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Chemically modified symmetric and asymmetric duplex RNAs: An enhanced stability to nuclease degradation and gene silencing effect

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Abstract

The present study accents on the relationship between dicing, nuclease stability, and RNAi activity of various types of chemically modified symmetric and asymmetric dsRNAs, covalently bound with amino-groups or cholesterol at one or both terminals. All modified dsRNAs were subjected to cleavage by recombinant Dicer enzyme. They possessed a high resistance to nuclease degradation in cell cultured medium and an excellent RNAi activity in viable cells. The best stability and RNAi activity was detected for 5′-sense amino-modified RNAs. These modifications manifested also a high long-term gene silencing effect within seven days post-transfection, while the RNAi activity of the native 21nt siRNA expired within two days. The conjugation of dsRNA with cholesterol at 5′-sense end resulted in easy intracellular delivery without transfection reagents. After a direct transfection in cells, the cholesterol-conjugated 27nt dsRNA possessed a higher RNAi activity than cholesterol-conjugated 21nt siRNA.

Keywords: Amino-modified dsRNA; Cholesterol-conjugated dsRNA; Dicing; Stability in serum; RNAi activity

The RNA interference (RNAi), discovered by Fire and Mello in 1998 [1], is a powerful tool for suppression of gene expression [2,3]. This phenomenon is a big challenge in the life science. The development of RNAi technology opened new trends in molecular cell biology and molecular medicine and enhanced the expectations to decide the problems of gene therapy of many incurable diseases (e.g., cancer, neurodegenerative disorders, acute viral infections, etc.) [4–11].

In RNAi, the long dsRNAs are subjected to cleavage by Dicer enzyme with production of short dsRNAs [from 21 to 23 nucleotides (nt), having a phosphate at 5′-end and a 2nt over-hang at 3′-end, named as short interfering RNAs (siRNAs)] [12,13] and subsequently bound with a protein complex, called RNA-induced silencing complex (RISC) [14]. This complex structure induces a sequence-specific degradation of homologous mRNA at a very low siRNA concentration, guided by the antisense strand of siRNA [15]. However, in mammalian cells, the long dsRNAs induce an interferon response [16,17]. In 2001, Tuschl and colleagues have reported that chemically synthesized siRNAs, consisting of a 19nt duplex in central region and a 2nt over-hang at 3′-ends, avoid the interferon response and induce a strong suppression of the homologues mRNA [18,19]. Twenty-one nt siRNAs are widely used in the practice, however, recently, it has been found that 27nt dsRNAs have much higher RNAi activity than 21nt siRNAs [20,21] and the efforts are directed to clarify this phenomenon.

Despite of all advantages and expectations of RNAi in mammals, this technology possesses several serious restrictions to be successfully applied in viable cells and animals.

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To solve the problems of RNAi technology, such as the intracellular delivery of siRNAs, their nuclease stability, side-effects, etc., 21nt siRNAs are chemically modified [22–29]. The modification of 21nt siRNA with 2′-OH [24,25], phosphate backbone [26,27], or sugar structure [28,29] results in excellent nuclease stability. The 21nt siRNAs conjugated with functional molecules, e.g., cholesterol [30,31], cell-penetrating peptides [32–34], sugars [35], and PEG [36], exhibit an improved intracellular uptake, and some of them could be used for application *in vivo*. However, most of the modified 21nt siRNAs possess a lower RNAi activity than the non-modified sequences.

In the present study, we designed several types of chemically modified symmetric and asymmetric 23–27nt dsRNAs targeted to *Renilla* luciferase gene and investigated their stability in serum, dicing, and RNAi activity in comparison with 21nt dsRNA. The structures of dsRNAs are shown in Table 1 (Supplementary Information). The amino-group was attached at 5′-end or 3′-end of dsRNAs. The abbreviation "Ds RNA" defines dsRNAs with a bland end at both terminals. "RO RNA" defines dsRNAs with a bland end at only 5′-sense strand and a 2nt dangling end at 3′-sense strand. "Si RNA" defines dsRNA with a 2nt dangling end at 3′-sense and 3′-antisense stands. It was established that most of the modified dsRNAs could be diced to the native 21nt siRNAs with removing of the modified site. All modified dsRNAs manifested a strong stability to nuclease degradation and a high gene silencing effect. Some of them possessed much higher stability in cell cultured medium and higher RNAi activity in comparison with 21nt siRNAs and non-modified 23–27nt sequences.

Materials and methods

All methods are described in details in Supplementary Information.

Results and discussion

Dicing of amino-modified dsRNAs

After 12 h incubation with recombinant Dicer enzyme, the non-modified 27nt dsRNA (Ds27A) and 5′-end amino-modified 27nt dsRNAs (Ds27B, Ds27C, and Ds27D) were subjected to dicing with production of 21nt siRNAs (Fig. 1). The other amino-modified 27nt dsRNAs (Ds27F, Ds27H, and Ds27I) were subjected to dicing with production of siRNAs with different lengths. The 3′-sense/3′-antisense amino-modified 27nt dsRNA was not diced during 12 h incubation with enzyme. The same phenomenon has been reported by Kim et al. [16]. The authors have observed that the dicing of 3′-sense/3′-antisense fluorescein-labeled 27nt dsRNA is difficult. The results suggest that the Dicer enzyme probably recognizes the 3′-ends and especially the 3′-sense end. In our previous study [37], we demonstrated that the 5′-sense amino-modified 27nt dsRNA was subjected to dicing analogous to the non-modified duplex. The same amino-modified 27nt dsRNA possessed the best RNAi activity—higher than the activity of the other amino-modified and non-modified duplexes. Based on this observation, we designed various types of 5′-amino-modified dsRNAs with different lengths (asymmetric—RO-type, and symmetric—Si-type) and investigated

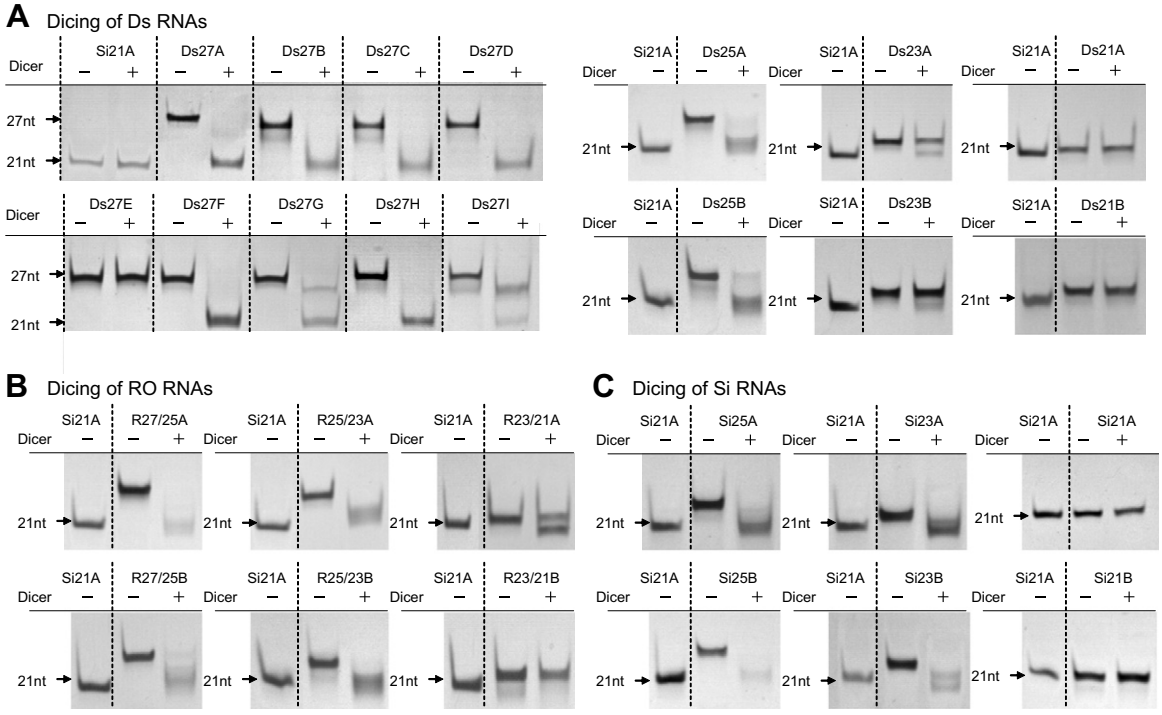


Fig. 1. Dicing of amino-modified and non-modified Ds RNAs, RO RNAs and Si RNAs by recombinant Dicer enzyme *in vitro*. Representative blots from four independent experiments are presented in the Figure.

their dicing, stability to nuclease degradation, and RNAi activity.

The dsRNA, consisting of relatively long strands (e.g., Ds27A, Ds25A, R27/25A, R25/23A, Si25A), was completely diced to 21nt siRNA within 12 h, whereas the dsRNA, consisting of relatively short strands (e.g., Ds23A, Ds21A, R23/21A, Si23A), was subjected to dicing with production of more than one product (e.g., with 21nt length and longer) (Fig. 1). It seems that there are limitations for the recognition of bland-ended 23nt RNA duplex by Dicer enzyme. However, if 23nt RNA has 2nt dangling 3′-ends (at both strands) or one dangling 3′-end (Si23A or RO25/23A) it could be diced easily. Obviously, the 2nt dangling end is important for RNA recognition by Dicer.

The dicing of amino-modified 23nt dsRNA (Ds23B) was slower in comparison with non-modified duplex (Ds23A). This is also evidence that the Dicer recognizes RNA duplex from 3′-end. The amino-group at 5′-sense strand slightly impeded the access of Dicer from 3′-antisense end, but does not affect significantly the process of dicing.

Stability of amino-modified dsRNAs to nuclease degradation

The stability of amino-modified dsRNAs to nuclease degradation were investigated on a cell cultured medium supplemented with 10% FBS. The modified and non-modified 23–27nt dsRNAs possessed a high resistance to nuclease degradation. In contrast, the 21nt RNAs rapidly degraded in the medium (Fig. 2A–C). Although the stability of dsRNAs depended on their lengths and sequences, the bland ended dsRNAs (Ds RNA or RO RNA) manifested a higher stability in comparison with Si RNAs with the same length and similar sequence. All amino-modified dsRNAs possessed a higher stability than Si21A duplex.

The degradation of dsRNAs in cell cultured medium could be explained with primary 3′ exonuclease and RNase A activities in plasma/serum. It is widely accepted that the hydrolysis of single-stranded oligonucleotides in plasma occurs exclusively by 3′ to 5′ exonuclease [38], while the hydrolysis of dsRNA in plasma/serum occurs by RNase A [39]. Eder et al. have observed that 3′ pyrimidine nucleotides are cleaved more rapidly than 3′ purines. Turner et al. have recently reported that siRNA duplex with UpA sequences close to one end are vulnerable to rapid cleavage. This produces a fragment of mass consistent with the presence of a 2′,3′-cyclic phosphate that is slowly hydrolyzed to a 2′-(3′-)phosphate on extended incubation. The substitution of these sites with 2′-*O*-methyl U residues prevents the cleavage and confirms that the major pathway for initial degradation is via cleavage by an RNase A-like activity.

We analyzed the mechanism of degradation of our dsRNAs (Fig. 2D). The Si21A, Ds19A, and Ds21A were incubated with cultured medium, containing 10% FBS, for 3 h and 5 h, respectively, and the products were analyzed by 20% PAGE. The hypothetical mechanism is shown in Fig. 2E. We suppose that the first step of RNA degradation is catalyzed by 3′ exonuclease. The enzyme cleaves two nucleotides from the 3′-dangling end with production of 19nt dsRNA. Presumably, the second step is catalyzed by RNase A. The enzyme cleaves the ApU-reach region with production of 17/18nt product. Using MALDI TOF-MS we calculated that the product consists of 17nt sense strand (containing phosphate) and 18nt antisense strand (data are not shown). In the case of Ds21A, most likely the duplex is subjected to RNase A-catalyzed cleavage of ApU-reach region. The product consists of 19nt sense strand (containing phosphate) and 19nt antisense strand.

RNAi activity of amino-modified dsRNAs

The RNAi activities of amino-modified dsRNAs were evaluated using luciferase gene reporter assay (Fig. 3). psiCHECK™-2 Vector was transfected in HeLa cells using LF2000. After 4 h incubation, dsRNA (in different concentrations) was transfected into the same cells using LF2000. The *Renilla* and *Firefly* luciferase gene expressions were analyzed 48 h after the Vector transfection (Fig. 3A). The non-modified 27nt dsRNA (Ds27A) strongly suppressed the expression of *Renilla* luciferase gene. The amino-modified 27nt dsRNAs (Ds27B-I) manifested different RNAi activities depending on the position of the amino-modification. The most active sequence was Ds27D (5′-sense modified) and less active were Ds27E (3′-sense/3′-antisense modified, non-Diced) and Ds27I (3′-sense/5′-antisense modified, with delayed Dicing). The 5′-sense modified Ds27D possessed the highest RNAi activity—several times higher than the activity of Si21A and Ds27A, while 5′-antisense modified Ds27C possessed lower RNAi activity in comparison with non-modified Si21A and Ds27A. Presumably, the RISC could recognize most easily the diced product of Ds27D than the diced product of Ds27C. The other amino-modified 27nt dsRNAs (e.g., 3′- and/or 5′-antisense modified) exhibited the same or lower RNAi activity in comparison with non-modified Ds27A (Fig. 3A1). A good liner correlation was observed between RNAi activity of amino-modified 27nt dsRNAs and their dicing.

The 5′-sense amino-modified asymmetric RNAs also possessed a higher RNAi activity in comparison with the same types of non-modified asymmetric RNAs (Fig. 3A2). In contrast, the 5′-sense amino-modified Si RNAs manifested almost the same RNAi activity as non-modified Si RNAs. It seems that the 5′-sense bland end is important to guarantee a high RNAi potency. The symmetric and asymmetric dsRNAs modified at 5′-sense bland end were selected as most stable, accessible for Dicer, and with highest gene silencing effect.

The long-term RNAi activity of amino-modified dsRNAs was also investigated using luciferase gene reporter assay (Fig. 3B). dsRNAs (50 nM) were transfected in HeLa cells using LF2000 and the cells were cultured within 1 week. Forty-eight hours before the luciferase activity assay, the psiCheck-2™ Vector was transfected into the

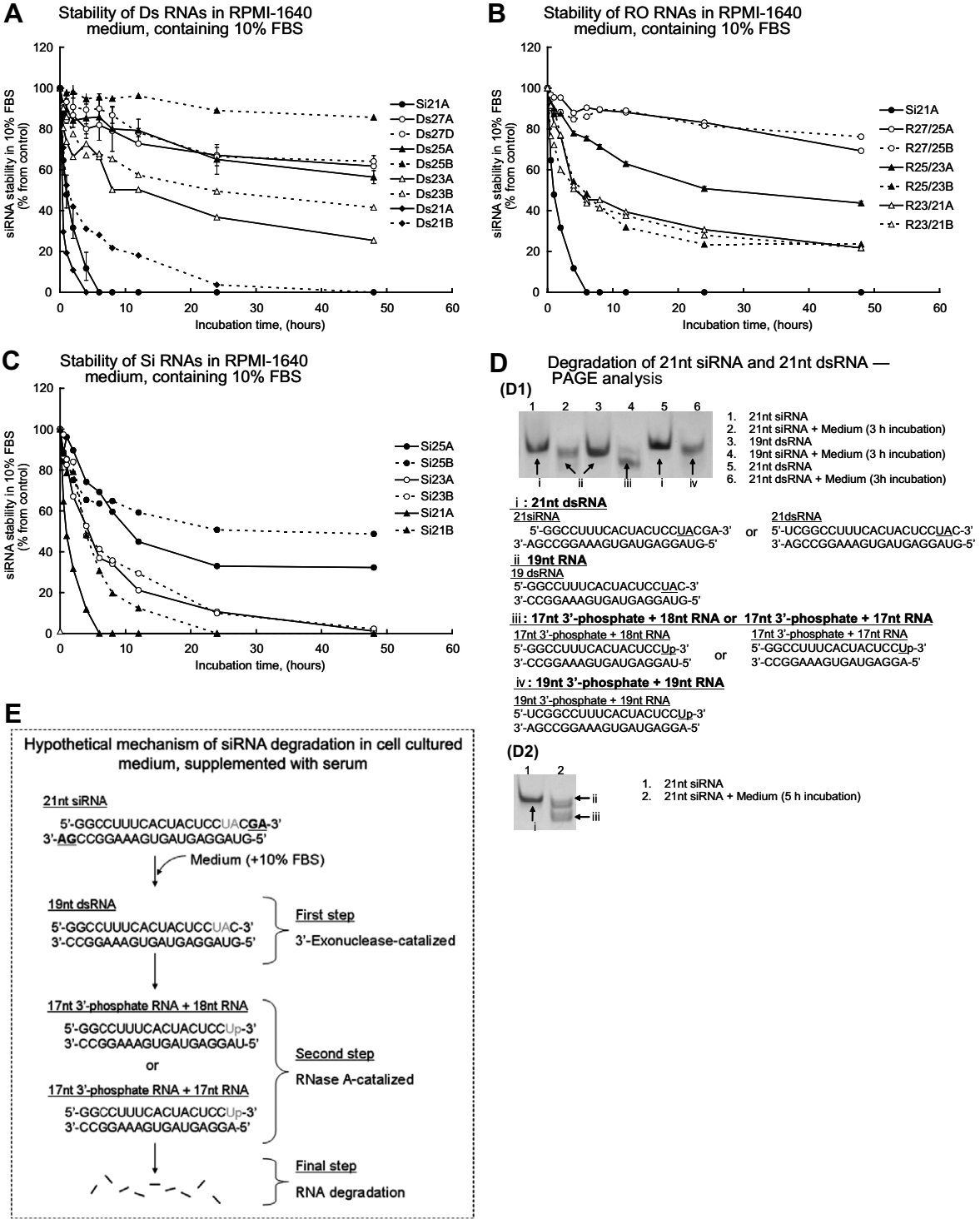


Fig. 2. (A–C) Stability of duplex RNAs to nuclease degradation in cell cultured medium. (D) Hypothetical mechanism of degradation of 21nt siRNA in biological fluids. Mean values from four independent experiments are presented into the charts (SD did no exceed 10%).

same cells using LF2000. The activity of Si21A decreased after 1 week cell transfection in comparison with the activity detected after 48 h cell transfection, presumably because of the low nuclease stability of Si21A (please, see Fig. 2). In contrast, the symmetric and asymmetric 27nt dsRNAs manifested a higher long-term RNAi activity in comparison with the activity detected after 48 h cell transfection, as well as in comparison with the activity of Si21A (Fig. 3B). All designed dsRNAs possessed a high stability to nuclease degradation in biological fluids (e.g., cell cultured medium, cells). This could explain, at least partially, their high long-term RNAi activity.

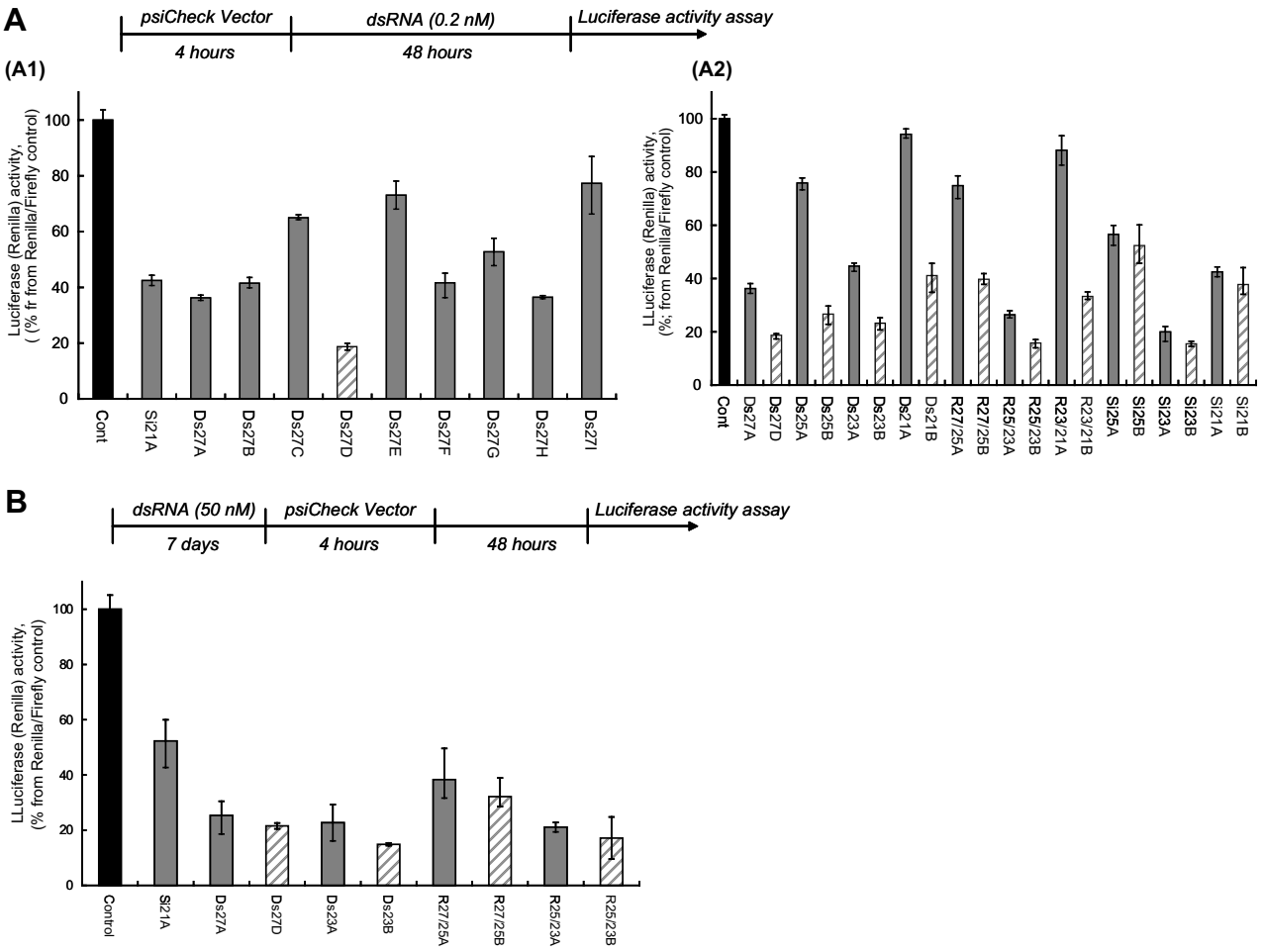


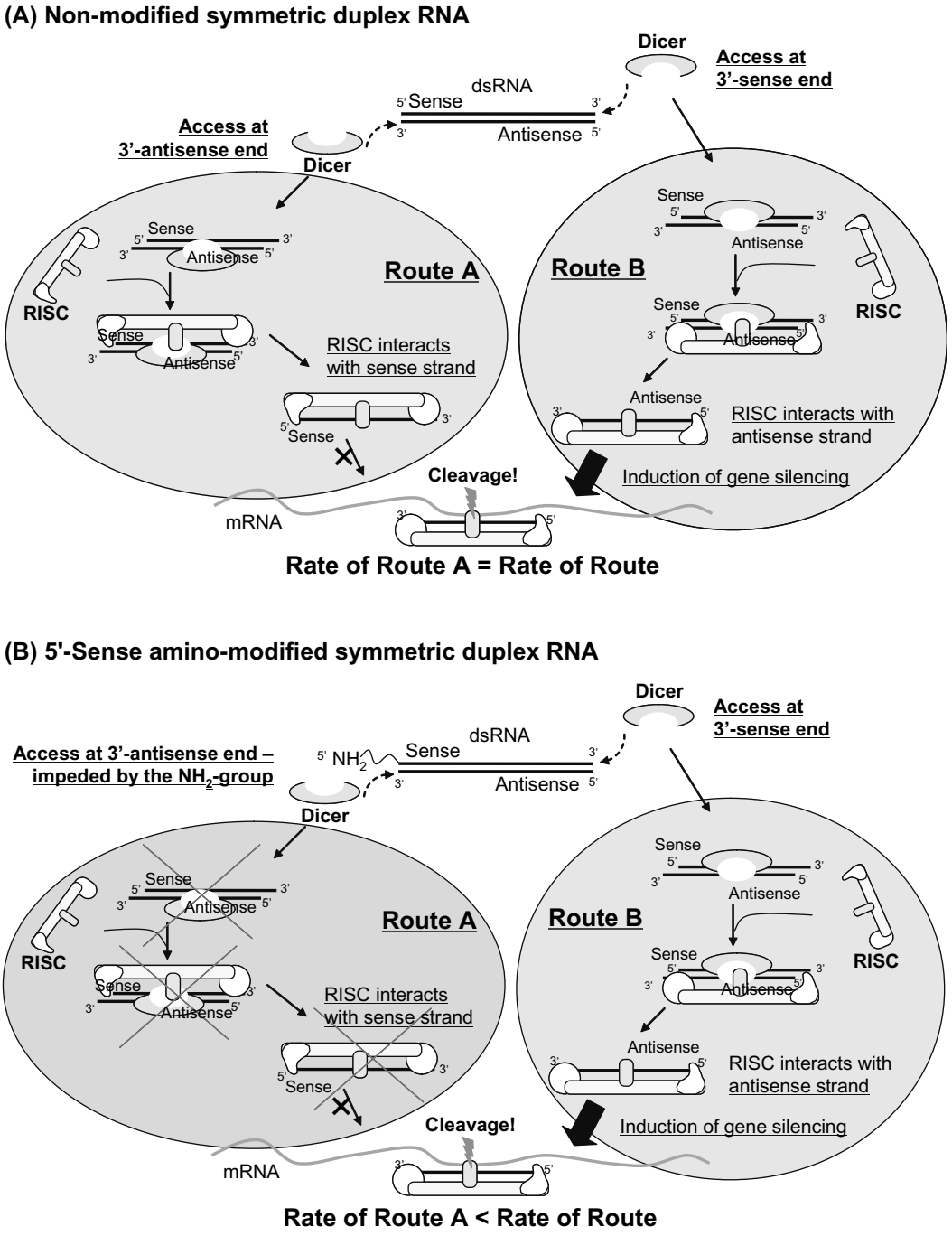
Fig. 3. Short-term (A) and long-term RNA interference (B) of *Renilla* luciferase activity in HeLa cells by amino-modified and non-modified duplex RNAs. In the short-term assay (A), the psiCHECK™-2 Vector was transfected into the cells using LF2000. After 4 h, dsRNAs (0.2 nM) were transfected into the same cells using LF2000 and the luciferase activity assay was carried out after 48 h. In the long-term assay (B), dsRNAs (50 nM) were transfected into the cell using LF2000 and the cells were cultured within 1 week in humidified atmosphere. Forty-eight hour before the luciferase activity assay, the psiCHECK™-2 Vector was transfected into the same cells using LF2000. The data are means ± SD values from four independent experiments in protocol (A) or three independent experiments in protocol (B).

Relationship between RNAi activity and dicing of duplex RNAs: hypothetical mechanisms

An excellent RNAi activity of dsRNAs could be obtained if the access of Dicer could be controlled. The different RNAi activities of amino-modified and non-modified symmetric and asymmetric duplex RNAs could be explained, at least partially, with their different access and recognition by Dicer.

Scheme 1A demonstrates the relationship between dicing and RNAi activity of long symmetric duplex RNAs (e.g., 27nt Ds RNA and 27nt SiRNA). The Dicer could interact in equal degree with 3′-sense (Route A) and 3′-antisense ends (Route B) with production of symmetric siRNAs. Therefore, the rate of Route A will be same as the rate of Route B. The amount of RISC-antisense complexes will be equal to the amount of RISC-sense complexes. However, only Route B (RICS-antisense complex) could induce a gene silencing of the target mRNA. Another 50% of RISC molecules (RISC-sense complex) will be inactive. The modification of 5′-sense end (with amino-, thiol-, fluoresceine-groups) impedes the access of Dicer to 3′-antisense end (Scheme 1B). In this case, the Dicer will interact predominantly with 3′-sense strand and the amount of RISC-antisense complexes will be higher than the amount of RISC-sense complexes. The rate of Route B will be higher than the rate of Route A. This hypothesis explains the higher RNAi activity of 5′-sense amino-modified dsRNAs in comparison with non-modified duplexes, obtained in our study.

Scheme 2 (see Supplementary Information) demonstrates the relationship between dicing and RNAi activity of long asymmetric duplex RNAs (RO-type). The Dicer could interact easily with 3′-sense dangling end (Route A) than with 3-antisense bland end (Route A) (Scheme 2A). In this case, the rate of Route B will be higher than the rate of Route A, which explains the higher RNAi



Scheme 1. Relationship between dicing and RNAi activity of symmetric duplex RNA and its 5′-sense amino-modification - hypothetical mechanism.

activity of asymmetric RO RNAs in comparison with symmetric ones with similar length and sequence. The modification of 5′-sense end with amino-group additionally impedes the access of Dicer to 3′-antisense end (Scheme 2B) and decreases the rate of Route A in comparison with the rate of Route B. This hypothesis explains the higher RNAi activity of 5′-sense amino-modified asymmetric duplex RNAs in comparison with non-modified duplexes.

Dicing, nuclease stability, cellular uptake and RNAi activity of cholesterol-conjugated 21nt siRNA and 27nt dsRNA—a comparative analysis

Two cholesterol-conjugated dsRNAs were used in this study—Ds27-Chol and Si21-Chol (Fig. 4A). Both conjugates had a complementally sequence for *Renilla* luciferase mRNA. The 5′-sense end was modified with cholesterol. The cholesterol-conjugated RNAs were investigated for

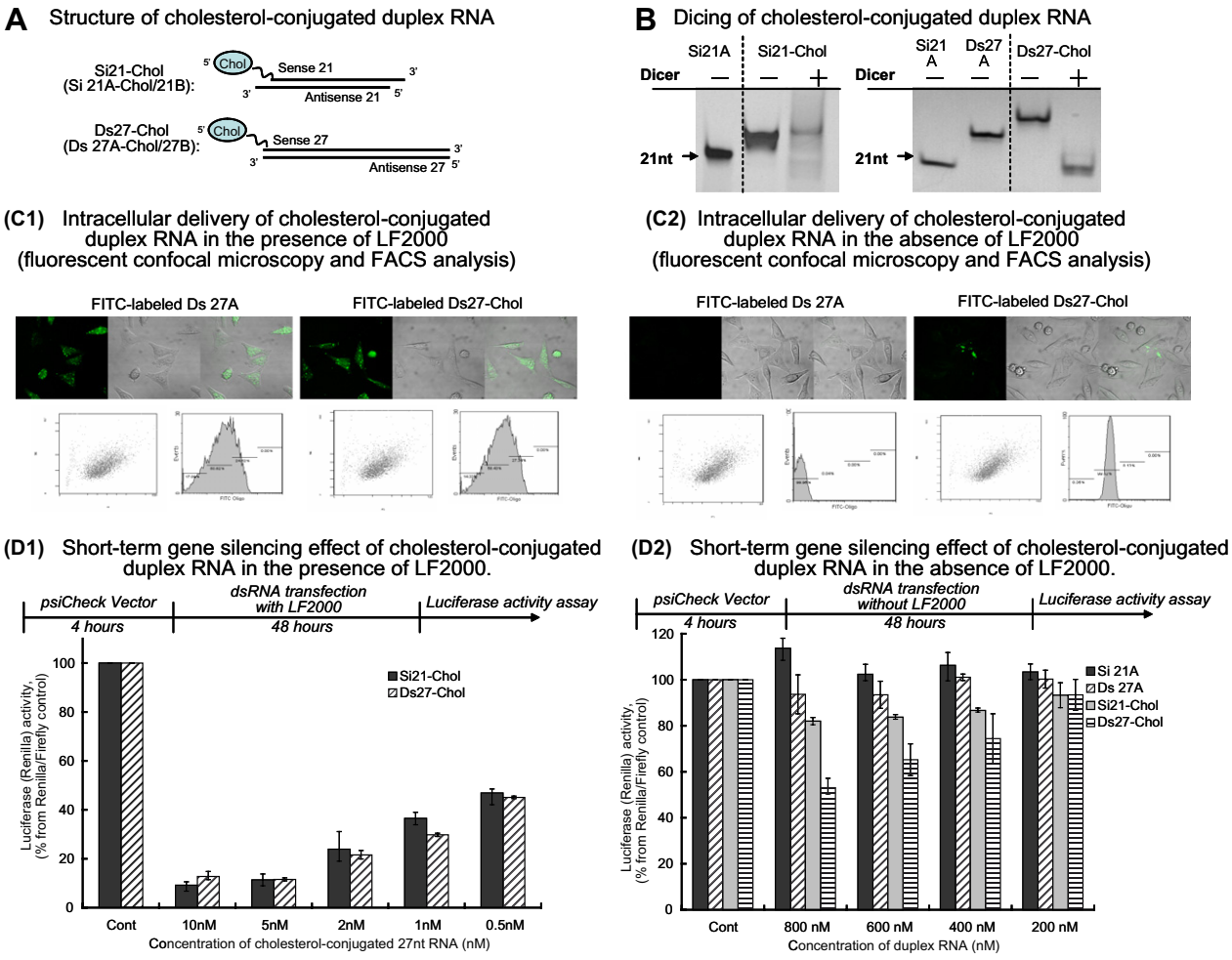


Fig. 4. Structure (A), dicing (B), membrane permeability (C), and RNAi activity (D) of cholesterol-conjugated RNAs in the presence or absence of LF2000. Mean ± SD values from four independent experiments are presented in the charts (D).

dicing, nuclease stability, intracellular uptake, and RNAi activity (Fig. 4B–D).

The Ds27-Chol was diced to 21nt siRNA. The Si21-Chol was not diced—the products were the same in the presence and absence of recombinant Dicer enzyme (Fig. 4B). Obviously, the conjugation of small molecules, as cholesterol, at the 5′-sense end of Dicer substrates (e.g., dsRNAs consisting of 23 or more nucleotides) does not restrict the access of the enzyme to RNA duplex.

The cholesterol-conjugated dsRNAs possessed a strong nuclease stability in 10% FBS—much higher that the stability of non-modified dsRNAs. Presumably, the cholesterol-conjugated dsRNAs make complexes with some proteins in cell cultured medium. This might be a reason for their higher stability to nuclease degradation in comparison with non-conjugated duplexes.

The Ds27-Chol possessed a high membrane permeability in the absence of transfectants (Fig. 4C). This observation is in agreement with the data of Soutscheck et al. The authors have reported an accumulation of cholesterol-conjugated siRNA in the liver of living animals without use of any transfection technique [30].

The RNAi activity of Ds27-Chol and Si21-Chol was investigated, using a luciferase reporter assay in the presence or absence of LF2000 (Fig. 4D). After transfection with LF2000, the RNAi activity of Ds27-Chol was almost the same as the activity of Si21-Chol. However, if the RNA-conjugates were applied to the cells in the absence of LF2000, Ds27-Chol manifested much higher RNAi activity than Si21-Chol. Since Ds27-Chol possessed a higher stability to nuclease degradation and a higher RNAi activity than Si21-Chol, we consider Ds27-Chol as most appropriate for *in vivo* application without transfection reagents.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2007.10.116.

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