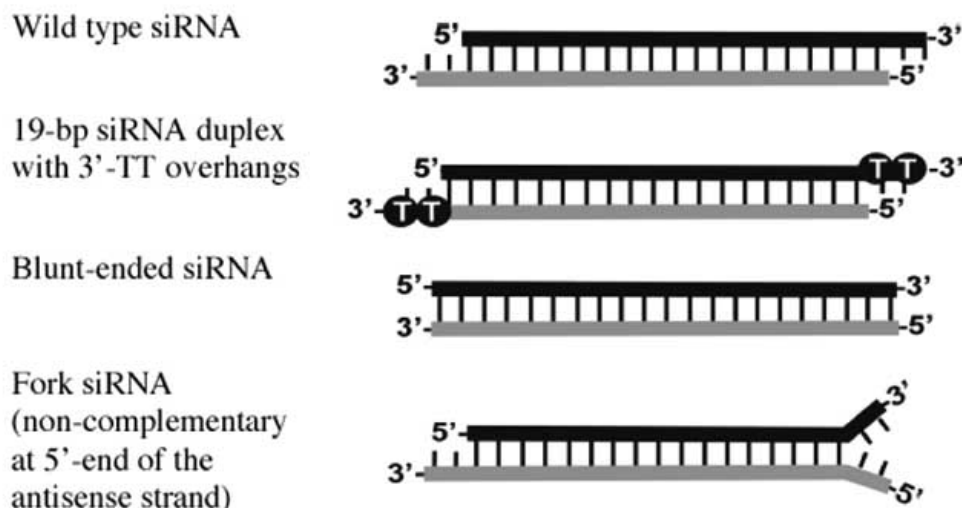
The siRNA duplexes of various lengths were tested for their ability to induce RNA interference. Recently, it was demonstrated that 27-bp RNA duplexes were the substrates for Dicer nuclease and were very active in cellular system [37, 38]. Also shorter 19-bp blunt siRNA duplexes exhibited remarkable silencing activity, while 17-bp constructs were non-effective, even in the presence of additional target-specific overhangs [31]. Interestingly, a 19-bp long siRNA duplex not fully complementary to the terminal positions of the target sequence (with up to four mismatches) still mediated efficient gene silencing, suggesting that the base pairing with the target mRNA is not the most decisive factor determining the minimal length of functional siRNA.

Important information was gained from chemical modifications of different regions of the siRNA duplex. It was found that the 3'-ends could be freely modified, while the 5'-ends, especially the 5'-end of the antisense strand, does not tolerate any modification [Fig. (2B)]. The recently published structure of the PIWI domain of RNA-binding Argonaute protein confirmed the importance of the 5'-phosphate group for the interaction within RISC [39, 40].

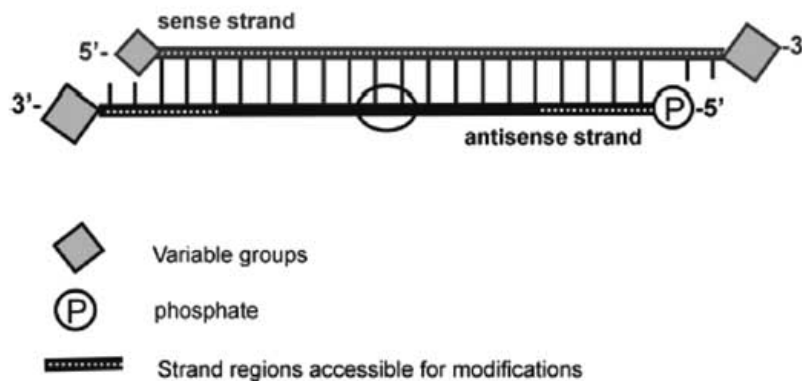
The effects caused by point mutations differed depending on the site of modification. Modifications within the sense strand did not affect the activity, even if several mismatches were introduced. Single modifications close to the ends of the antisense strand did not reduce the activity significantly on the contrary to those introduced in the middle [41, 42]. These observations are in accordance with the *in vitro* analysis showing that the cleavage of target mRNA takes place at the phosphate opposite of nucleotides 10 and 11 of the guide strand of siRNA (starting from the 5'-end phosphate moiety) [29]. Notably, the synthetic siRNAs are effectively phosphorylated at the 5'-end by intracellular kinases [16, 17, 43].

One has to notice that different siRNA duplexes, designed according to the mentioned above rules (duplex length, presence of 3'-overhangs, sequence complementarity in the centre and presence of the phosphate group at the 5'-terminus of the antisense strand), act differently in silencing of the same target gene. It is well known that even small siRNA sequence shift significantly alters silencing activity [44]. Such observations support conclusion that siRNA sequence itself, and not a local secondary structure of the target mRNA molecule, is crucial for duplex potency. However, there are some reports supporting opposite dependence [45-47]. Important information about siRNA activity determinants was obtained from studies on the mechanism of incorporation of the RNA strand into RISC. It was found that the unwinding of siRNA duplex is not symmetrical and the strand finally incorporated is that with weaker hybridization at its 5'-end [27, 28]. Decisive influence on activity of siRNA is attributed to the nucleotide composition – preferably with more A and U units close to the 5'-end of the strand dedicated as a guide strand. Moreover, sequences able to form structures of higher order should be avoided. These rules were used for algorithms of web browsers of potentially active sequences of siRNAs,

A



B



**Fig. (2).** (A) Structural diversity of active siRNA duplexes. (B) Possible chemical modification sites in siRNA duplex. Modifications located at 3'-ends of the sense and antisense strands as well as at 5'-end of the sense strand are accepted to maintain siRNA silencing activity. Phosphate group at the 5' terminus of the antisense strand is required for functional siRNA duplex. Modifications at this site are allowed only when they are attached to the terminal phosphate group. Introduction of modifications at the centre of the antisense strand (opposite to scissile phosphate, marked with an ellipse) leads to a loss of the silencing activity of the siRNA duplex.

offered by vendors synthesizing siRNAs (e.g. Dharmacon, Ambion) [48, 49] or by independent web browsers [50, 51]. One of such tools is Deqor program [52] searching for longer sequences suitable for generation of esiRNA molecules. There is no algorithm assuring high activity of the selected siRNA. Despite of the careful selection of the target sequence and its specificity, siRNAs may affect the expression of many other genes in a cell, causing so called "off-target effects" [53-58]. This fact emphasizes the importance of detailed search for intracellular processes in which siRNA-like molecules are involved. There is also some hope that observed off-target effects, which are concentration-dependent, can be limited by application of new modifications making the siRNA probes more effective.

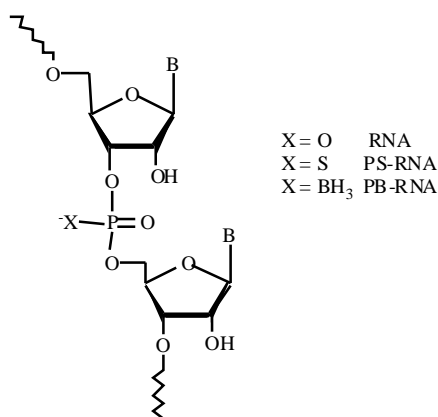
#### CHEMICAL MODIFICATIONS OF siRNA MOLECULES

RNA interference induced by synthetic siRNA molecules is a transient process. In mammalian cells re-expression of

the target gene usually occurs after few days [59]. Therefore, an improvement of cellular stability of the siRNA molecules is of interest to design *in vivo* effective gene silencers. Although variety of chemical modifications of RNA strands are intended mostly to enhance activity and specificity, an increased stability of siRNA in living systems is highly appreciated. Up to now several review papers summarizing the influence of the chemical modifications within siRNA strands on the *in vitro* and *in vivo* activity and specificity of siRNA molecules were published [60-63].

#### Chemical Modifications Improving Resistance of siRNA Molecules Towards Nucleases

Natural nucleic acids are easily degraded by intracellular nucleases. This process may be slowed down, or even terminated, by chemical modification of oligonucleotide strands of siRNA duplexes. Chemical modifications of siRNA molecules fall into several categories, including modifications of the internucleotide phosphodiester bond



**Fig. (3).** Structures of RNA ( $X=O$ ), phosphorothioate RNA ( $X=S$ ) and boranophosphate RNA ( $X=BH_3$ ) fragments.

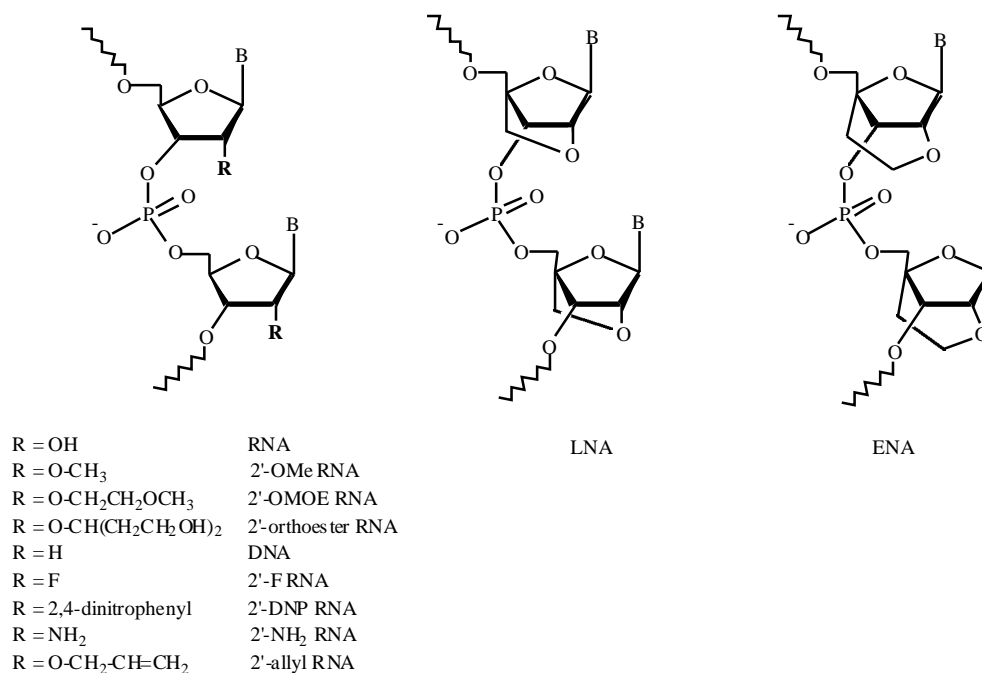
[Fig. (3)], ribose ring (substituent at 2'-carbon atom, LNA, ENA, see Fig. (4)), nucleobases [Fig. (5)] as well as those with different "tags" attached at either 5'- or 3'-ends [Fig. (6)].

### Modifications of the Phosphodiester Bond

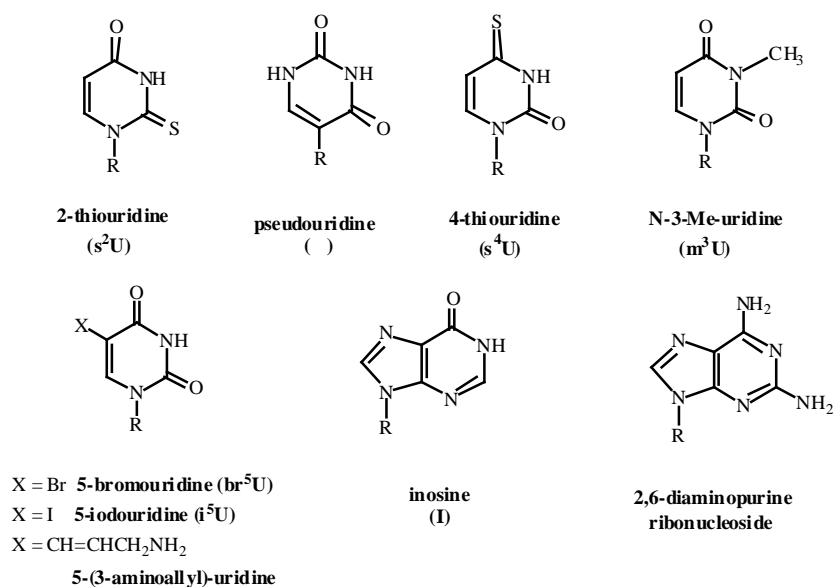
Replacement of sulfur atom for one of the non-bridging oxygen atoms in phosphate internucleotide bond is widely used to protect exogenous oligonucleotides against nucleolytic hydrolysis [64-66] [Fig. (3)]. Such strategy was widely used to protect antisense DNA oligonucleotides tested for therapeutic applications [67-69]. The diastereomeric mixture of the chemically synthesized phosphorothioate oligonucleotides forms thermodynamically less stable duplexes with

complementary RNA strands [70]. The stability of the PS-siRNA duplexes in serum is very high (up to 72 h of incubation) in comparison to PO-siRNA duplexes [71-73]. In general, phosphorothioate modifications of siRNA duplexes do not interfere with their silencing activity [41, 44, 70, 74], although it was also shown that PS-siRNAs alter silencing from medium to high level [72]. In experiments reported by Harborth *et al.* [44], phosphorothioate bonds introduced at the 5'- and 3'-ends of both, antisense and sense strands of siRNA, or at juxtaposed position of the oligonucleotide chains did maintain strong efficiency for silencing of lamin A/C. On the contrary, Chiu and Rana reported that the presence of the phosphorothioate modifications in the sense strand (which is not involved in the mRNA hydrolysis) reduced the activity of siRNA by ca. 40 % [71]. Numerous reports presenting applications of various PS-siRNA molecules for effective silencing of the target genes are summarized in Table 1. Some of them describe PS-siRNAs with increased toxicity, especially those containing more than half of the internucleotide bonds modified [41, 44, 72]. This unwanted effect was avoided by the use of the siRNA in lower concentrations [72]. An increased nuclease resistance of PS-modified siRNAs is beneficial for their pharmacokinetic properties [72]. The *in vivo* studies showed that the siRNA duplexes consisting of antisense PO- and sense PS-RNA strands were present in blood of animals at higher concentration than their unmodified PO/PO counterparts [73].

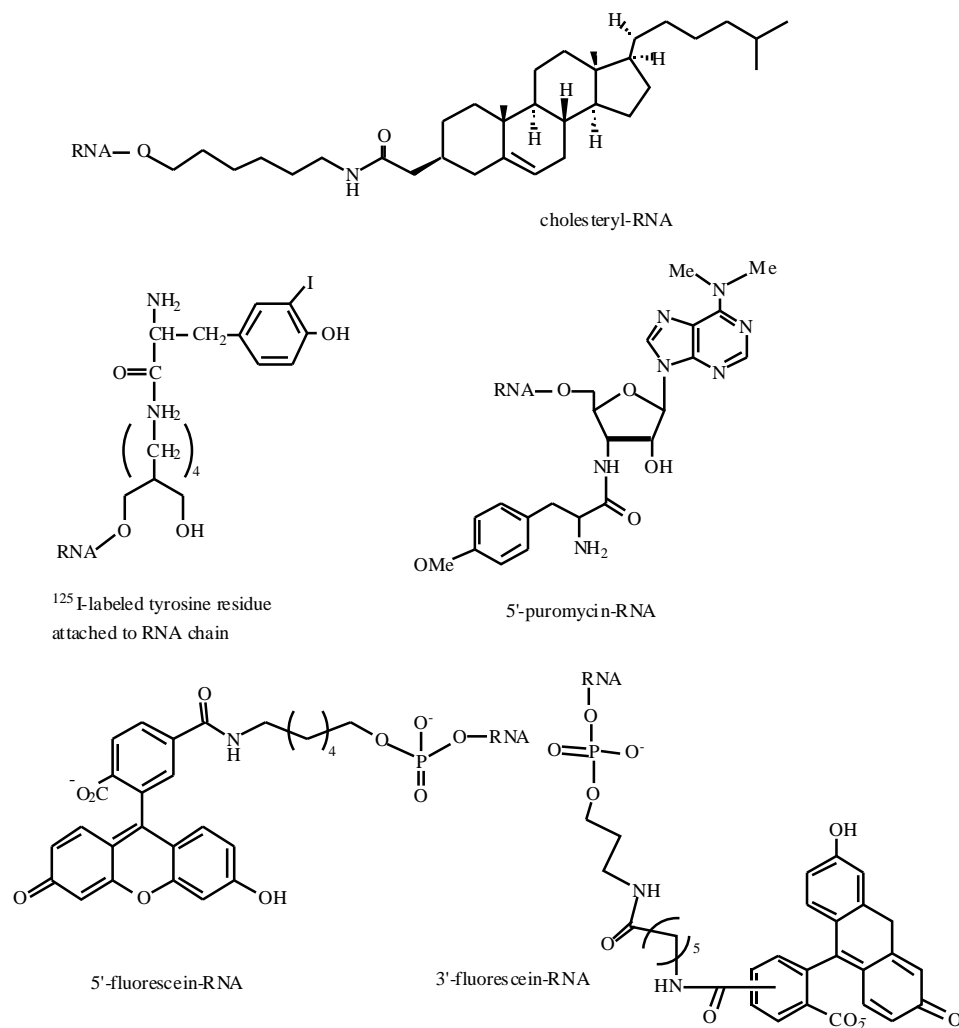
Boranophosphate analogues of siRNA obtained enzymatically using stereospecific T7 RNA polymerase represent another interesting tool for potential therapeutic application [75]. These compounds obtained as stereodefined diastereoisomers of  $S_P$  configuration at the P-chiral center (all- $S_P$  oligomers) were more lypophilic (yet carrying a negative



**Fig. (4).** Structures of RNA analogues possessing modifications in the 2'-position of the ribose ring. B constitutes nucleobase (uracil, cytosine, adenine or guanine) residue.



**Fig. (5).** Structures of base-modified ribonucleosides used for siRNA structure/activity studies. R constitutes ,D-ribofuranoside residue.



**Fig. (6).** Structures of RNA conjugates with 3'-attached cholesteryl residue, radioactively labeled tyrosine residue or with fluorescein attached at 3'- or 5'-terminus of the sense strand.

Table 1. Selected Examples of Silencing Activity of PS-Modified siRNA Molecules

Duplex ID	Site of modification (underlined) 5' 3' (sense) 3' 5' (antisense)	Target gene/cells	siRNA concentr./ time	siRNA activity (% of degraded mRNA or silenced protein)	Ref.
hCav PS4	<u>gg</u> oooooooooooooooo <u>TT</u> <u>TT</u> oooooooooooooooo <u>gg</u>	hCav/HeLa	200 nM/ 48 h	>95 (>95% native siRNA)	[70]
hCav PS8	<u>gggg</u> oooooooooooooooo <u>ggTT</u> <u>TTgg</u> oooooooooooooooo <u>gggg</u>			~ 93	
hCav PS12	<u>ggoooo</u> oooooooooooooooo <u>ggooooTT</u> <u>TTggoooo</u> oooooooooooooooo <u>ggoooo</u>			~90	
hCav PS16	<u>ggoooooo</u> oooooooooooooooo <u>ggooooTT</u> <u>TTggoooooo</u> oooooooooooooooo <u>ggoooooo</u>			~85	
hCav PS21	<u>ggoooooo</u> oooooooooooooooo <u>ggooooTT</u> <u>TTggoooooo</u> oooooooooooooooo <u>ggoooooo</u>			~75	
Thio siRNA-2a	oo <u>ggg</u> oooooooooooo <u>ggg</u> ooUU UUoooooooooooooooooooo	Luc/HeLa	10 nM/ 48 h	95 (82% native siRNA)	[72]
Thio siRNA-2b	o <u>ggg</u> oooooooooooo <u>ggg</u> ooUU UUoooooooooooooooooooo			86	
Thio siRNA-2c	<u>ggg</u> oooooooooooo <u>ggg</u> ooUU UUoooooooooooooooooooo			87	
Thio siRNA-2d	<u>ggg</u> oooooooooooo <u>ggg</u> ooUU UUoooooooooooooooooooo			94	
Thio siRNA-2e	o <u>ggg</u> oooooooooooo <u>ggg</u> ooUU UUoooooooooooooooooooo			84	
Thio siRNA-2f	ooo <u>ggg</u> oooooooooooo <u>ggg</u> ooUU UUoooooooooooooooooooo			81	
SS/AS-P-S	oooooooooooooooooooo <u>TT</u> <u>TT</u> oooooooooooooooooooo	EGFP-C1/ HeLa	50 nM/ 42 h	42 (95% native siRNA)	[71]
SS-P-S/AS	<u>ooooooooooooooooooooTT</u> TToooooooooooooooooooo			62	
DS-P-S	<u>ooooooooooooooooooooTT</u> <u>TT</u> oooooooooooooooooooo			47	
hTF167i P1+1	<u>g</u> oooooooooooooooooooo <u>UA</u> <u>GC</u> oooooooooooooooooooo	TF/HeCaT	100 nM/ 24 h	~ 88 (90% native siRNA)	[41]
hTF167i P0+2	oooooooooooooooooooo <u>UA</u> <u>GC</u> oooooooooooooooooooo			~ 88	
hTF167i P2+2	<u>gg</u> oooooooooooooooooooo <u>UA</u> <u>GC</u> oooooooooooooooooooo			~ 82	
hTF167i P2+4	<u>gg</u> oooooooooooooooooooo <u>ggUA</u> <u>GCgg</u> oooooooooooooooooooo			~ 80	

(Table 1) Contd....

Duplex ID	Site of modification (underlined) 5' 3' (sense) 3' 5' (antisense)	Target gene/cells	siRNA concentr./ time	activity (%) of degraded mRNA or silenced protein)	Ref.
s/as-ps(even#)	ooooooooooooooooooooTT TT <u>oooooooooooooooooooo</u>	Lamin A/C / 3T3	100 nM / 68 h	~90 (90% native siRNA)	[44]
s/as-ps(19-21)	ooooooooooooooooooooTT TT <u>oooooooooooooooooooo</u>			~ 87	
s/as-ps(2)	ooooooooooooooooooooTT TToooooooooooooooo <u>oo</u>			~85	
s-ps(even#) /as	<u>oooooooooooooooooooo</u> TT TToooooooooooooooooooo			~90	
s-ps(19-21) /as	oooooooooooooooooooo <u>TT</u> TToooooooooooooooooooo			~90	
s-ps(2) /as	<u>oooooooooooooooooooo</u> TT TToooooooooooooooooooo			~90	
s-ps(even#) /as-ps(even#)	<u>oooooooooooooooooooo</u> TT TT <u>oooooooooooooooooooo</u>			~87	
s-ps(19-21)/as-ps(19-21)	oooooooooooooooooooo <u>TT</u> TT <u>oooooooooooooooooooo</u>			~80	
s-ps(2)/as-ps(2)	<u>oooooooooooooooooooo</u> TT TToooooooooooooooo <u>oo</u>			~ 85	
s-ps(19-21)/as-ps(19-21)	oooooooooooooooooooo <u>TT</u> TT <u>oooooooooooooooooooo</u>	Lamin A/C / HeLa	100 nM / 46 h	~95 (~95% native siRNA)	

charge) [76] and were two times more resistant towards nucleases than their PS-counterparts. Their activity in green fluorescent protein (GFP) silencing was found equal or higher as compared to the native siRNAs, and higher in respect to the corresponding PS-siRNAs, unless the modifications were present in the middle of the antisense strand. Notably, the boranophosphate analogues were non-toxic for cells, even when 76 % of the internucleotide bonds underwent modification [75].

#### C2'-Ribose Modifications [Fig. (4)]

Increased stability against nucleases can result from modification or substitution the 2'-OH group in the ribose ring of the ribonucleotide units of siRNA. As demonstrated earlier in antisense strategy [77], alkylation of the 2'-OH group significantly enhances the stability of siRNA molecule and increases affinity of the modified strand into complementary unmodified RNA. Typically, the 2'-OH group is converted into 2'-O-methyl (2'-OMe) [31, 74], 2'-O-(2-(methoxy)-ethyl) (2'-OMOE) [41] groups or *via* 2'-acetoxymethyl orthoester chemistry (ACE) into orthoester group [2'-O-CH(OCH<sub>2</sub>CH<sub>2</sub>OH)<sub>2</sub>] [74].

Appreciated modification of the 2'-position of the ribose moiety involves 2'-OH to 2'-H substitution by application of 2'-deoxyribonucleotides. In general, DNA oligomers are more resistant towards serum and intracellular nucleases,

compared to oligoribonucleotides. Incorporation of DNA monomers at the ends of RNA molecules to the certain extent increases stability of the siRNA [29, 78].

However, there are contradictory opinions about activity of so called hybrid siRNAs - constructs consisting of RNA strand hybridized with entire DNA strand. While some groups reported such hybrids as completely inactive [29, 78], others estimated them as even more potent than "pure RNA" duplexes [79, 80].

In the case of highly stable siRNA duplexes containing fully modified 2'-OMe RNA strands (antisense as well as sense strand) there are many observations of a lost of their silencing function [31, 70]. The stability of siRNA constructs with 2'-OMe units in juxtaposed positions is comparable with those, containing fully modified strands, with retained ability to induce the RNAi process [31]. Similar effect was observed when several 2'-OMe-ribonucleotides were present at the 3'-ends of the RNA strands [41, 79-81]. The 2'-OMOE substituents are tolerated only if present in the sense strand. If they are located at the ends of the antisense strand, the siRNA activity is dramatically reduced, while their presence in the middle of the duplex does not affect significantly duplex activity [81]. The limited applicability of the 2'-O-alkyl substituents may be related to their steric hindrance causing distortion of the siRNA molecule from the preferred RNA A-type helical structure [31, 41].



The electronegative groups at the 2'-position strongly stabilize the C3'-*endo* conformation of the ribose ring, preferred for RNA strands [82 - 84], and modified in this way oligomers form exceptionally thermodynamically stable duplexes [70, 84]. RNA analogues containing 2'-fluoro-2'-deoxyribonucleotides are among the most prominent in respect to their thermodynamic stability and resistance against nucleases [68]. Promising pharmacokinetic and silencing results were obtained with constructs containing fluorine atom in the 2'-position, both in phosphate and phosphorothioate siRNA strands [44, 70, 71, 74, 81]. Duplexes containing in 21-nt antisense strand all C and U residues (15 in total) replaced with 2'-fluoro-2'-deoxyuridine and 2'-fluoro-2'-deoxycytidine, and additionally possessing four such replacements in the sense strand, tested in HeLa cells were practically indistinguishable in their silencing activity from non-modified duplexes, even if they were modified in combination with phosphorothioate bonds [44]. The 2'-F siRNA constructs were tested *in vitro* in lymphocytes-T culture without the use of transfecting agent. They were found to be stable towards RNase A and reduced HIV-1 viral infection [85]. The 2'-F siRNA worked well *in vivo* in mice co-transfected with GFP coding plasmids. Interestingly, despite of enhanced stability against nucleases, in this case no prolongation of gene silencing was observed [84].

Distinct improvement of pharmacokinetics was observed for siRNAs carrying several different C2' modifications introduced simultaneously. For example, high activity (>500-fold enhanced in comparison to the native siRNA) and improved stability was found for siRNA with all 2'-OH groups replaced in alternate positions with 2'-OMe and 2'-F substituents [32]. Constructs with additional modifications resulting from the presence of inverted abasic nucleosides at the ends of the sense strand effectively prevented HBV infection in mice [87, 88].

Attractive group of modified siRNA molecules consists of 2'-O-arylated compounds with 2,4-dinitrophenyl group (DNP-RNA) [60]. The arylation could be done after enzymatic synthesis; the products were stable towards nucleases and did not require any carrier to cross the cellular membranes. Notably, they exert good RNAi activity and their cytotoxicity is rather low, making them good candidates for further evaluation [60].

Some other modifications of 2'-position of ribose ring in siRNA units were tested including 2'-amino group [74, 78] and 2'-O-allyl group [41]. While introduction of 2'-amino-2'-deoxyuridine or cytidine units into antisense strand of siRNA triggered silencing activity, those modifications in the sense strand were well tolerated in RNA-induced gene silencing in *C. elegans* [74].

An approach based on conformational restraints imposed on the ribose ring employing locked nucleic acids (LNA) [89] and ethylene bridged nucleic acids (ENA) [90] [Fig. (4)] has to be also mentioned. These bicyclic molecules differ in the number of carbon atoms in the bridges. LNA nucleotides possess one-, while ENA – two-carbon bridge. Initial studies showed that such modifications significantly enhance thermodynamic stability of the duplex (increase of the T<sub>m</sub> parameter was 2-4 °C per modification). Surprisingly, the

ENA units present at both ends of siRNA completely abolished its activity [42]. On the other hand, the presence of the LNA units in these positions seemed to be well accepted [70]. Interestingly, observed activity of LNA-siRNA duplexes (which melt at the temperature > 95 °C), suggests high efficiency of the RISC helicase, which is able to unwind the strands of such unusually stable siRNA duplexes.

### Base Modifications Affecting siRNA Structure and Activity

As demonstrated by Chiu and Rana in experiments performed with bulge siRNA strands in HeLa cells [43], the perfect A-type helix of siRNA is not required for effective interference. However, perfect mRNA/guide strand RNA helix recognition seems to be crucial for its assembly at RISC complex. Therefore, chemical modifications located in the guide (antisense) strand of siRNA, due to possible changes of the mRNA/guide siRNA local conformation, may tune gene silencing by RNA interference. Some nucleobases are known to affect the ribose conformation in similar way as substituents at 2' position of the ribose ring. For example, naturally occurring modified nucleosides such as 2-thiouridine (s<sup>2</sup>U) and pseudouridine ( ) stabilize the C3'-*endo* sugar ring puckering [91]. Therefore, their presence in siRNA molecules was expected to result in enhanced thermal stability of the siRNA duplexes. Recently, we have screened several modified siRNAs possessing combinations of s<sup>2</sup>U and for their thermal stability and silencing efficiency in GFP/RFP dual fluorescence assay [43, 92]. Duplexes with several s<sup>2</sup>U modifications exhibited extremely high thermal stability, similar to that observed for LNA-containing siRNAs, and comparable interference with non-modified duplexes. Point mutations with 2-thiouridine units allowed for manipulation with the duplex "asymmetry" by enhancing the Watson-Crick interactions and, in consequence, increasing the thermodynamic stability of one terminus of the siRNA. The structures of modified nucleobases are shown at Fig. (5).

Very few examples of base-modified siRNA strands were tested for their silencing potential. One of the first was the report of Parrish *et al.*, where five base-modified analogues including 4-thio-, 5-bromo-, 5-iodo- and 5-(3-aminoallyl)-uridine as well as inosine were incorporated to the sense or antisense strand of siRNA and tested in *C. elegans* [78]. While 4-thiouridine and 5-bromouridine nucleotides located either in the sense or antisense strand did not alter silencing activity of corresponding constructs from the wild type duplexes, the remaining modifications triggered RNAi most likely by steric hindrance or by cationic nature of the substituent.

To investigate an impact of base-pairing interactions on siRNA duplex stability and silencing activity, 5-bromouridine, 5-iodouridine and 2,6-diaminopurine nucleoside [Fig. (5)], (which are known for enhancing the A-U base pair interactions [82]), were incorporated into siRNAs and tested in dual fluorescence assay performed in human HeLa cells [71]. All modifications stabilizing duplex structure, including 5-bromo- and 5-iodouridine and 2,6-diaminopurine nucleoside, as well as those destabilizing duplex structure as N-3-methyl-uridine, reduced activity of the siRNA molecules, especially if modifications were introduced into the

strands possessing C2'-fluoronucleosides. Thus, enhancing interactions between base pairs increasing mRNA targeting efficiency is not always beneficial enough for effective induction of RNAi.

The N-3-methyluridine-modified siRNA was used to elaborate structural requirements for RNAi [71]. The methyl group of N-3-methyluridine, positioned in the major groove of the helix formed by the antisense strand and its target mRNA, inhibited RNAi by steric hindrance [71]. The use of this siRNA modification allowed to postulate that availability of the major groove of mRNA/guide siRNA duplex is required for effective RNA-RISC interaction.

In conclusion, the bulky substituents distorting the RNA conformation reduce siRNA silencing activity, while modifications anchoring the C3'-endo puckering of the ribose ring enhance the RNAi process. Difference in thermodynamic stability of the 5'-ends of the siRNA duplex determines which strand of the duplex is incorporated into the RISC complex. Desired "asymmetry" to a certain extent can be achieved by proper selection of the sequence, although there are some limitations in respect to specificity issue.

### Modifications at the Ends of siRNA Strands

It was already mentioned that modifications of the 5'-end of the antisense strand are not allowed because the phosphate group at this position is necessary for antisense strand accommodation at RISC [13, 16, 17, 39, 40, 43]. Although chemically synthesized RNA usually does not possess 5'-terminal phosphate, it is supposed that the 5'-OH groups of siRNA strands are phosphorylated intracellularly [13, 16]. Some reports demonstrate higher activity of chemically modified siRNA molecules, which are phosphorylated prior transfection [32]. It is suggested that chemical modifications may affect recognition of 5'-ends of siRNA strands by intracellular kinases and that prior phosphorylation helps to overcome this problem.

The 5'-end of the sense strand and both 3'-ends can be used for attachment of functional groups bearing fluorescence [43, 44] or radioactivity [73] as well as groups facilitating cellular uptake and transport through membranes (e.g. cholesterol, lithocholic acid, lauryl acid or long alkyl chains) [74, 93, 94]. The cholesterol-modified siRNAs were delivered into the cells, both *in vitro* [93] and *in vivo* [94], without addition of polycationic transfection reagents.

Alternatively, this effect might be achieved by attachment of TAT peptide (47-57) - derived oligocarbamate to the 3'-end of antisense strand of siRNA [95]. It was proved that these oligomers could reach cell nuclei, but to date there are no reports on application of such constructs *in vivo*.

Attachment at the 3'-ends of siRNA molecules such pendant groups as puromycin [43], biotin [43], inverted nucleosides [87, 88], allyl residue [41] or aminoalkanes [31] has to be mentioned, albeit such modifications did not affect the silencing activity of resulting siRNA constructs.

Harborth *et al.* used fluorescently labeled siRNA duplexes to correlate their cellular uptake efficiency and effectiveness in gene silencing [44]. Fluorescein was conjugated to lamin A/C siRNA duplex at the 5'-end or 3'-end of the sense or antisense strands. The fluorophore was

separated by a 6-carbon linker from the 5'-phosphate and by longer alkyl linker from the 3'-phosphate. The obtained results were contradictory to those reported earlier that 3'-end labeling does not interfere with duplex activity [43] and that a free terminal 5'-phosphate of the antisense strand is essential for RNAi [13, 16, 17, 43]. It was also demonstrated that cellular uptake of such fluorescently labeled siRNA duplexes was not always correlated with their silencing activity [44].

### SUMMARY

Over the last five years substantial progress in the field of specific silencing of gene expression through the process of RNA interference has been achieved. Nowadays, the siRNA duplexes are widely used for studying functions of the genes in different cell lines. Moreover, there is enormous increase in the number of studies *in vivo*, including clinical studies on the use of siRNA in therapy of age-related macular degeneration (AMD) [96, 97]. However, despite promising results, before the siRNAs become real alternative for already approved therapeutics, further studies are required mostly directed towards more detailed understanding of side-effects and enhancement of specificity. These goals can be possibly accomplished by chemical modifications within the nucleotide units. To date, the most important results were obtained in respect to the increase of siRNA stability *in vivo* by introduction of the C2'-F ribose modification. On the other hand, effective transfection without the use of transfecting reagents was achieved due to the presence of the cholesteryl tag at the 3'-end of the sense strand. The siRNA duplexes consisting of DNP-RNA strands seem to be interesting extension to the original siRNA class, as these duplexes have effective gene silencing activity and high stability. Since none of the reported modifications largely enhanced RNAi activity of the siRNAs constructs, the research on structure/RNAi activity relationship is a matter of exciting challenge.

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### ABBREVIATIONS

3'-UTR	=	3'-Untranslated region
AMD	=	Age-related macular degeneration
ATP	=	Adenosine triphosphate
bp	=	Base pair
CMV	=	Cytomegalovirus
DNA	=	2'-Deoxyribonucleic acid
DNP	=	2,4-Dinitrophenyl residue
dsRNA	=	Double stranded RNA
ENA	=	Ethylene bridged nucleic acid
FDA	=	Food and Drug Administration

GFP	=	Green fluorescent protein
HIV	=	Human immunodeficiency virus
LNA	=	Locked nucleic acid
miRNA	=	Micro RNA
MOE	=	2'-(methoxy)-ethyl residue
mRNA	=	Messenger RNA
nt	=	Nucleotide
Pol III	=	DNA-dependent RNA polymerase III
PS-siRNA	=	Phosphorothioate modified siRNA
RFP	=	Red fluorescent protein
RISC	=	RNA induced silencing complex
RNA	=	Ribonucleic acid
RNAi	=	RNA interference
shRNA	=	Short hairpin RNA
siRNA	=	Short interfering RNA
Tm	=	Melting temperature
VEGF	=	Vascular endothelial growth factor

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