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42- and 63-bp anti-MDR1-siRNAs bearing 2′-OMe modifications in nuclease-sensitive sites induce specific and potent gene silencing

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ABSTRACT

DsRNAs longer than 30 bp induce interferon response and global changes in gene expression profile in mammalians. 21 bp siRNA and 25/27 bp dsiRNA acting via RNA interference mechanism are used for specific gene silencing in this class of organisms. We designed selectively 2′-O-methyl-modified 42 and 63 bp anti-MDR1-siRNAs that silence the expression of P-glycoprotein and restore the sensitivity of drug-resistant cancer cells to cytostatic more efficiently than canonical 21 bp siRNAs. We also show that they act in a Dicer-independent mode and are devoid of immunostimulating properties. Our findings suggest that 42 and 63 bp siRNAs could be used as potential therapeutics.

1. Introduction

RNA interference (RNAi) is an evolutionarily conserved cellular mechanism of sequence-specific gene silencing mediated by diverse classes of double-stranded RNAs [1]. Long dsRNAs inside the cell are cleaved by the RNAse-III class nuclease Dicer into 21–23-bp dsRNAs with 2-base 3′-overhangs and 5′-phosphates, termed short interfering RNA (siRNAs) and assemble into the RNA-induced gene silencing complex (RISC) [2–4]. Because of the outstanding potency and specificity in comparison with other loss-of-function technologies, RNAi has quickly developed into a potent biological tool for the specific inhibition of gene expression.

Long dsRNAs efficiently induce RNAi in non-mammalian eukaryotes, but in mammalian cells, dsRNAs longer than 30 bp activate the innate immunity system and induce global changes in the gene expression profile [5]. Tuschl and colleagues demonstrated that short 21-bp RNA duplexes, mimicking the products of dsRNA processing by Dicer, can be used for specific gene silencing, avoiding non-specific interferon response [3].

Another approach is to use longer synthetic RNA duplexes (typically 25–30 bp) that are substrates for Dicer [6]. These longer duplexes are effectively processed by Dicer into short 21 bp RNA duplexes, enter RISC and trigger RNAi [7]. Recent studies have revealed that longer synthetic RNA duplexes effectively silenced gene expression in many cell types without inducing interferon response, whereas they could trigger an innate immune response in several cell types [8]. The chemical modifications known to prevent the recognition of longer RNA duplexes by the innate immune system without influencing silencing activity have been assumed [6,9]. These chemical modifications can block the immune response and we can suppose that theoretically nothing prevents the use of partly modified dsRNAs of any length. Therefore, we decided to check this theory in our study using various experiments.

In our study, we used selectively modified RNA duplexes 21, 42 and 63 bp in length targeted *MDR1* mRNA and examined their ability to silence the expression of the target gene and the specificity of their action. We showed that 42 and 63 bp siRNAs induced more effective RNAi at lower concentrations than classical 21 bp siRNA without non-specific immune effects. To our surprise, no substantial processing of dsRNAs by Dicer was detected inside the cells. Our results remove the length limits for the design of RNAi effectors, and add another example in the list of novel RNAi-inducing molecules differing from the classical siRNA.

Abbreviations: RNAi, RNA interference; siRNA, small interfering RNA; MDR, multiple drug resistance; FBS, fetal bovine serum; MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide; IFN, interferon

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2. Materials and methods

2.1. siRNA synthesis and duplex annealing

Modified siRNAs were synthesised on an automatic ASM-800 DNA/RNA synthesiser (Biosset, Russia) using ribo- and 2′-O-methylribo β-cyanoethyl phosphoramidites (Glen Research, USA). The nuclease-sensitive sites in siRNA were protected by introducing 2′-O-Me-analogues of ribonucleotides. After standard deprotection, oligoribonucleotides were purified by denaturing polyacrylamide gel electrophoresis (PAGE) and isolated as sodium salts. The purified oligoribonucleotides were characterised by MALDI-TOF mass spectrometry on PEFLEX III (Bruker Daltonics, Germany). The following siRNAs were used in the present study: a 21 bp monomer (siM) homologous to the mRNA region 557–577 of the *MDR1* gene (sense strand 5′-GGCUUmGACmAAGUUmGUm AUmAUmGG-3′; antisense strand 5′-AUmAUmACmAACUUmGUCm AAGCCmAA-3′), the 42 bp dimer (siMDR-D) and the trimer (siMDR-T), which contain the sequence of the monomer repeated two and three times, respectively. The siRNA-Scr 21 bp monomer (siScr-M), with no significant homology to any known mRNA sequences from mouse, rat or human (sense strand 5′-CmAAGUCUCGUm AUmGUmAGUmGGUU-3′; antisense strand 5′-CCmACUmACmAUm ACGAGACUUmGUU-3′), the 42 bp dimer (siScr-D) and 63 bp trimer (siScr-T), which contain the sequence of the Scr monomer repeated two and three times, respectively, was used as the control. siRNA duplexes were obtained via annealing of the antisense and sense strands at equimolar concentrations in buffer A (15 mM HEPES-KOH pH 7.4, 50 mM potassium acetate, and 1 mM magnesium acetate) and stored at −20 °C.

2.2. Cell culture and transfection

Multiple drug-resistant human cell line KB-8–5 growing in the presence of 300 nM vinblastine was generously provided by Prof M. Gottesman (NIH, USA). The cells were grown in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin, 100 μg/ml streptomycin and 0.25 μg/ml amphotericin at 37 °C in a humidified atmosphere containing 5% CO_2/95% air. Cells in the exponential phase of growth were plated in 96-well plates at a density of 1×10^3 cells/well, in 24-well plates at a density of 0.4×10^5 cells/well or in 6-well plates at a density of 1.5×10^5 cells/well 1 day before the experiment; 24 h later, cells were transfected with siRNA using Oligofectamine according to the manufacturer's protocol. Untreated cells or those treated with Oligofectamine only were used as controls.

2.3. Real-time reverse transcription PCR (RT-PCR)

KB-8–5 cells were plated in 24-well plates (0.4 × 105 cells/well) and 24 h later the cells were transfected with siRNA using Oligofectamine. Total RNA was extracted from the cells using the SDS-phenol method 24 and 48 h post-transfection [10]. Quantification of specific mRNAs by RT-PCR was done essentially as described in [11]. The amount of mRNA of each gene was normalized to the amount of *GAPDH* mRNA used as an internal standard. To assess the expression of *GAPDH* gene the following primers were used: GAPDH forward: 5′-GTGAAGGTCGGAGTCAAC-3′ and GAPDH reverse: 5′-TGGAATTTGCCATGGGTG-3′. The relative level of gene expression was calculated using the Bio-Rad iQ5 2.0 software (Bio-Rad Laboratories Inc., United States).

2.4. Western blotting

KB-8–5 cells in an exponential phase of growth were plated in 24-well plates (0.4×10^5 cells/well); 24 h later, the cells were transfected with siRNAs using Oligofectamine. 4 and 6 days post-transfection, the culture medium was removed and the cells were lysed in 60 μl of Sample buffer (Sigma–Aldrich, USA). Western blotting was performed as described with [12] (detailed information in Supplementary material).

2.5. MTT assay

The number of living cells was determined after 120 or 144 h of transfection using the colorimetric method based on the oxidation of 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) in the mitochondria of living cells as described in [13,14] (detailed information in Supplementary material).

2.6. Northern blotting

KB-8–5 cells in an exponential phase of growth were plated in 6-well plates (1.5×10^5 cells/well); 24 h later, the cells were transfected with siRNAs (200 nM) using Oligofectamine according to the procedure recommended by the manufacturer. After incubation of the cells with siRNAs for 24, 48 and 96 h, the culture medium was removed and the total RNA was extracted from the cells using *mir*Vana miRNA Isolation Kit. Northern blotting was performed via analogy with [15] (detailed information in Supplementary material).

3. Results

3.1. Design of siRNAs

We designed a set of siRNAs with different length (siMDR) targeted to the region 557–577 nt of the human *MDR1* mRNA. Previously, we have demonstrated that 21 bp siRNA targeted to this region has the ability to efficiently inhibit the *MDR1* expression both at the mRNA and protein (P-glycoprotein) levels [16]. Furthermore, this siMDR is capable of reversing the multiple drug resistance phenotype of cancer cells and inducing their death in the presence of earlier tolerable concentrations of cytostatics [16]. Here we constructed longer siRNAs (42 and 63 nt in length) containing the sequence of the previously studied siMDR (here and after siMDR-M or monomer) repeated two and three times, and designated as siMDR-D and siMDR-T or dimer and trimer, respectively. siRNAs with the same lengths: 21 bp siScr-M, 42 bp siScr-D and 63 bp siScr-T, which had no significant homology to any known mRNA sequences from mouse, rat or human, were used as negative controls.

The nuclease sensitive CpA, UpA and UpG sites in siRNAs were protected by introducing 2′-O-methyl analogues of ribonucleotides according to the algorithm, which was described by us earlier [17]. The modifications in the sites of potential Dicer cleavage were omitted.

3.2. Long selectively 2′-O-methylated siRNAs do not trigger interferon response

It is well established that 2′-OMe modifications efficiently prevent the induction of interferon response by 21 bp siRNA containing immunostimulation sequences [18] and 25/27 bp dsiRNAs [19]. We evaluated the ability of selective 2′-O-methyl modification to prevent the induction of interferon (IFN) response by longer siRNAs. In these experiments, the mRNA levels of two IFN responsive genes, *β-actin* and *protein kinase R* (*PKR*), were used as markers of interferon response and the level of *GAPDH* mRNA was used as internal standard. Poly(I:C), a well-known IFN inducer, was used as a positive control. The data

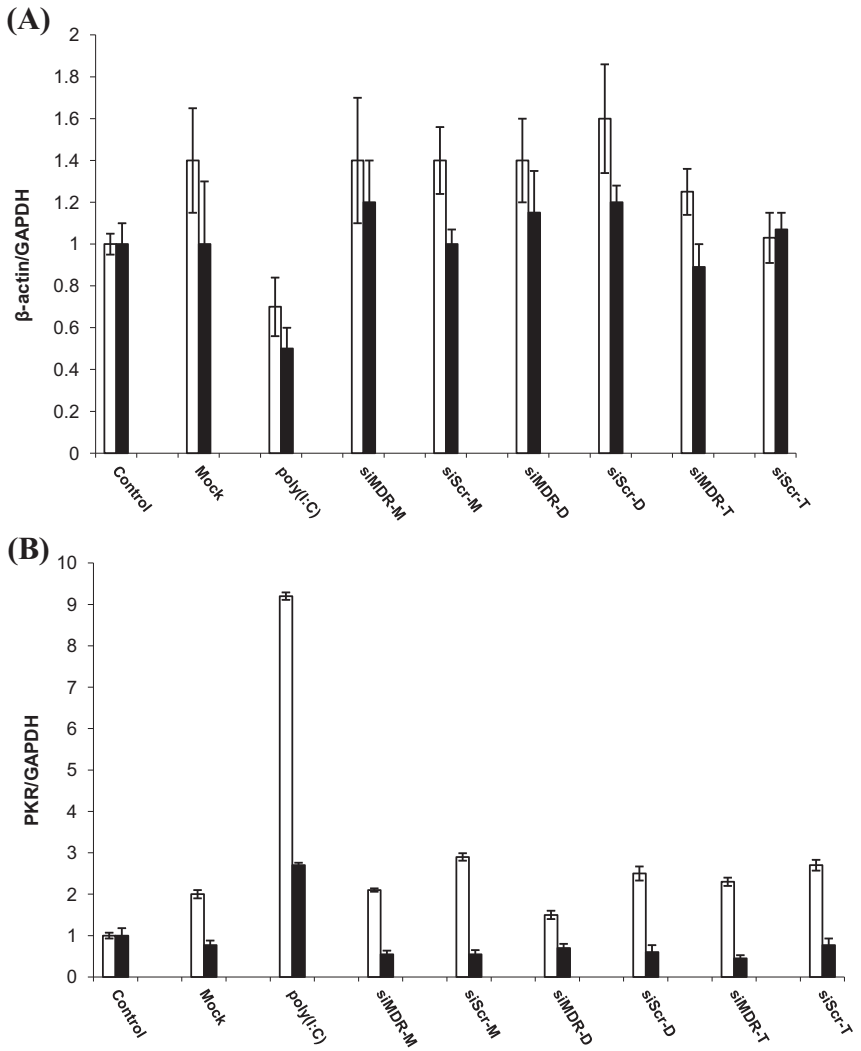

Fig. 1. Expression of interferon response marker genes encoding β-actin (A) and PKR (B) measured by RT-PCR 24 h (white bars) and 48 h (black bars) after transfection of KB-8-5 cells with siRNAs (200 nM) or poly (I:C) (250 ng/mL). The level of *GAPDH* mRNA was used as an internal standard. Error bars indicate the standard deviations (SD) of three independent sets of experiments.

obtained (Fig. 1) demonstrated that the level of *PKR* mRNA increased by a factor of 4.5-fold 24 h after and 3-fold 48 h after the transfection of KB-8–5 cells with 250 ng/mL poly(I:C), while the expression level of *β-actin* mRNA was reduced by 2-fold in the same samples. Treatment of the cells with 200 nM siMDR-M, siMDR-D, siMDR-T or siScr-M, siScr-D, siScr-T did not significantly alter the expression of the marker genes. Thus, we can conclude that tested siRNAs do not trigger a non-specific IFN response in the cells.

3.3. siRNA-mediated silencing of P-glycoprotein expression

Silencing activity of the 21-, 42- and 63 bp siRNAs was compared by their ability to reduce the level of P-glycoprotein using Western blot analysis in KB-8–5 cells. Cells were treated with increasing concentrations of siMDR-M (25–200 nM), siMDR-D (12.5–100 nM), siMDr-T (8–66.6 nM) and 200 nM siScr-M, 100 nM siScr-D and 66.6 nM siScr-T. Concentrations of siMDR-D, siMDR-T and siScr-D, siScr-T were selected in such a way in order to keep the equal "dose" of the 21 nucleotide sequence complementary to the target in parallel samples (here and after "dose-equivalent concentrations"), presuming the processing of siMDR-D and siMDR-T into two and three molecules of siMDR-M, respectively. The P-glycoprotein level was assayed 96 and 144 h after the treatment since the maximum reduction of P-glycoprotein level by siMDR-M was observed at these time points earlier [17]. It was found (Fig. 2) that treatment with specific siRNAs led to a concentration-dependent decrease in the P-glycoprotein level. The silencing effect of siMDR-M (up to 60%) at 96 h after transfection at concentrations of 100–200 nM was similar to that of siMDR-D at the dose-equivalent concentrations. However, when assayed 96 h after transfection, siMDR-D at low concentrations (30–50% silencing) and siMDR-T across the entire range of concentrations (40–20% silencing) induced less pronounced gene silencing than siMDR-M (60% silencing) (Fig. 2). The silencing effect was substantially increased (up to 80%) at 144 h after transfection with 100–200 nM of siMDR-M and was comparable to that of siMDR-D and siMDR-T at dose-equivalent concentrations. It should be noted that siMDR-T at low concentrations (8–16 nM) 144 h after transfection was more active than siMDR-D and siMDR-M: the inhibitory effect was 80%, 62% and 58% for 8 nM concentration, respectively. The P-glycoprotein level in the KB-8–5 cells treated with 200 nM siScr-M, 100 nM siScr-D and 66.6 nM siScr-T remained unaffected.

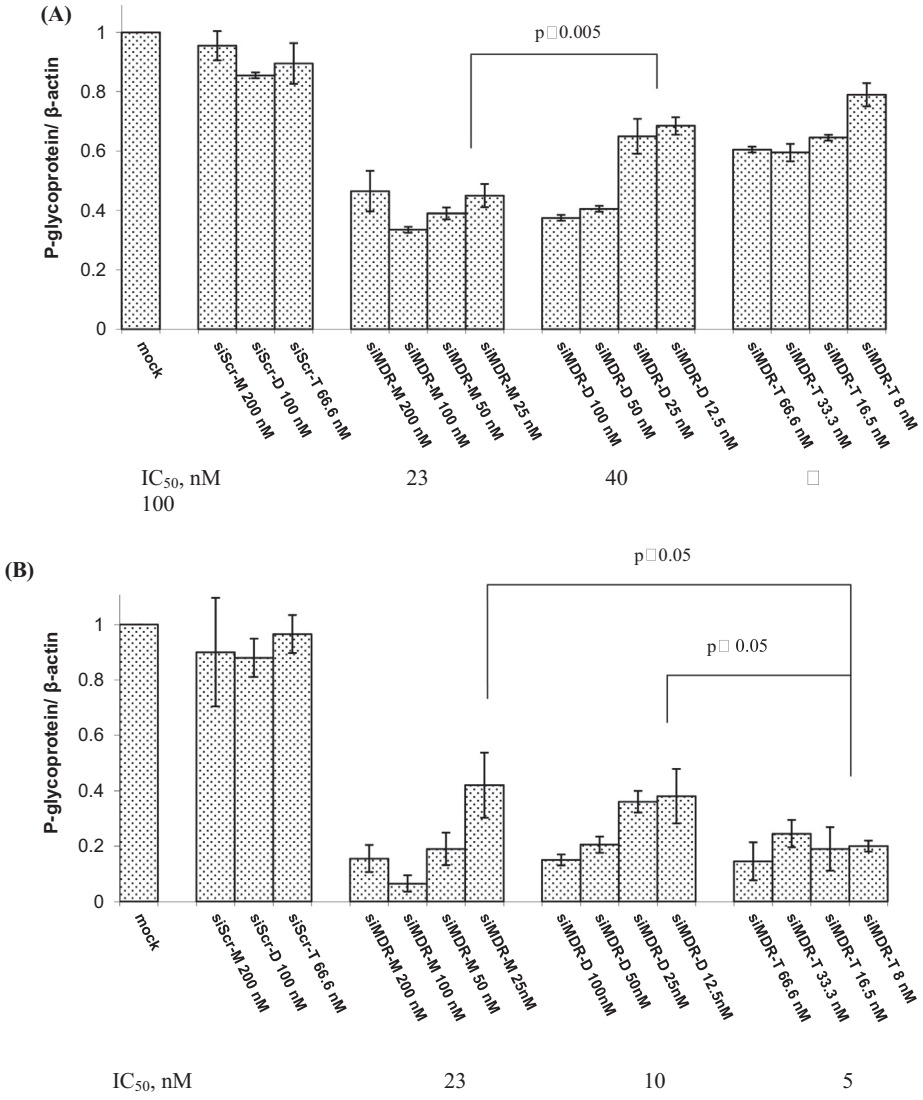

Fig. 2. siRNA-mediated silencing of the P-glycoprotein in KB-8–5 cells. P-glycoprotein levels were determined by Western blot analysis 96 h (A) and 144 h (B) after transfection with different concentrations of siRNAs. The relative P-glycoprotein level was normalised against the level of β-actin protein. Error bars indicate the standard deviations (SD) of three independent sets of experiments.

3.4. Silencing of MDR1 expression by siRNAs revert the multiple drug resistance of cancer cells

Since the therapeutic goal of *MDR1* gene silencing is restoration of the sensitivity of drug-resistant cancer cells to anti-cancer drugs, we examined the ability of anti-MDR siRNAs to decrease a number of living cells in the presence of earlier tolerable concentrations of vinblastine (300 nM) using the MTT assay. Cells were treated with increasing concentrations of siMDR-M (25–200 nM), siMDR-D (12.5–100 nM), siMDR-T (8–66.6 nM) and maximal concentrations of the control siRNAs: 200 nM siScr-M, 100 nM siScr-D and 66.6 nM siScr-T. Amounts of living cells were estimated 122 and 144 h after transfection. The number of cells in the sample treated with transfection reagent only was taken as 100%. The data indicated that treatment with siRNAs led to concentration-dependent cell death (Fig. 3). The treatment of KB-8–5 cells with 200 nM siMDR-M, 100 nM siMDR-D and 66.6 nM siMDR-T caused 50% reduction of viable cells at 122 h and the almost complete disappearance of viable cells at 144 h after transfection. It should be noted that transfection with siMDR-T resulted in the more efficient reduction of viable cell number (up to 75%) at low concentrations after 144 h of incubation as compared with samples treated with siMDR-D and siMDR-M at dose-equivalent concentrations. siScr-M, siScr-D, and siScr-T had no effect on the cell viability in the presence of 300 nM vinblastine.

3.5. siRNA processing in the cells

dsRNAs longer than 25–30 bp are known to be efficiently processed by the cellular enzyme Dicer when introduced into cells [7]. Modification patterns of 25–27 dsRNAs including alternating 2′-OMe residues that do not span the expected Dicer cut site were found to be compatible with intracellular processing [6]. Here, we investigated whether the longer 42 and 63 bp selectively modified siRNAs are processed in the cells into canonical 21 bp siRNAs and whether the cleavage is necessary for the silencing activity. siMDR-M, siMDR-D and siMDR-T were transfected into KB-8–5 cells and after 4, 24 and 48 h the cells were washed extensively to remove unbound siRNA; the total RNA was isolated from the cells using the SDS-phenol method. Northern blot analysis revealed that siMDR-D and siMDR-T remained intact inside the cells after transfection for a prolonged period of time and the products of their

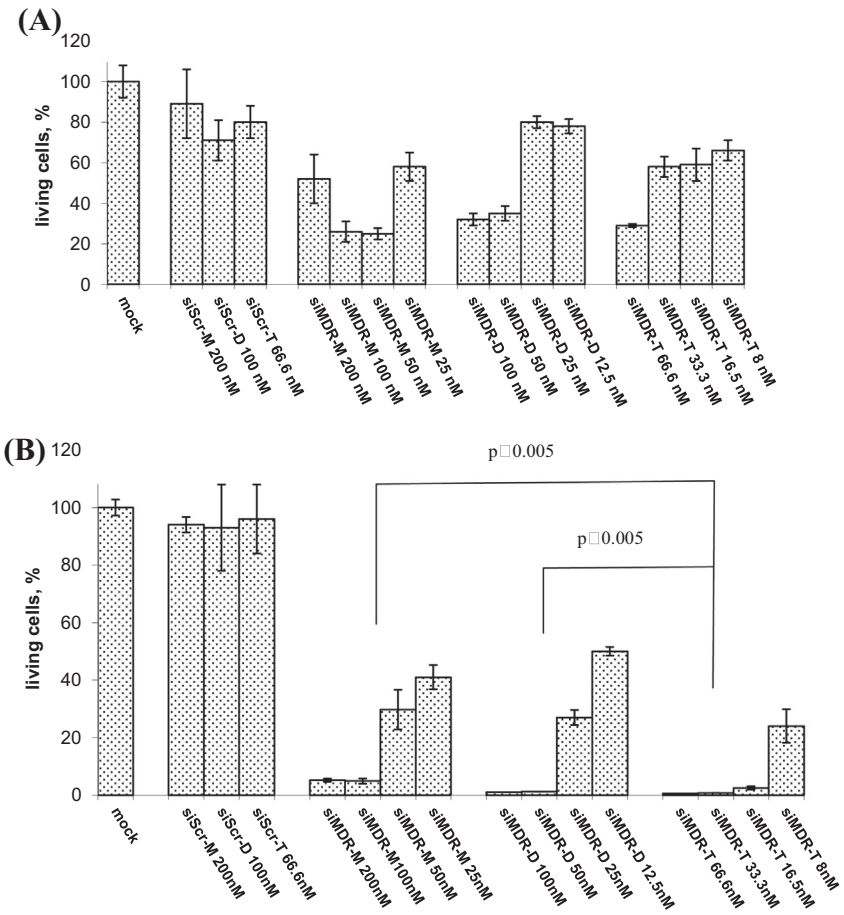

Fig. 3. Amounts of living cells were analysed using MTT-assay 120 h (A) and 144 h (B) after transfection with siRNA and subsequent incubation in the presence of 300 nM vinblastine.

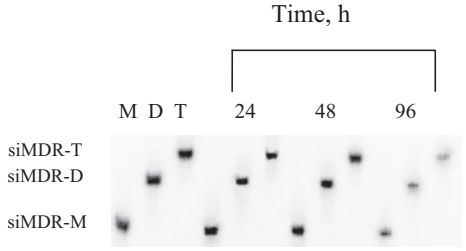

Fig. 4. Northern blot analysis of siMDR-M, siMDR-D and siMDR-T transfected into the KB-8–5 cell line. Sense strands of siMDR-M (M) siMDR-D (D) and siMDR-T (T) were used as controls.

cleavage by Dicer were not detected (Fig. 4). At the same time, these siRNAs showed potent RNAi activity, suggesting that long siMDR-D and siMDR-T duplexes could trigger RNAi in a processing-independent manner.

4. Discussion

Recent reports have proven that longer duplexes (25–30 bp in length) are processed by Dicer into 21 bp siRNAs and loaded into RISC more efficiently than canonical siRNAs and demonstrate better gene silencing [7]. It has been shown that the RNAi effector complex containing Dicer and accessory proteins interacts with an Argonaut protein (Ago) and actively loads the bottom strand of the siRNA into the RISC. Such a model implies an increased affinity of Dicer and Dicer/TRBP for longer dsRNAs, as Dicer requires a minimum stem length for efficient cleavage [6–8,20]. Moreover, it was recently demonstrated [21] that 25–27 bp dsRNAs can be directly loaded into Ago2 and show better efficacy as compared with canonical 21 bp siRNAs.

Therefore, in our study, we compared the silencing activity of 21 bp siRNA with the activity of 42 and 63 bp siRNAs containing two and three copies of the same 21 bp sequence, respectively.

The main problem in the application of the long non-modified dsRNAs as mediators of RNAi in mammalian cells and mammalians is the induction of interferon response and the subsequent global changers in gene expression profile [9,22,23]. Chemical modification patterns preventing the activation of innate immunity and compatible with the Dicer cleavage were described for 27/25 bp dsiRNA [19]. Our data revealed that 2′-OMe modification of CpA, UpA and UpG motives in 42 bp and 63 bp siRNAs effectively prevents activation of the innate immunity response and did not change the expression levels of two key interferon response genes, *PKR* and *β-actin,* in KB-8–5 cells. Thus, selectively modified long dsRNAs could be used in mammalian cells for specific gene silencing.

Gene expression assays at the protein level clearly demonstrate the efficacy and specificity of all tested anti-MDR1-siRNAs. Exposure to siRNAs caused a concentration-dependent reduction of the P-glycoprotein level. The concentration-dependent results are consistent with our previous observations using anti-MDR1 siRNAs, which can effectively inhibit target gene expression at a concentration of 200 nM [24]. To compare the biological activity of siRNAs with different lengths we used concentrations of the equal

"dose" of the 21 nucleotide sequence of siMDR-M repeated two or three times (dose-equivalent concentrations), presuming the processing of siMDR-D and siMDR-T into two or three molecules of siMDR-M, respectively.

We found that siMDR-M (IC_{50} 23 nM) was notably more active than siMDR-D and siMDR-T at 96 h after transfection. The biological activity of siMDR-M exceeded that of siMDR-D (IC_{50} 40 nM) at low concentrations and the biological activity of siMDR-T (IC_{50} was not achieved) at all of the concentrations used.

The data obtained 144 h after transfection showed that the silencing effect of longer siMDR-T (IC_{50} 5 nM) was more pronounced than the effects of siMDR-D (IC_{50} 10 nM) and siMDR-M (IC_{50} 23 nM). The inhibitory effect of siMDR-T at concentrations of 33.3–66.6 nM measured at 144 h after transfection was similar to that of siMDR-D and siMDR-M at dose-equivalent concentrations. However, transfection of siMDR-T at low concentrations (8–16.6 nM) resulted in significantly more efficient reduction of P-glycoprotein level. The siRNA-mediated effect was specific since no effect on the protein levels was observed in cells treated with the same concentrations of siRNA with a scrambled sequence both at 96 and 144 h after transfection.

The results that were consistent with P-glycoprotein suppression data were obtained in the experiments on KB-8–5 cell viability. These cells are characterised by over-expression of the *MDR1* gene, and have the ability to grow in the presence of 300 nM vinblastine. Silencing of the *MDR1* gene by siRNAs induces the death of KB-8–5 cells at concentrations of vinblastine that were previously tolerated by cells. Transfection with 50–25 nM siMDR-M resulted in the more efficient reduction of viable cells (up to 42%) after 122 h of incubation as compared with siMDR-D and siMDR-T at dose-equivalent concentrations. However, siMDR-T was found to be substantially more effective than siMDR-D and siMDR-M 144 h after transfection when used at low concentrations: up to 75% reduction of viable cells was achieved by the samples transfected with 8 nM siMDR-T. This difference in active concentrations of siRNAs is therapeutically relevant and demonstrates the preference of longer selectively modified interfering RNAs.

The slow development of the silencing effect caused by siMDR-D and siMDR-T in comparison with the effect of siMDR-M may be related to the time required for the processing of longer siRNAs or to the difference in the kinetics of Dicer/TRBP-mediated RISC loading and direct Ago2 loading. The potential explanation for the difference in canonical siRNA versus longer siRNA gene silencing potency may be related to the minimum length requirement of siRNA that can be directly loaded into Ago2 leading to bias strand selection towards the guide strand, persistence of the guide strand and improved guide strand gene silencing.

We compared the activity of siRNA at equal 21 bp «dose» concentrations, hypothetically assuming that they will be completely processed into 21 bp duplexes inside the cells. However, the data obtained (Fig. 4) revealed that selectively modified 42 and 63 bp siRNAs were not cleaved by Dicer to yield detectable levels of 21 bp products when transfected into the cells and the intact antisense strands were present in the cells for prolonged periods of time (at least up to 48 h). Despite the fact that the potential sites of cleavage in the tested siRNAs are modification-free, the effect may be connected to the 2′-O-methyl modifications of the flanking regions of these siRNAs, which prevent the formation of degradation products. The direct comparison of our modification patterns with those of Dicer substrates described in [21] shows that besides the cleavage site, the smaller part of dsiRNA, which has to be cut off during processing, does not contain modifications. In our structures, the sequences located after the cleavage site are designed as a copy of interfering RNA and contain modification. We can suppose that modifications in the flanks can reduce the efficiency of cleavage. The observed sequence-specific gene silencing can be rationalised in at least two ways: firstly, the effect can be induced by the very small quantities of processed siRNA, which are not detectable by the Northern blot in the presence of the majority of non-processed longer species; secondly, the ability of long dsRNA and pre-miRNA to form the direct complexes with Ago2 [21,25] makes Dicer-independent gene silencing possible. The latter suggestion can be supported by the recent study, where a 38 nt-long molecular structure that was not processed by Dicer, but induced efficient gene silencing via the RNAi mechanism, was described [26]. The authors implied that longer dsRNAs can be incorporated into an active RISC complex without Dicer processing. The data on the comparison of the efficiency of siRNA action at different time points are consistent with both explanations: both the need of time for processing and the difference in the kinetics of Dicer/TRBP-mediated RISC loading and direct Ago2 loading could account for the delay in the development of the silencing effect by longer siRNA. However, taking into account the data of Northern Blot analysis we can conclude that the delay in the development of the silencing effect by longer siRNA is related to the difference in the kinetics of Dicer/TRBP-mediated RISC loading and direct Ago2 loading.

In conclusion, we designed selectively modified 42 and 63 bp anti-MDR1 dsRNAs and showed their potent RNAi activity and specificity. These siRNAs were found to silence the expression of P-glycoprotein and restore the sensitivity of drug-resistant cancer cells to vinblastine more efficiently that canonical siRNA with the same sequence. For this reason, we believe that these structures could be used for the development of efficient therapeutics.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.febslet.2014.02.015.

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