

RORC2 Gene Silencing in Human Th17 Cells by siRNA: Design and Evaluation of Highly Efficient siRNA

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Abstract

Background: RNA interference-based gene silencing has recently been applied as an efficient tool for functional gene analysis. RORC2 is the key transcription factor orchestrating Th17 cells differentiation, the cells that are known as the pathogenic elements in various autoimmune diseases. The aim of this study was to design efficient siRNAs specific for RORC2 and to evaluate different criteria affecting their functionality.

Methods: Three siRNA duplexes specific for RORC2 mRNA were designed. Th17 cells were produced from IL-6 and IL-1 treated cord blood CD4⁺ T cells. The T cells were transfected with three different designed siRNAs against RORC2 and the expression of RORC2 gene was measured using quantitative real time PCR.

Results: Different levels of RORC2 down regulation were observed in the presence of each of the designed siRNAs. Efficient siRNA with 91.1% silencing activity met the majority of the established bioinformatics criteria while the one with 46.6% silencing activity had more deviations from these criteria.

Conclusion: The more bioinformatics criteria are considered, the more functionality were observed for silencing RORC2. However, the importance of the type of criteria per se should not be neglected. Although all recommended criteria are important for designing siRNA but their value is not the same.

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Introduction

The discovery of RNA interference (RNAi) provided an unexpected new approach for studying gene functions in many different cell types¹. Using RNAi, researchers are able to silence the expression of every single gene in mammalian cells with extremely high and specific inhibitory effect²⁻⁶. Recently, numerous human genes have been knocked down in cultured somatic cells using small interfering

RNAs (siRNAs)⁷⁻¹¹. The silencing effect of RNAi is so potent that can mediate gene-silencing even at a concentration of 1-3 double strands per cell^{6,12}.

siRNA, with characteristic 2-3 nucleotide 3' overhangs, is produced in cytoplasm by Dicer and incorporated into the RNA-induced silencing complex (RISC)^{2,7,13-17}. Then, with its anti-sense or guide strand, it searches for

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complementary mRNAs and promotes their enzymatic degradation^{1,3-6,8,11,13-20}.

Essential principles for designing functional siRNA

At the beginning, the Tuschl rules were the only criteria accessible until Reynolds *et al* reported their algorithm for rational design of effective siRNAs and since, several other algorithms have been emerged¹³. Reynolds *et al*, Ui-tei *et al*, Amarzguoui and Prydz mostly emphasized on position preferences in siRNA sequence^{15,19,21-23}.

To design siRNA duplexes for target mRNAs, identification of unique targets containing the sequence AA(N19)UU or presence of one adenosine (A) at the beginning of the targeted region, at least 100 bases downstream of the AUG, is recommended^{3,9,14,24}.

Additional parameters that were evaluated for correlation with high functionality include: (I) the potential for intra-molecular secondary structures (hairpins), (II) the stability of duplex ends, and (III) specific sequence motifs within the duplex^{3,17,18,21,24-26}.

Several studies identified significant correlations between the target mRNA secondary structure and its accessibility with the functionality of siRNAs¹⁴. Indeed, the local structure of mRNA at the target site could be an important factor²⁷. This means that secondary structure of mRNA may act as an obstacle to RNAi, but how strong this effect is in comparison with other factors remains to be determined²⁴. Nevertheless, according to a number of reports, if mRNA secondary structure plays a role in siRNA efficiency, it is not the primary determinant of function¹⁴. However, now it is recommended to avoid target regions where the mRNA can form a hairpin structure²⁷.

Many reports have demonstrated that introduction of siRNAs into mammalian cells may lead to unintended gene suppression via RNAi-independent and/or RNAi-mediated mechanisms. For example, duplexes longer than 23 bp in length, particular sequence motifs, such as GUCCUCAA and UGUGU,

and terminal end structures induce IFN response through Toll-like receptor 3 (TLR-3)^{28,29}.

The most common mechanism by which off-target gene silencing takes place is through knocking down of genes with identical or partially identical sequence homologies²⁸. The selected siRNA should have multiple mismatches to all potential non target mRNA sequences but, as a threshold, it is recommended that siRNAs less than 84% (16/19×100) homology in sequence to any part of total mRNA sequences other than the target can be selected for RNAi^{22,23}. Thus, all siRNA candidates must be filtered through one of the local sequence alignment algorithms like Basic Local Alignment Search tool (BLAST) or Smith-Waterman^{23,28}. A combination of different bioinformatics criteria, which have been proposed for designing siRNA so far, are listed in table 1.

Nowadays, there are a number of online services which help researchers to design highly efficient siRNAs against mRNA of every desired gene. Although the current rational design algorithms accurately predict target gene knockdown, the real parameters which contribute to the siRNA specificity are not well known³⁰. For designing highly functional siRNAs, combining these criteria would enable us to design more effective siRNA sequences^{23,31}. However, it seems that bioassay systems are the only accepted way to clear the efficiency of a given siRNA.

In contrast to more evaluated "primer design tools", programs specified for designing siRNA are too young and not all of their bioinformatics criteria are assessed for wide variety of genes to validate their predictive values. Hence, the aim of the current study was to design siRNAs specific for RORC2, the key transcription factor of pro-inflammatory Th17 cells, using distinct algorithms and a combination of different bioinformatics criteria, and to validate the predictive value from degree of functionality of the selected siRNAs *in vitro*.

Table 1. A combination of the most important criteria for siRNA function prediction

Criteria	Reference	Criteria	Reference
No homology of seed region	(17,22,29,30,39-41)	No G in 15th position of S strand	(21,22,26)
No AS strand homology	(28)	GC content 30-52%	(3,17,18,21-26)
Off target homology <84%	(23)	A/U in 13th position of S strand	(3,17,18,21-26)
>100bp downstream of start codon	(3,9,14,24)	G/C in 19th position of AS strand	(3,17,18,21,24-26)
No 5'-UGGC in AS strand	(29)	No 5 tandem A/U or G/C	(14,21,29)
No G/U mismatches (wobble) of AS strand with off-target	(11,42)	A in position 3 of S strand	(3,17,18,21-26)
Central mismatch with off-target	(13,42)	A/U in 19 th position of S strand	(3,17,18,21-26)
Mismatch in 3' of AS strand with off-target	(13,28)	U in 10 th position of S strand	(3,17,18,21-26)
No SNP included on target	(17,28)	More G/C in 5' end of S strand	(15,19,21,23)
No C in 16 th position of S strand	(21-23)	No 9 tandem G/C in 5' of S strand	(15,19,21,23)
Up to 5 A/U in position 15-19 of S strand	(15,19,21-23)	AA or NA(N19) target	(3,9,14,24)
Tm <60 °C	(26)	No immune-modulator motif (GUCCUUC AA or UGUGU)	(29)
A/U in 1 st position of AS strand	(3,17,18,21,24-26)	No quarter A, G, C or T on target	(14,21,29)
G/C in 1 st position of S strand	(15,19,21-23)	Average of internal stability (AIS) for positions 9-14 of AS strand: -3.6 to -13.6kcal/M	(43)
No secondary structure on target	(13,27)	Binding energy of AS strand 5' end less than [9kcal/M]	(15,43)
More A/U in 5' of AS strand	(15,19,21,23)	differential stability of siRNA duplex ends (DSSE)>0	(28)

Materials and Methods

Protocol for in silico design of siRNA specific for RORC2 gene

Retrieving the target mRNA sequence and preparing candidate siRNA list: The mRNA sequence of RORC2 could be retrieved using RefSeq accession number: NM 001001523 in National Center for Biotechnology Information (NCBI) Entrez Gene database.

Based on the conserved nature of coding sequences and the lower (compared to UTRs) probability of unknown polymorphisms²⁸, coding sequence (CDS) of RORC2 was pinpointed for designing siRNAs in the present study. Protein binding sites on mRNA in the 5' un-translated region (UTR), 3' UTR, start codon, introns and splice junctions should be avoided^{3,14,28}.

A list of academic and commercially provided algorithms for designing siRNA which were applied in the current study is shown in table 2. Using these online services, a lot of target sequences and related siRNA candidates were obtained. These predicted siRNAs

were then screened by the following criteria for finding the most efficient ones:

A) Homology search

To minimize the chance of off-target effects, the most widely used algorithm, BLAST (<http://blast.ncbi.nlm.nih.gov>) was applied in this study. Both the sense and Antisense (AS) strands of a candidate siRNA were checked because the sense strand can also cause off-target cleavage by accidental incorporation into RISC²⁸.

Applying default search parameters in BLAST search may not be applicable for very short sequences such as siRNA homology test. Hence, in this study the parameters were adjusted according to the Birmingham *et al* guideline²⁸.

B) Single nucleotide polymorphism (SNP)

Another important issue which should be concerned during siRNA designation is that even one nucleotide mismatch with target sequence may cause a dramatic decrease or loss of functionality in siRNA²⁸. Due to presence of two Single Nucleotide Polymorphisms

Table 2. A list of the most popular siRNA design centers

Tool	Developer	Web site	Reference
siDirect	University of Tokyo	http://genomics.jp/sidirect/index.php?type=fc	(23,28)
siRNA Target Finder	Gene script	http://www.genscript.com/ssl-bin/app/rnai	(23)
siRNA Design Software		http://www.cs.hku.hk/sirna/	(23)
OptiRNA	University of Nebraska-Lincoln	http://optirna.unl.edu/	(23,28)
siDRM	University of Minnesota	http://sirecords.umn.edu/siDRM/	(28)
siRNA Selection Server	Whitehead Institute	http://jura.wi.mit.edu/bioc/siRNAext/	(23)
siRNA Wizard	Invivogen	http://www.sirnazizard.com/design_advanced.php	(23)
Block-iT RNAi Designer	Invitrogen	https://rnaidesigner.invitrogen.com/	(23,28)
siRNA Target Finder	Ambion	http://www.ambion.com/techlib/misc/siRNA_finder.html	(23,28)
siDESIGN Center	Dharmacon	http://www.dharmacon.com/sidesign	(22,23,28)

(SNPs) in exon 3 nucleotide 264 (rs34830957) and in exon 4 nucleotide 827 (rs41263732) in *RORC2* gene, proposed siRNAs specific for these two areas were discarded.

C) Evaluation of internal energy and secondary structures

For each siRNA candidate, functionality score was calculated based on differential end stability (the relative thermodynamic stability of the two ends of the duplex), instability in the central region of the siRNA and nucleotide composition preferences at each special position. These criteria are defined as properties that enhance AS strand selection by RISC, target annealing and cleavage, respectively.

We evaluated the thermodynamic features of candidate siRNAs using Sfold software (<http://sfold.wadsworth.org>) which includes a statistical sampling algorithm to produce a probability profiling of single stranded regions in RNA secondary structure^{14,32} and Genbee service (http://www.genebee.msu.su/services/rna2_reduced.html). Some of thermodynamic aspects of siRNA such as T_m (the predicted melting temperature of the siRNA hairpin loop) were calculated based on nearest neighbor method using Oligo 6.0 software and "Fermentas" online service (<http://www.fermentas.com/reviewer/app?page=OligoProperties&service=page>).

D) Seed match search

According to the short length of this region, it is impossible to anticipate off-target seed homologies by BLAST program and it needs

specific software. For this purpose, we applied some web-based search tools which are available for identification of all anticipated seed matches for any given siRNA sequence in following URLs²²: <http://informaticseskitis.griffith.edu.au/SpecificityServer>, <http://www.dharmacon.com/seedlocator/default.aspx>

Candidate siRNAs which had the least possible seed homology were selected. Eventually, Uracil (U) residues in the 2 nucleotides 3' overhangs were replaced by deoxythymidine (T). It is reported that, this replacement significantly reduces the cost of RNA synthesis and also enhances nuclease resistance while doesn't lead to loss of activity^{3,9}.

Isolation of naive CD4⁺ T cells

Mononuclear cells were separated from 100 ml cord blood sample using Ficoll-Hypaque density gradient (biosera). Naive CD4⁺ T cells were isolated using human naive CD4⁺ T cell isolation kit II (Miltenyi Biotec) according to manufacturer's instruction. Briefly: CD45RO⁺ activated/memory T cells and non-CD4⁺ T cells were magnetically labelled by a cocktail of biotin-conjugated antibodies against CD8, CD14, CD15, CD16, CD19, CD25, CD34, CD36, CD45RO, CD56, CD123, TCR γ/δ , HLA-DR, CD235a (Glycophorine A) and anti-biotin micro-beads. Then, highly pure naive CD4⁺ T cells were isolated following depletion of magnetically labeled cells^{33,34}. Purity was confirmed by FACSCalibur (Becton Dickinson) after immune staining with FITC conjugated anti human CD4 and PE conjugated anti human CD45RA antibodies (BD).

Cell culture and differentiation

CD4⁺ T cells were treated with 200 μ l PBS including 5 μ g/mL anti-CD3 antibody and 2 μ g/mL anti-CD28 antibody (ebioscience) and incubated at 4°C overnight. Naïve CD4⁺ T cells ($4\text{--}5 \times 10^5$) were then cultured in X-VIVO 15 serum free medium (Lonza) in the presence of IL-6 (30 ng/mL), IL-1 β (100 ng/mL) (ebioscience), anti IFN γ (10 μ g/mL) and anti IL-4 (10 μ g/mL) (ebioscience) to inhibit unwanted deviation to Th1 or Th2 subsets. Culture media and all their contents had been refreshed after 3 days. After 6 days, cells were washed and their viability was checked by trypan blue³³⁻³⁵.

Transfection of the cells with siRNA

Cells were transfected with siRNA oligonucleotides on day 3, using TransIT-TKO Transfection Reagent (Mirus) as instructed by manufacturer. For $3\text{--}5 \times 10^5$ cells per well, 4 μ l TransIT-TKO Transfection Reagent, 50 nM of siRNA (final concentration) and 50 μ l of serum-free medium OptiMEM were applied. In order to silencing RORC2 gene expression, differentiating human CD4 T cells were treated with three selected siRNA sequences, "Amb", "Opti" and "siDir", separately.

In addition, one treatment, named "mix", was performed using a combination of these three siRNA duplexes in equal ratios. Untransfected T cells were applied as control and all other treated cells were compared with this group. T cells transfected with Label IT[®] RNAi Delivery Control kit (Mirus) as scrambled siRNA and T cells accompanied with transfection reagent without siRNA (mock control) were used as toxicity controls. The cells were incubated overnight and then, medium and all of its contents were refreshed and culture continued until day 6.

Real time quantitative RT-PCR

On the 6th day, total RNA from cultured CD4⁺ T cells was extracted using RNeasy mini kit (Qiagen) and cDNA was synthesized by QuantiTect reverse transcription kit (Qiagen) as instructed by manufacturer. The resulting transcripts were then quantified by

real time PCR on a Rotor-Gene 6000 real time DNA amplification system (Corbett life science) with QuantiFast SYBR Green PCR kit (Qiagen). Pre-designed primers (QuantiTact primer Assay; Qiagen) specific for RORC2 and IL-17 genes were used. Template cDNA was held in 95°C for 5 min and RCR performed in 40 cycles of 95°C for 10 s (denaturation) and 60°C for 30 s (combined annealing & extension). For each sample, transcript quantity was normalized to the amount of beta-actin (ACTB) expression. The results were analysed by relative quantification method^{36,37}.

Statistical analysis

One Way ANOVA applied for comparisons of the treated groups with siRNA duplexes. Pearson correlation was used to evaluate the degree of relationship between RORC2 and IL-17. Replicates have been aggregated and their mean and relative standard deviation (rel. SD) values have been shown in graphs using percentage of expression.

Results

In order to evaluate the actual predictive values of different bioinformatics criteria proposed so far, we combined nearly all reported criteria and sequence preferences (Table 1) for designing the most efficient siRNA(s) specific for the key transcription factor of Th17 cells, called *RORC2*. In the current study, applying different siRNA designing tools, numerous siRNA candidates have emerged. According to the criteria listed in table 1, a number of candidates were filtered out and only 17 siRNA candidates from 7 designing tools had all the criteria. Among these, 4 siRNAs had been designed by more than one tool proposing the more probability of being highly functional.

Based on the highest scores earned through passed steps, the best three siRNA candidates (the highest scores) were selected, one of them, named "Amb", was designed using "Ambion", "RNAi design software" and "Dharmacon", the second one, named "siDir",

Table 3. Selected siRNA duplexes specific for RORC2 gene

	siRNA name	Start position	siRNA sequence
1	OptiRNA	872	5'-CCUCCUGACAGAGAUAGATT-3' 3'-TTGGAGGGACUGUCUCUAUCU-5'
2	siDirect	1197	5'-CCGCACGGUCUUUUUUGAATT-3' 3'-TTGGCGUGCCAGAAAAAACUU-5'
3	Ambion	1393	5'-GUAGAACAGCUGCAGUACATT-3' 3'-TTCAUCUUGUCGACGUCAUGU-5'

was designed using "siDirect" and "Ambion", and the third one, named "Opti", was solely proposed by "OptiRNA" software (Table 3).

Highly pure naïve T cells were isolated from cord blood samples in the current study. Figure 1 shows that based on flowcytometric analysis, more than 95% of isolated cells were CD4⁺/CD45RA⁺ cells which represent the naïve T cells.

Following transfection of the T cells with Label IT[®] RNAi Delivery Control kit, transfection efficiency was identified quantitatively by flow cytometric analysis (Figure 2). Transfection efficiency was 89% without any significant toxicity in all samples.

Silencing procedure was performed using 50 nM final concentration of each siRNA, for CD4⁺ T cells cultured in X-VIVO 15 serum free medium treated with IL-1 β and IL-6. At the end of the 6th day, total RNA of the cells was extracted and qRT-PCR was performed to evaluate *RORC2* and *IL-17* genes expression.

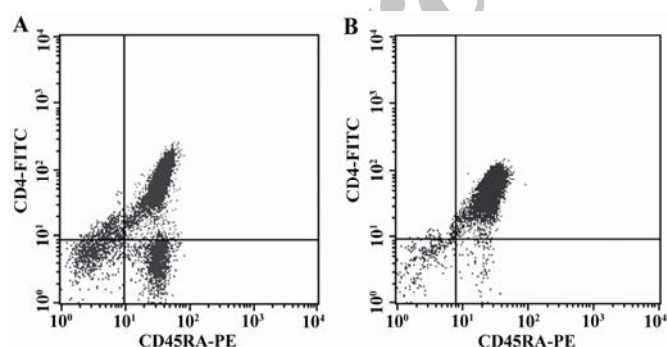


Figure 1. Flow cytometric analysis of naïve CD4⁺ T cells population isolated from cord blood samples. A) Dot plot diagram shows three distinct cell populations exist in cord blood mononuclear cells separated via ficoll hypaque density gradient, before applying human naïve CD4⁺ T cell isolation kit; B) Dot plot diagram shows more than 95% purity of CD4⁺/CD45RA⁺ T cells in cell samples obtained from human naïve CD4⁺ T cell isolation column

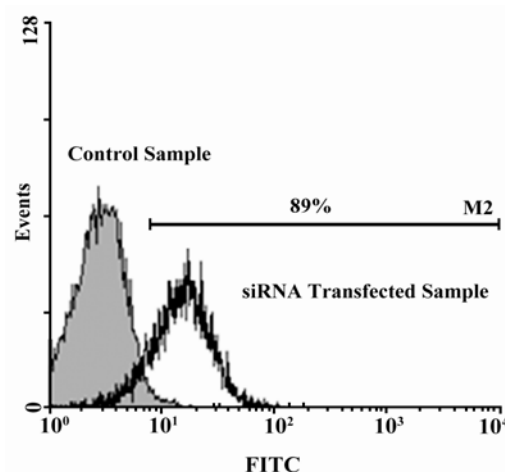


Figure 2. Human CD4⁺ T cells, 24h after transfection with flourescein-labeled siRNA. Flow-cytometric analysis of transfected T cells shows 89% transfection efficiency. Gray curve represents untransfected cells

The expression level of RORC2 has not significantly suppressed in "siCONTROL" and "mock" control compared with "untransfected" control. This represents that performed treatments for siRNA transfection had not had unspecific inhibitory effect on RORC2 gene expression. Hence, and in order to evaluate the pure effect of specific siRNAs on RORC2 gene inhibition, all treated samples were compared with "siCONTROL".

Figure 3 shows that the silencing effect of siRNA designed via OptiRNA software (Opti; start position 872) was 89.6% on RORC2 gene expression. This siRNA had only 3 deviations from all studied criteria (Table 2). Other two siRNA duplexes named "siDir" (start position 1197) and "Amb" (start position 1393), were also functional with 55.3% and 38% knocking down effect, respectively. These siRNA duplexes had lost 6 and 8 bioinformatics criteria from all which are listed in table 3. Applying a combination of these three siRNAs (Mix) led to 99.5% knocking down in

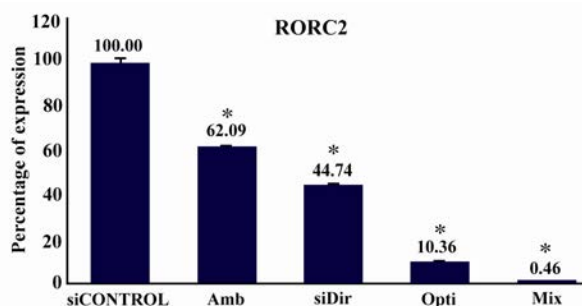


Figure 3. Quantitative real time-PCR evaluation of siRNAs inhibitory effects on RORC2 transcript level. All siRNA duplexes significantly suppressed RORC2 gene expression (* $p < 0.05$) compared to siCONTROL. The most silencing was obtained when a combination of siRNAs (Mix) was applied. Data, are shown in relative percentage scale, are the mean of three sets of similar experiments. Relative SD is shown by the bar. Scrambled siRNA (siCONTROL): transfection with Label IT® RNAi Delivery Control

RORC2 gene expression. Suppressive effect of all studied siRNA duplexes in the present study was significant ($p < 0.05$) compared with siCONTROL.

In order to confirm RORC2 suppression and functional analysis of affected Th17 cells, gene expression of IL-17 was measured as well. Measurement of IL-17 transcript levels in T cells whose RORC2 expression was knocked down revealed that, suppression of RORC2 gene was strongly effective for inhibition of IL-17 gene expression ($p < 0.05$) (Figure 4), and there was a significant correlation between RORC2 inhibition and decrease of IL-17 expression ($R = 0.84$; $p = 0.000$).

Discussion

Among the best three siRNA candidates designed and evaluated in current project for silencing RORC2 in human Th17 cells, "Amb" stood on the 3rd rank having eight deviations from all criteria. Accordingly, the inhibitory effect of this siRNA on RORC2 gene expression was only 38% which was the least inhibition compared with other siRNAs. According to the Ui-Tei *et al* classification, siRNAs with lower than 20% activity are classified as highly ineffective and those with more than 70% inhibitory effect are considered as highly effective¹⁵. Therefore, as the

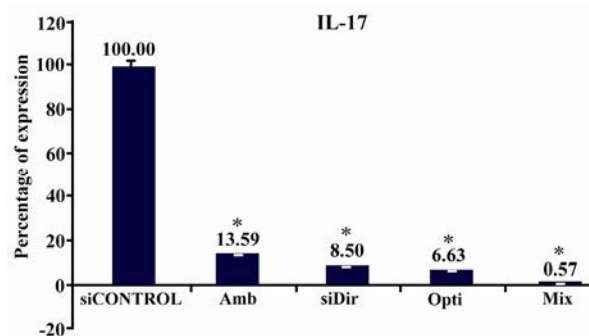


Figure 4. Quantitative real time-PCR evaluation of RORC2 suppression effects on transcript levels of IL-17. With all degrees of suppression in RORC2 gene, decrease of mRNA levels of IL-17 was significant (* $p < 0.05$) compared to siCONTROL. The most silencing was again seen in "Mix" sample. Data, are shown in relative percentage scale, are the mean of three sets of similar experiments. Relative SD is shown by the bar. Scrambled siRNA (siCONTROL): transfection with Label IT® RNAi Delivery Control

silencing effect is less than 50% but more than 20%, we can consider this one as a low functional C siRNA.

In three independent studies it has been reported that the presence of more G/C in 5' end of Sense Strand (SS) is important for increasing the functionality of siRNA^{15,19,21,23}. In the same studies and a study done by Amarzguoui *et al*, it is reported that the presence of G/C in 19th position of anti sense (AS) strand is correlated with high functionality^{3,17,18,21,24-26}. In addition, in a number of studies, siRNAs possessing A/U in 19th position of sense strand (SS) are reported to have more RNAi activity^{3,17,18,21-26}. Other groups in different studies reported that possessing a NA(N19) sequence on target mRNA may lead to highly effective RNAi activity^{3,9,14,24}.

Considering the fact that "Amb" does not meet these criteria while applying the same sequence preferences in designing the other two siRNAs was correlated with more silencing activity, it appears that taking these rules into consideration for designing a highly effective siRNA is necessary.

In the present study, the siRNA named "siDir" was the second most effective siRNA regarding both the bioinformatics criteria (with 6 deviations) and according to its suppressive effect on RORC2 (55.3%) *in vitro*.

Therefore, this one could be considered as class B siRNA with slightly more than 50% suppression.

A number of studies have reported that highly effective siRNAs have preferentially an A/U residue in 13th position of their AS strand^{3,17,18,21-26}. Other studies independently have stated that occurrence of 5 tandem A/U or G/C residues in siRNA sequence is correlated with ineffective RNAi activity^{14,21,29}. In three studies, possessing more A/U in 5' end of AS strand was reported to be consistent with higher RNAi activity^{15,19,21,23}.

These sequence preferences were considered in designing "Amb", the least effective siRNA, while for designing "siDir" these criteria were ignored. According to the higher efficiency of "siDir", it seems that these criteria do not greatly impact on effectiveness of siRNA activity. Considering the low efficiency of "siDir" compared to "Opti", which meets these criteria, taking these items into consideration is recommended.

Three groups independently have reported that presence of quarter A, G, C or U on target mRNA sequence conversely correlated with siRNA functionality^{14,21,29}. This criterion was considered neither for designing of "Amb" nor for "siDir". Regarding the higher effect of "siDir" compared with "Amb", it seems that other mentioned criteria had more impact on their activity. It should be noted that, as the same criterion was considered for designing the most effective siRNA in the current study, "Opti", the possible importance of it should not be neglected.

In the present study, the most effective siRNA was "Opti", having only three deviations from all considered *in silico* criteria and 89.6% silencing activity on cultured CD4⁺ T cells. With more than 70% silencing effect, this one could be classified as a highly effective siRNA or class A.

Many groups have reported that the presence of an A residue in 3rd position and an U residue in 10th position of sense strand is critical for increasing RNAi activity^{3,17,18,21-26}.

In the current study, these two criteria were considered neither for selecting "Opti" nor for "siDir" and "Amb". Interestingly, in these three siRNAs, instead of a U residue in position 10 of SS, nucleotide G was present at this position. Although "siDir" and, especially, "Amb" were not highly effective, regarding to the high efficiency of "Opti" it suggests that these sequence preferences are not essential criteria at least for silencing RORC2.

In a number of studies it is stated that accessibility of siRNA to its specific target sequence is a critical factor defining high RNAi functionality. Therefore, it is recommended that siRNAs should be specific for the regions on target that are less likely to have stable secondary structure^{13,27}.

Although, the computation of target site accessibility is considered as a factor necessary to improve the prediction of effective siRNAs³², the secondary structure of target site may not be critical¹³. In the present study, "siDir" was the only selected siRNA that met this criterion. "Opti" and "Amb" were specific for regions with more chance to form secondary structure. Although "Amb" was not so effective regarding the high efficiency of "Opti", it suggests that target accessibility is not a crucial factor for defining a high functional siRNA.

On the other hand, considering the fact that target mRNA structure is a parameter that cannot be confidently modeled¹⁸ and secondary structure prediction for a single mRNA molecule is a typical problem of computational biology³⁸, it is likely that "Opti" is specific for an accessible site which was falsely predicted as an inaccessible site.

Regarding IL-17, it is demonstrated that the expression of this cytokine was dramatically decreased following siRNA mediated knocking down of RORC2 expression, showing a significant correlation ($R=0.84$; $p=0.000$) between RORC2 and IL-17 gene expression. This result confirms the inhibitory effect of siRNAs designed in this study as the main function of Th17 cells, that is IL-17 expres-

sion, was blocked after RORC2 gene suppression.

Conclusion

According to our results, although there are some criteria for designing functional siRNA, the value of these criteria is not the same based on the target gene. Usually, using a mixture of different siRNAs against distinct positions on a certain gene leads to more efficient silencing.

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