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SiRNA Design

Bachelor's thesis

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Outline

1. Abstract

RNAi interference (RNAi) induces sequence-specific degradation of mRNA and posttranscriptionally down-regulates gene expression. Target mRNAs are found on the basis of homology with short double stranded RNA, called small interfering RNA (siRNA). RNA interference is a widespread experimental tool for gene silencing. Successful silencing highly depends on the selection of siRNA sequence, because not all siRNAs are efficient. Scientists identified a lot of guidelines for design efficient and specific siRNAs. The purpose of this assay is to summarize siRNA design guidelines and to make overview of siRNA designing tools. This assay also involves overview of modifications influencing siRNA features.

Keywords: RNAi, siRNA, algorithm, nucleotide modification

Abstrakt

RNA interference (RNAi) indukuje sekvenčně specifickou degradaci mRNA, a tím posttranskripčně reguluje genovou expresi. Cílová mRNA je vyhledávána na základě homologie s krátkou dvouvláknovou RNA, zvanou siRNA. RNA interference je široce rozšířenou metodou využívanou k cílenému snižování genové exprese. Jestli RNAi úspěšně proběhne, závisí na pečlivém výběru sekvence siRNA, neboť ne všechny siRNA vykazují vysokou účinnost. Vědci objevili mnoho vlastností, které musí siRNA mít, aby účinně a specificky navozovala RNAi. Cílem této práce je shrnout pravidla a zpracovat přehled programů pro siRNA design. Tato práce též zahrnuje přehled modifikací, které mohou ovlivnit vlastnosti siRNA.

2. Introduction

RNA interference (RNAi) induces sequence-specific dsRNA-mediated mRNA degradation. This process was discovered in 1998 in *Caenorhabditis elegans* where injection of dsRNA led to silencing of genes homologous to delivered dsRNA (Fire et al. 1998). Subsequently, RNAi was observed in many other organisms - insects, frogs, mice and plants. In plants and invertebrates RNAi appears to have a role in protection against viruses, which generate dsRNA during their life cycle. In mammals, there is no evidence that RNAi could serve in antiviral immunity (Cullen 2006).

RNAi has become widespread tool frequently used for studying gene function. In contrast to homologous recombination, using RNAi to reduce gene expression is easy and fast solution.

3 .RNAi

3.1. RNAi pathway

During the initial step of the RNAi, dsRNA is digested by RNase III Dicer to 21- 23 nt long dsRNAs with two-nucleotide 3´overhangs and phosphorylated 5´ends called small interfering RNAs (siRNA) (Zamore et al. 2000, Elbashir et al. 2001 A). SiRNA is loaded into the ribonucleoprotein effector complex called RNA-induced silencing complex (RISC), which is responsible for mRNA degradation (Meister et al. 2004). Argonate proteins are important component of the RISC. Argonaute proteins contains PAZ domain, which indentifies two-nucletide 3´overhang of the siRNA (Lingel et al. 2004). In mammalian cells, Argonaute 2 (Ago 2) protein is catalytic component (Meister et al. 2004). During RISC formation, siRNA is loaded as a duplex and then the sense strand (SS, so-called passenger strand) is degraded. Target mRNA is found on the basis of the complementarity to the guiding antisense strand (AS) and cleaved at the site corresponding to the centre of siRNA antisense strand (Zamore et al. 2000, Elbashir et al. 2001 A).

DsRNA adopts a right-handed A-form helix conformation that is more tightly packaged than a B-from helix typical for DNA. Helical geometry could influence the RISC formation and loading of the guide strand. The antisense strand-target mRNA duplex also must be in A-form helix for successful mRNA cleavage (Chiu and Rana 2002).

3.2. MiRNA pathway

In addition to the above described RNAi pathway, miRNA pathway exists (Lagos-Quintana et al. 2001). In this pathway, short dsRNAs called miRNAs rise from short hairpins coded in genom. Hairpin is cleaved out from transcript by RNase III Drosha in nucleus and then it is transported to cytoplasm by Exportin 5. In cytoplasm, Dicer cleaves out the loop structure. MiRNAs have the same features as siRNAs (length, overhangs) but they often have mismatches in the complementarity of strands. Matured miRNA is loaded into the effector RISC-like complex. In mammalian cells, RISC and RISC-like complex differ in type of Ago protein. RISC contains Ago2, which is able to cleave target mRNA, while RISC-like complex can also contain Ago1, 3 or 4, which lacks cleaving activity (Meister et al. 2004).

Endogenous miRNAs are usually only partially homologous to the 3´UTR of target mRNAs and they repress translation instead of the mRNA cleavage (Ambros 2001). Then the whole complexes with mRNAs cluster together and create cytoplasmic structures called P-

bodies. However, if target mRNA will be cleaved or not depends on rate of homology with si(mi)RNA and type of Ago protein in effector complex not on short dsRNA origin.
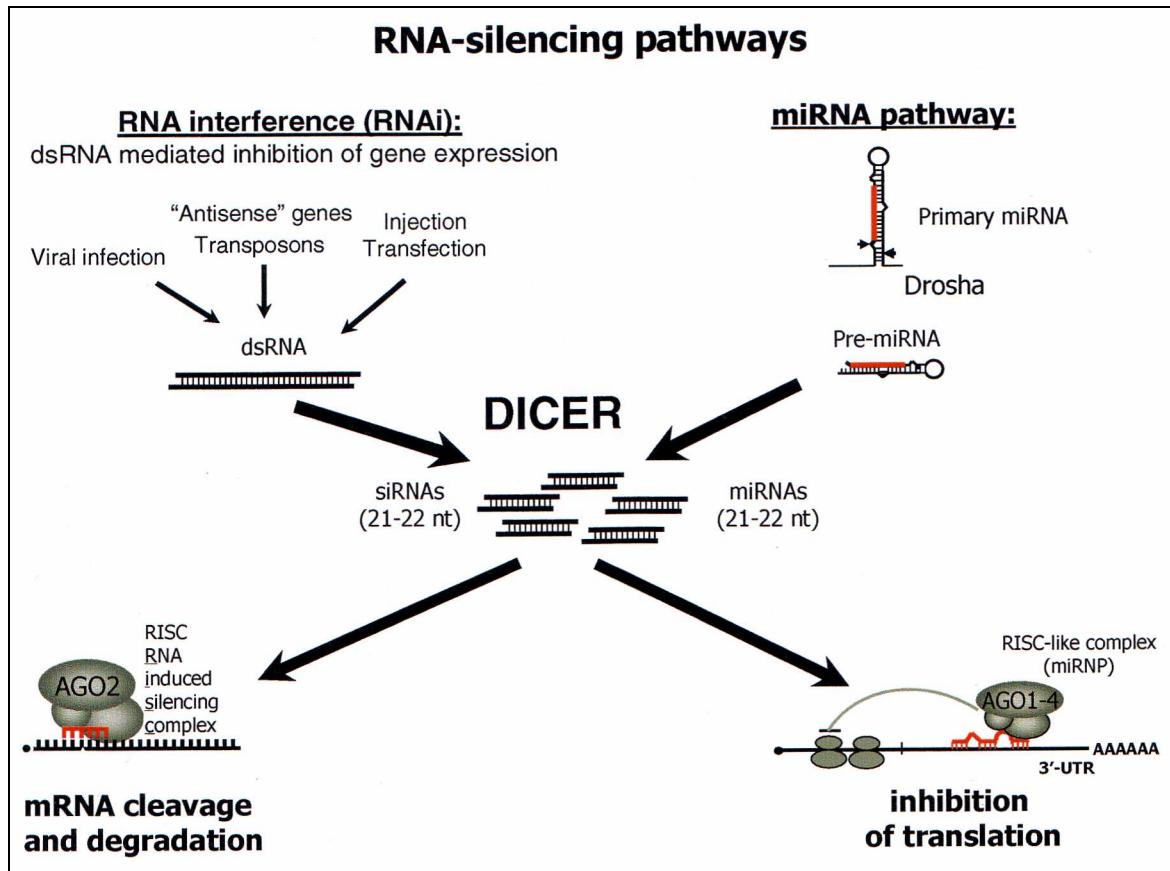
Figure 1: Mechanism of RNAi and miRNA pathway
Both RNAi and miRNA pathway produce short dsRNAs, which down-regulate gene expression in posttranscriptional manner. SiRNAs are usually perfectly complementary to target mRNA and cleave it, while miRNA are usually only partially complementary to target mRNA and inhibit translation. However, destiny of mRNA depends on complementarity not on short dsRNA origin.

3.3. RNAi as a scientific tool

RNAi is a useful experimental tool frequently used in the gene function studies. One way to induce RNAi is transfection of chemically synthetized siRNAs. Though natural siRNAs have phosphorylated 5´ends because the phosphate group is essential for its function it is not necessary to phosphorylate chemically syntetized siRNAs, which do not have phosphorylated 5´ends. 5´OH ends are phosphorylated by cellular kinases after transfection (Elbashir et al. 2001 A). Disadvantage of transfected siRNAs is that their silencing effect is transient. Stable silencing can be achieved by vector-based RNAi. Vectors can express long or short hairpins. In mammalian somatic cells only short hairpin expressing systems have to be used. Somatic mammalian cells recognize long dsRNA as viral product and trigger

immune response. Long hairpins are useful and very efficient in invertebrates and in the mammalian oocytes and stem cells where immune response to the long dsRNA misses.

4. SiRNA design

4.1. Efficient siRNA guidelines

In the first siRNA experiments, siRNA sequences were chosen at random. However, only some of them significantly decreased target gene expression suggesting that the rate of silencing may depend on siRNA sequence.

4.1.1. The oldest rules and target mRNA patterns

Systematical analysis revealed the most functional siRNAs had two-nucleotide 3´overhangs. Experiments also indicated that siRNAs with UU or TT 3´overhangs are more efficient (Elbashir et al. 2001 A). SiRNAs started to be designed with 3´TT overhangs, because synthesis was cheaper and deoxyribonucleotides probably increased nuclease resistance. *Elbashir* et al. suggested that symmetric 3´overhangs help to form RISCs with antisense and sense strands in equal ratio. So, an ideal target mRNA contained AA(N19)TT motif where N indicate the target sequence. If no AA(N19)TT motif was found, NA(N19)TT or NA(N21) motif was used as target sequence. Then, siRNA SS sequence corresponded to (N19)TT or N21. In NA(N19)TT or NA(N21) motifs, nucleotide at 3´end of AS was converted to T because this nucleotide at this position was not recognized sequence-specifically and had not to be complementary to target mRNA site. In NA(N21) motif 3´overhang of SS was also converted to TT to generate symmetric siRNAs. Sense strand did not affect mRNA recognition and that is way its 3´overhang had not to agree with nucleotides in mRNA target site. Symmetry also simplified chemical synthesis (http://www.rockefeller.edu/labheads/tuschl/sirna.html). Exampes of siRNAs corresponding to motifs are shown in table 1.

SiRNAs were designed to avoid targeting 5´ and 3´ UTRs because UTR-binding proteins or translation initiation complexes could disrupt RISC binding. However, no detail studis confirming importance of this rule exist (Pei and Tuschl 2006).

4.1.2. Thermodynamic profile

Subsequent studies revealed the importance of siRNA thermodynamic profile, which is crucial for siRNA incorporation into the RISC. SiRNA strand with less thermostable 5´end is preferably loaded into the RISC, so the low stability of siRNA antisense strand 5´end correlates with siRNA efficiency. AS with A or U at the most of 1 – 7 positions appears to be more efficient because less hydrogen bonds between A and U than between C and G results in low stability (Khvorova et al. 2003, Schwarz et al. 2003).

4.1.3. GC content

Already first studies indicated that higher siRNA activity correlates with lower GC content (Elbashire et al. 2001 B, Holen et al. 2002). The GC content of the most effective siRNAs usually varies between 30 – 52 % in functional siRNAs (Reynolds et al. 2004). However, GC pair at the 3´end of AS together with AU at the 5´end gives rise to thermodynamic asymmetry which influences strand loading during RISC formation.

4.1.4. The other target mRNA patterns

In 2004, Ui-Tei summarized rules how the rate of silencing depends on siRNA sequence. Effective siRNAs have to satisfy following conditions (Ui-Tei et al. 2004): (i) A/U at the 5´end of the antisense strand; (ii) C/G at the 5´end of the sense strand; (iii) A/U rich 5´end of the antisense strand; (iv) no more than 9 GC pairs straight-line. SiRNAs with the opposite first three features have little or no effect on gene expression. So suitable target mRNA has N2(G/C)N17(A/U)N2 pattern. *Ui-Tei* et al. confirmed presented rules by testing of 57 potentially highly effective and 16 potentially ineffective siRNAs. They also confirmed that these rules are required not only for effective silencing by transfected siRNA but also for vector-based RNAi. The same year, Reynolds et al. revealed further sequence preferences. They presented that the more effective siRNAs target mRNA with N4(A)N6(T)N2(A/T/C)N5(A/U)N2 pattern (Reynolds et al. 2004). In 2006, Pei and Tuschl summarized their findings and they published that the most important single nucleotide preferences are A/U at the position 1 in AS, C/G (C is more common) at the position 19 and A/U (A is more common) at the position 10. These siRNAs target mRNA with N2(G/C)N8(A/T)N8(A/T)N2 motif. A/U at the position 10 in the sense strand is at the cleavage site and could promote passenger strand and subsequently mRNA cleavage (Pei and Tuschl 2006).

Indeed, all mentioned patterns demand A/U richness at the 5´end of antisense strand.

Table 1: Target mRNA patterns:

Examples of target mRNA (recommended nucleotides are violet, arbitrary nucleotides are red) and corresponding siRNAs (recommended nucleotides are blue). Presented sequences are illustrative and do not agree with any mRNA and/or published siRNAs.

Pattern	mRNA siRNA - sense strand antisense strand
AA(N19)TT (Elbashir et al. 2001 A)	5´…ACGG**AA**CGAGTGGAATAACCTCATT**TT**GGT… 3´ 　　　5´ CGAGUGGAAUAACCUCAUU**UU** 3´ 　　　3´ **UU**GCUCACCUUAUUGGAGUAA 5´
NA(N19)TT (Elbashir et al. 2001 A)	5´…TAGG**CA**CGAGTGGAATAACCTCATT**TT**GGT… 3´ 　　　5´ CGAGUGGAAUAACCUCAUU**UU** 3´ 　　　3´ G**U**GCUCACCUUAUUGGAGUAA 5´
NA(N19)TT with converted nucleotide in 3´end of AS (Elbashir et al. 2001 A)	5´…TAGG**CA**CGAGTGGAATAACCTCATT**TT**GGT… 3´ 　　　5´ CGAGUGGAAUAACCUCAUU**UU** 3´ 　　　3´ **UU**GCUCACCUUAUUGGAGUAA 5´
NA(N21) (Elbashir et al. 2001 A)	5´…TAGG**CA**CGAGTGGAATAACCTCATACAGGT… 3´ 　　　5´ CGAGUGGAAUAACCUCAUACA 3´ 　　　3´ G**U**GCUCACCUUAUUGGAGUAT 5´
NA(N21) with converted 3´ overhang of SS	5´…TAGG**CA**CGAGTGGAATAACCTCATA**CA**GGT… 3´ 　　　5´ CGAGUGGAAUAACCUCAUU**UU** 3´ 　　　3´ G**U**GCUCACCUUAUUGGAGUAA 5´
N2(G/C)N17(A/T)N2 (Ui-Tei et al. 2004)	5´…TAAG**CA**CGAGTGGAATAACCTCAT**T**TTGGT… 3´ 　　　5´ **C**GAGUGGAAUAACCUCAU**U**UU 3´ 　　　3´ GU**G**CUCACCUUAUUGGAGUA**A** 5´
N4(A)N6(T)N2(A/T/C)N5(A/T)N2 (Reynolds et al. 2004)	5´…TAGG**CA**CG**A**GTGGAA**T**AA**CC**TCAT**T**TTGGT… 3´ 　　　5´ CG**A**GUGGAA**U**AA**C**CUCAU**U**UU 3´ 　　　3´ GUGC**U**CACCUU**A**UU**G**GAGUA**A** 5´
N2(G/C)N8(A/T)N8(A/T)N2 (Pei and Tuschl 2006)	5´…TAAG**CA**CGAGTGGAA**T**AACCTCAT**T**TTGGT… 3´ 　　　5´ **C**GAGUGGAA**U**AACCUCAU**U**UU 3´ 　　　3´ GU**G**CUCACCUU**A**UUGGAGUA**A** 5´

4.1.5. "Non-traditional" short dsRNAs

In 2005, Kim et al. published that 27 bp long dsRNAs can enhance RNAi efficacy. These RNAs enter in the RNAi one step earlier than siRNA and serve as Dicer substrate (Dicer-substrate siRNA, DsiRNAs). DsiRNA is designed with two-nucleotide 3´overhang at the antisense strand, which facilitates prediction of the Dicer cleavage site. The opposite end of the DsiRNA is blunt or it has 3´ DNA overhang (Kim et al. 2005, Rose et al. 2005).

4.2. Influence of target mRNA structure

SiRNA efficiency may be influenced by target mRNA structure although the first studies did not consider importance of secondary mRNA structure. SiRNAs targeted to highly structured regions of mRNA are less effective than those targeted to regions without complicated structure (Yoshinari et al. 2003). Influence of the mRNA structure on siRNA

efficacy was observed in experiment studying HIV-I. Efficient siRNA targeting Nef mRNA became functionless in some viral clones. Mutations in Nef mRNA but not in target site changed mRNA conformation, weakened siRNA/target-RNA dupex and abolish RNAi (Westerhout et al. 2005).

Ameres et al. described how target mRNA structure affects RNAi. They found the RISC has low binding affinity to dsRNA in contrast with ssRNA. Hairpin structure with high internal energy in target site can weaken RISC binding so much that RNAi is eliminated (Ameres et al. 2007).

4.3. Nonspecific effects

4.3.1. Off-targeting

Unfortunately, siRNAs have potential to function as miRNAs and down-regulate many non-targeted genes with partial homology with siRNA sequence. This undesirable effect is known as off-target effect (Jackson et al. 2003, Persengiev et al. 2004). Complementarity between 3´UTR of mRNAs and seed region of the antisense strand (nucleotides at positions 2-7 or 2-8) is associated with off-targeting (Birmingham et al., 2006). The number of influenced non-targeted genes depends on siRNA concentration. Interestingly, Long dsRNA does not evoke off-targeting because a pool of siRNAs with different sequences arises after Dicer digestion (Stein et al. 2005). In this case, concentration of the particular siRNAs is lower in respect of total siRNA concentration and off-targeting is reduced or even not detectable. So, a pool of siRNAs differing in sequences is and solution how to reduce off-target effect using chemically synthetized siRNAs (Cullen 2006).

The sense strand RISC loading may be the second source of the off-target effects. Even if a siRNA sequence satisfies conditions mentioned above, small percent of sense strand could be incorporated into RISC and served as a guide strand and change expression of many genes (Jackson et al. 2003). In this case, chemical modifications of SS could solve this problem (see chap. 4.4.)

4.3.2. Immune response connected with RNAi

RNAi could be triggered by dsRNA. As mentioned in chapter 3.3., dsRNA is not useful for gene silencing in mammalian somatic cells. The presence of just a small amount of dsRNA longer than 30 bp induces series of reactions known as an intreferon response. Interferon response, which naturally protects cells against virus invasion, involves activation of protein kinase R (PKR), retinoic acid-inducible gene-I (RIG-I) and/or family of 2´-5´-oligoadenylate synthetases (2´5´-OAS). Activated PKR phosphorylates the eukaryotic

translation initiation factor EIF2α, which leads to the global repression of protein synthesis and, eventually, apoptosis. Active 2´5´-OAS leads to activation of RNase L, which triggers nonspecific mRNA degradation. RIG-I is involving in interferon production.

In some cases, dsRNA shorter than 30 bp also triggers immune response. Blunt- ended short dsRNA can be recognized by the RIG-I helicase while siRNAs containing short 3´overhangs usually do not induce interferon response (reviewed in Marques and Williams 2005). However, some siRNAs can evoke immune response if they contain immunostimulatory sequence such as GUCCUUCA and UGUGU (Judge et al., 2005, Hornung et al. 2005). Generally, GU-rich motifs are more immunostimulatory then other motifs (Heil et al., 2004). However, several imunnostimulatory siRNAs do not contain these motifs and many GU-rich siRNAs do not stimulate immune response.

4.4. Chemically modified siRNAs

Many studies demonstrate that some chemical modifications can improve properties of siRNAs. Modifications may increase specificity and stability of siRNAs and prolong duration of silencing. Some chemical modifications have no or little influence on siRNA efficacy but others abolish RNAi (Tab. 2). It is not possible to modify 5´end of AS. Free phosphorylated 5´end of anti-sense strand is essential for siRNA function. Blocking of the 5´end of AS leads to the loss of RNAi activity whereas blocking of AS 3´end and/or one or both ends of SS has no significant effect (Chiu and Rana 2002, Czauderna et al. 2003). Modifications at other positions have not so strong influence on siRNA function. 2´OH group of the ribose is not essential for RNAi. Modification of this group could enhance RNA stability because 2´OH, which distinguishes RNA from DNA, is required for nucleophilic attack during degradation by RNases (Chiu and Rana 2003). More stable siRNAs down-regulate gene expression for longer period and could be more successfully delivered in potential *in vivo* applications.

Table 2: Effect of siRNA modifications to RNAi

Passenger strand	Guide strand	RNAi function
5´-end capping	3´-end capping	Not affected
3´-end capping	Unmodified	Not affected
Unmodified	5´-end capping	Severely affected
Pyrimidines that contain 2´-fluoro ribose	Pyrimidines that contain 2´-fluoro ribose	Not affected
2´-deoxyribose	Unmodified	Moderately affected
Unmodified	2´-deoxyribose	Severely affected
2´-O-methyl ribose	Unmodified	Moderately affected
Unmodified	2´-O-methyl ribose	Severely affected
Unmodified	2´-O-methyl ribose at position 2 from the 5´end	Reduced off-target RNAi
2´-O-(2-methoxyethyl)	Unmodified	Not affected
Unmodified	2´-O-(2-methoxyethyl)	Moderate-severe effect depending on the position of the base
Phosphorotioate backbone	Unmodified	Not affected
Unmodified	Phosphorotioate backbone	Moderately affected
Phosphorotioate backbone	Phosphorotioate backbone	Moderately affected
3-methyl-U	3-methyl-U	Severely affected
Unmodified	C5-halogenated pyrimidine	Not affected

RNAi function: not affected. >50% gene silencing; moderately affected. 20-50% gene silencing; severely affected. <20% gene silencing. (adopted from Rana 2007)

4.4.1. Phosporothioate backbone

RNA stability can be also enhanced by thioate linkage (P-S, Figure 2). Phosphorothioate backbone increases resistance to ribonucleases. Experiments revealed that P-S linkage lower RNAi. Modified SS decreases RANi activity less than linked AS or both strands (Chiu and Rana 2003). An alternative backbone modification increasing stability is the boranophosphonate linkage (Figure 2).

4.4.2. 2´-fluoro modifications (Figure2)

2´-fluoro-cytidine (2´-FC) and 2´-fluoro-uridine (2´-FU) was the other tested modification, which could protect siRNAs from degradation. Chiu and Rana discovered that siRNAs with 2´-FC, FU modified SS or both strands are stable than unmodified siRNAs or siRNAs with 2´-FC, FU modified AS. Subsequently experiments evidence that 2´-FC, FU modifications do not decrease RNAi (Chiu and Rana 2003).
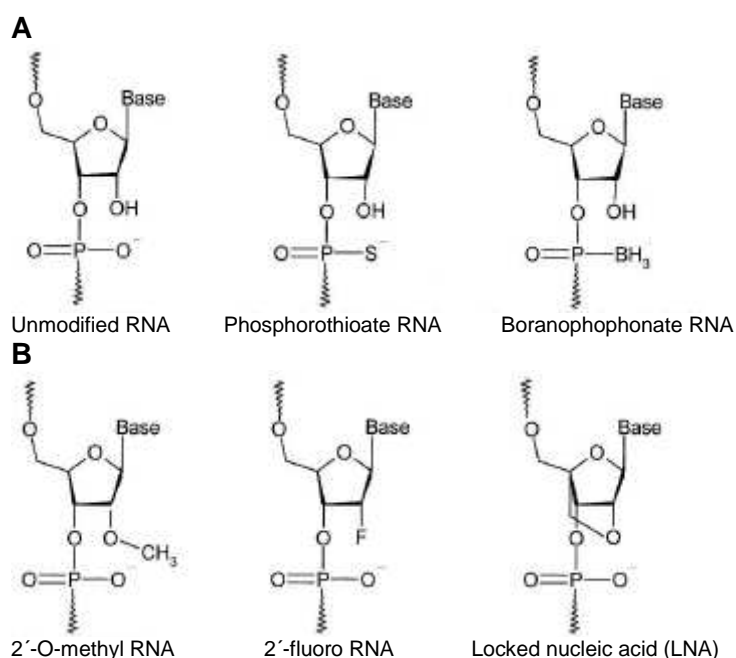
4.4.3. 2´-O-methyl modification

As expected, 2´-O-methyl modified siRNAs have also higher resistance against serum-derived nucleases (Czauderna et al. 2003). Unfortunately, 2´-O-methyl modification

considerably decreases RNAi when more nucleotides are modified. Modification of a whole strand (one of them or both) severely affects silencing (Chiu and Rana 2003).

However, 2´-O-methyl modification can reduce off-targeting. Jackson et al. hypothesized that modifications of siRNA weaken or disrupt RISC-mRNA interaction if complementarity between mRNA and guide strand of siRNA is imperfect. They showed that 2´-O-methyl group at the second nucleotide from the 5´end of the antisese strand reduces off-target effect while down regulation of targeted mRNA is unaffected (Jackson et al. 2006). 2´-O-methyl modification also improves stability of siRNAs in serum.

Figure 2: Chemical modifications introduced in siRNAs
(A) Phosphodiester modifications. (B) 2´sugar modifications. (adopted from De Paula et al. 2007)

A

Unmodified RNA   Phosphorothioate RNA   Boranophophonate RNA

B

2´-O-methyl RNA   2´-fluoro RNA   Locked nucleic acid (LNA)

4.4.4. SisiRNA

Small internally segmented interfering RNA (sisiRNA, Figure 3) can reduce nonspecific down-regulation caused by the SS RISC loading. Sense strand of sisiRNA consisted of two shorter 10nt and 12nt strands so it could not be incorporated into RISC and occasional silencing activity of sense strand was eliminated (Bramsen et al. 2007). A nick in passenger strand has no influence at generation of the functional RISC (Leuschner et al. 2006). SisiRNAs resolve situation if target sequence is restricted to thermodynamically unfavorable region for example if region with a single nucleotid mutation or junction between fused genes is targeted. SisiRNAs are more flexible and can rescue the function of chemically modified antisense strands. However, unmodified sisiRNAs do not function *in vivo* probably

because of low stability. Bramsen et al. used locked nucleic acid (LNA) modification to stabilize sisiRNAs (Bramsen et al. 2007).

Figure 3: Example of sisiRNA used in Bramsen´s experiment
Sense strand of sisiRNA consists of shorter strands and that is way can not be loaded RISC and induce off-targeting.

```
5´SS + 3´SS        5´- GACGUAAACG GCCACAAGUUCU - 3´
AS                 3´- CGCUGCAUUUGC-CGGUGUUCA - 5´
```
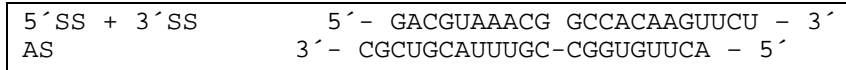
4.4.5. Locked nucleic acid (LNA)

The term locked nucleic acid involves family of conformationally locked nucleotide analogues LNA that contains a methylene bridge connecting the 2´-oxygen with the 4´-carbon of the ribose ring (Figure 2). This connection locks the ribose ring in the 3´-endo conformation, which is typical for A-form helix. Except stability enhancement LNA modification can decrease nonspecific down-regulation as well as sisiRNA. LNA modified 5´end of SS blocs SS loading into RISC and decreases off-target effect (Elmén et al. 2005).

Chemical modifications can improve properties of siRNAs. These siRNAs are more stable because modifications protect them against RNase. Modifications can also lower off-targetting, so modified siRNAs are also more specific. Despite of chemically modifies siRNAs advantages, siRNAs sequence selection is more important. Chemically modified siRNA can considerably improve results of experiment but are they are not applicable in vector based RNAi.

4.5. Algorithms predicting efficient siRNAs

Although many rules are mentioned above, it is not possible to determine whether an siRNA is highly effective without experimental verification. Many companies interested in biomedical research offer experimentally validated siRNAs targeting assorted genes. They also provide pre-designed siRNAs guarantying silencing. For example, Ambion guarantees that at least two of three pre-designed siRNAs targeting the same gene will reduce target mRNA levels by 70% or more (www.ambion.com/RNAi).

Many companies and many research institutes developed a number of algorithms predicting potentially efficient siRNAs (Tab.3).

Tab. 3: SiRNA predicting tools, their sponsors and website location

Name	Sponsor	website location
Ambion siRNA Targget Finder	Ambion Inc	http://www.ambion.com/techlib/misc/siRNA_finder.html
siDESIGN Center	Dharmacon Inc	http://www.dharmacon.com/sidesign/default.aspx
LASSO	Ecole do Mines di Paris	http://cbio.ensmp.fr/dsir
siRNA Target Finder	GenScript Corp	http://www.genscript.com/ssl-bin/app/rnai
Imgenex siRNA Designer	Imgenex Crop	http://imgenex.com/sirna_tool.php
EMBOSS siRNA	Institute Pasteur	http://mobyle.pasteur.fr/cgi-bin/MobylePortal/portal.py?form=sirna
IDT RNAi Design (SciTools)	Integrated DNA Technologies Inc	http://www.idtdna.com/Scitools/Applications/RNAi/RNAi.aspx
BLOCK-iT RNAi Designer	Invitrogen Corp	http://www.rnaidesigner.invitrogen.com/sirna/
DEQOR	Max Plank Institute	http://cluster-1.mpi-cbg.de/Deqor/deqor.html
siMAX	MWG-Biotech Inc	http://www.mwg-biotech.com/html/s_synthetic_acids/s_sirna_design.shtml
BIOPREDsi	Novartis Institutes for BioMedical Research	http://www.biopredsi.org/start.html
Promega siRNA Target Designer	Promega Corp	http://www.promega.com/siRNADesigner/program/
QIAGEN siRNA Design Tool	QIAGEN Inc	http://www1.qiagen.com/Products/GeneSilencing/CustomSiRna/SiRnaDesigner.aspx?
SDS/MPI	University of Hong Kong	http://i.cs.hku.hk/~sirna/software/sirna.php
OptiRNA	University of Nebraska-Lincoln	http://optirna.unl.edu/
SiDRM	University of New Mexico	http://sirecords.umn.edu/siDRM/
WI siRNA Selection Program	Whitehead Institute	http://jura.wi.mit.edu/bioc/siRNAext/
RNAxs	University of Vienna	http://rna.tbi.univie.ac.at/cgi-bin/RNAxs
RNAi explorer	Gene Link	http://www.genelink.com/sirna/RNAicustomorder.asp
siDirect	University of Tokio	http://genomics.jp/sidirect/
SVM RNAi 2.0	Chang Bioscience Inc	http://www.changbioscience.com/stat/sirna.html
sIR	University of Texas	http://biotools.swmed.edu/siRNA/
Microsynth siRNA Design Tool	Microsynth AG	http://www.microsynth.ch/sirnadesign/sirnadesign.html
E-RNAi	German Cancer Research Center	http://www.dkfz.de/signaling2/e-rnai/
OligoWalk	University of Rochester Medical Center	http://rna.urmc.rochester.edu/cgi-bin/server_exe/oligowalk/oligowalk_form.cgi

The first generation of siRNA prediction algorithms searched through target mRNA to find siRNAs according to parameters described in the chapter 4.1.

More advanced algorithms are based on bioinformatics analyses of experimentally tested siRNA. These algorithms use neural network trained on database of variously effective siRNAs. Then they find out siRNAs with properties similar to more efficient siRNAs from database in entered mRNA. Algorithms trained on the sets of siRNAs are for example BIOPREDsi, DEQOR, OptiRNA or LASSO.

In addition to efficacy, siRNA must be specific. The most prediction tools comprise or at least allow BLAST searching, which helps to find siRNA with lower probability of off-targeting. Off-targeting 5´half of antisense strand sequence is critical in off-targeting rise. It is assumed that three or more mismatches in 5´half of AS are enough to disrupt mRNA binding.

Previously, mRNA structure was not consider during siRNA design but several studies showed that siRNA efficacy could highly depends on target mRNA structure (see chap. 4.2.). Only a few siRNA design tools comprise mRNA structure prediction (for example siRNA Target Finder). Scientists from the University of New Mexico developed SDS/MPI algorithm, which connects to another siRNA finding tools, which predicts siRNAs regardless of mRNA structure. SDS/MPI selects the best siRNAs found out by this tools regarding mRNA structure. The newest tools as RNAxs or OligoWalk combine training on the set of siRNAs and predicting target mRNA structure.

Beside tools predicting siRNA sequences, tool searching DsiRNA candidates exists. Its name is IDT RNAi Design.

It is not possible to say, which tool is the best. Every algorithm place more emphasis on different parameters, so different tools select different siRNA candidates. Comparing results from more tools may help identify better candidates then single tool.

5. Conclusion

During ten years since RNAi was discovered, scientists gathered a lot of knowledge about RNAi mechanism and its experimental use. Many guidelines and tools for predicting highly specific siRNAs exist and many modifications increasing siRNA specificity have been discovered, so it is easy to design efficient and considerably specific siRNA. Even the most carefully chosen siRNA is not absolutely specific and is could slightly influence several genes. Despite this fact, RNAi is popular, useful and relatively easy scientific tool.

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