Biological efficacy of antisense oligonucleotides complementary to overlapping regions of the mRNA target

N. N. Dygalo, T. S. Kalinina, and G. T. Shishkina*

Institute of Cytology and Genetics, Siberian Branch of the Russian Academy of Sciences, 10 prosp. Akad. Lavrent´eva, 630090 Novosibirsk, Russian Federation. Fax: +7 (383 2) 33 1278. E-mail: dygalo@bionet.nsc.ru

Phosphorothioate oligonucleotides complementary to target mRNA are stable in biological milieu and are capable of decreasing levels of this mRNA and the protein encoded by this mRNA (antisense knockdown). The results of our study are compared with the data published in the literature on the efficacy of three antisense 18—21-mer oligonucleotides, which are targeted to the start codon or nearby sequences of α_{2A}-adrenoceptor mRNA, on receptor expression, and functions regulated by these receptors. The highest biological efficacy was shown by the oligonucleotide, which is complementary to the mRNA region and contains the largest number of unpaired bases in the theoretically calculated conformation corresponding to the free energy minimum. Targeting of both ends of the antisense on unpaired bases of the target also leads to the enhancement of its biological efficacy.

Key words: oligonucleotides, phosphorothioates, mRNA, α_{2A} noradrenaline receptor, antisense knockdown, brain.

The antisense technology is based on sequence-specific hybridization of a particular region of target gene mRNA to a synthetic oligonucleotide (antisense), which blocks the normal synthesis of a protein product.[1—4] This approach employs new possibilities for revealing or refining the functions of proteins, which were opened up once complete nucleotide sequences of the genomes of a series of organisms, including man, became available. For this purpose, two other approaches are also used, which are aimed at selective changes in gene expression (transgenic and knockout animals creating the situation of irreversible gene hyperexpression and, conversely, zero gene expression in the course of organism development).[3] These approaches exhibit limited (in the case of a transgene under the control of its natural promoter), if any, tissue specificity in target gene expression. These characteristic features of transgenic and knockout animals impose certain limitations on the interpretation of the results obtained with the use of these models, among which the possibilities of ontogenetic and functional compensation of permanent hyperexpression or zero gene expression are most apparent. An antisense causes only a temporary decrease in target gene expression, which may last hours or days depending on the character of the influence. Hence, the antisense technology is much more free from the effects of compensatory processes.

The antisense technology, which was first successfully used in 1978,[1] is still a young field of research in which the molecular mechanisms of repression of target gene expression as well as the problems of internalization of oligonucleotides into cells and their intracellular transport and pharmacokinetics are being extensively elucidated.[2,3] Presently, it is believed that the mechanism of action of an antisense involves the complementary interaction between the oligonucleotide and a region of target gene mRNA. The resulting duplex is a substrate of RNase H, which destroys the duplex thus decreasing the amount of target gene mRNA. Because of this as well as due to direct suppression of the translation mechanism by the duplex, the amount of the protein product of the target gene must be decreased. In spite of extensive experimental and theoretical studies with consideration for the mRNA folding, the choice of the antisense structure remains essentially empirical.[3—5]

However, the choice of the antisense sequence or, in other words, the region of target gene mRNA complementary to which the antisense oligonucleotide is synthesized is of key importance for its efficacy. Many researchers start their studies with the use of an oligonucleotide, which overlaps with the start codon and, in most cases, continue investigations with this oligonucleotide if there is evidence that it suppresses target gene expression.[5] Thus, the only one sequence was assayed in 82% out of 2026 published studies, in which the antisense approach was successfully used.[6] However, only a small proportion of the synthesized oligonucleotides suppress target gene expression with high efficacy.[4] The potential efficacy of an oligonucleotide is generally tested *in vitro*.

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Trials on animals, which are labor intensive and elaborate, are very rarely performed with the simultaneous use of several oligonucleotides. Hence, the aim of the present study was to compare the effects, which were obtained with the use of different antisense oligonucleotides targeted to nearby and even overlapping transcript regions of the same gene, which may be useful in elucidating the reasons for the difference in the *in vivo* efficacy of these nucleotides.

Brain α_{2A}-adrenoceptors present a possibility of performing this analysis. These receptors attract the attention of researchers because most of α_2-adrenoceptor-mediated clinically important functions, for example, such as lowering of the arterial blood pressure and anesthetization, are realized through the α_{2A}-subtype. Nonspecific stimulators of α_2-adrenoceptors are used for lowering of the arterial blood pressure and in anesthesia in medicine and veterinary surgery. Their antagonists are proposed for treatment of depression, schizophrenia, male sexual disorders, and Parkinson's and Altzheimer's diseases.[7,8]

α_{2A}-Adrenoceptors belong to the multigene family whose individual members cannot be distinguished with the use of the presently available ligands.[7,8] Hence, the antisense technology provides a possibility of elucidating their functions. This approach was employed by several research groups. The biological effects of three different phosphorothioate oligonucleotides complementary to the region containing the start codon or the nearby transcript region of this gene were examined. All three sequences overlap with each other to a greater or lesser extent. These sequences are targeted to the positions −11—+7 (antisense-1),[9,10] +1—+21 (antisense-2),[11,12] and +4—+21 (antisense-3)[12—14] of α_{2A}-adrenoceptor mRNA.

In spite of the differences in the targets, all sequences exhibit efficacy with respect to animals' behavior. However, noticeable effects were obtained with the use of different doses of these antisenses. When injected into brain even in a dose of 0.06 nmol, the antisense-1 activated the motor activity of neonatal rat pups after treatment for 3 days,[9] whereas an analogous effect was observed in mature rats when 72 nmol of the antisense-3 were injected.[13—14] In the case of mature rats, the hypothermic reaction was also weakened in response to stimulation of α_2-adrenoceptors.[13—14] At the same time, even two nmoles of the antisense-1 injected into brain of mature rats for 2 days was sufficient for a fourfold increase in the number of entries into the open arms of the elevated plus-maze.[10] The direct brain injection of the antisense-2 (15 nmol) for 3 days led to a substantial (by a factor of 2—2.5 times) enhancement of resistance of mature rats to the hypnotic action of the stimulator of these receptors.[11] The above-considered data were obtained in different laboratories, which hinders their direct quantitative comparison. However, the actual qualitative differences (by several orders of magnitude) in the doses of different oligonucleotides, which can induce similar deviations of the estimated parameter from the control level, allow one to arrange these antisenses in order of increasing biological efficacy according to the ordinal numbers assigned to these antisenses in the present study: 1 > 2 > 3.

This order of the antisenses is confirmed also by their relative efficacy in suppression of target gene expression. The antisense-1 injected into the brain stem of mature rats in doses of 0.06—3 nmol led to a specific decrease in the level of α_{2A}-adrenoceptor mRNA, which was determined by the reverse transcription-polymerase chain reaction, and also depressed receptor protein expression in this brain region depending on the dose, which was estimated by the radioligand binding assay.[9—10] Three nmoles of these antisense injected during 3 days led to a 80% decrease in the mRNA level and to an almost twofold decrease in the amount of the receptor protein.[10] Infusion of the antisense-2 in rat brain during one week (24 nmol per day) led to a decrease in expression of these receptors in brain regions adjacent to the site of infusion by 50—80% with respect to the level observed upon injection of the control oligonucleotide (estimated by autoradiography).[12] This efficacy of the antisense-2 with respect to the target gene was also confirmed by *in vitro* assays.[11] At the same time, the antisense-3 injected into brain during one week in a total amount of 72—168 nmol led to a decrease in the level of the receptor protein only in the regions most closely located to the site of infusion, and the decrease was no larger than 20—30% with respect to the control oligonucleotide.[12—13]

It should be noted that the antisense-2 consisting of 21 residues and the control oligonucleotide of the same length, which was designed based on the antisense sequence by the replacement of four bases, caused a pronounced toxic effect upon the injection of the total dose of 168 nmol during 7 days.[12] The shorter 18-mer antisense-3 and the corresponding control oligonucleotide taken in the same dose had no noticeable toxic effects.[12] The concentrations of the oligonucleotides thus accumulated upon the above-described injection in brain exceed the threshold above which sequence-independent effects are often observed.[4] The difference in the side effects of the 18- and 21-mer sequences may be associated with their lengths, which is responsible for higher ability of extended polymers to nonspecifically interact as polyanions with proteins and also for the higher probability that degradation products of a longer oligonucleotide contain more ectopic mRNA targets.[4] The latter is exemplefied by the fact that the most voluminous GenBank database (http://www.ncbi.nbm.nih.gov/BLAST/) contains 43 nucleotide sequences complementary to, at least, 15 positions of the antisense-2, whereas there are only 16 such sequences for the antisense-3. Presently, it is commonly

assumed that it is necessary to optimize the length of the oligonucleotide in the range of 16—20 bases and to use this oligonucleotide in doses, which do not produce toxic concentrations, with the aim of enhancing the specificity of action of the antisense.[4] Thus the antisense-2, which is toxic in high concentrations, did not exhibit this property at a concentration of 5 μmol L^{-1} and efficiently suppressed target gene expression.[11]

The efficacy of the antisense-1 in suppression of target gene expression normalized to the amount of the injected oligonucleotide is more than an order of magnitude higher than the efficacy of the antisense-3. The antisense-2 exhibits efficacy intermediate between those of the antisense-1 and the antisense-3. This order of efficacy is supported also by the direct comparison of the suppression efficacy of target gene expression by the antisense-2 and the antisense-3, which are most similar in structure and activity in the series of three oligonucleotides under consideration, in one assay.[12]

Apparently, the differences in efficacy of the antisenses are associated with the characteristic features of the secondary structure of the mRNA target. The most probable calculated secondary structure of the region of α_{2A}-adrenoceptor mRNA, whose overlapping regions are targeted by the antisense oligonucleotides, is shown in Fig. 1. We obtained this structure with the use of the program (http://wwwmgs.bionet.nsc.ru/mgs/gnw/garna/)[15] based on the genetic algorithm of optimization of the results of calculations. The structure reflects the theoretical conformation of the region corresponding to the free energy minimum of the mRNA molecule. This algorithm was verified by the *in vitro* experimental data[15] and the results of our calculations were constantly reproduced upon variations in the initial parameters. However, this structure is, apparently, only the most probable conformation of those actually present *in vivo*. Eleven out of 18 (or more than 60%) nucleotides of the antisense-1 are targeted to the bases of the mRNA target, which are unpaired in this conformation. The proportion of these positions in the least efficient antisense-3 is only ~20%, whereas the proportion of these positions in the antisense-2 exhibiting intermediate efficacy is 30%. Besides, both termini of the antisense-1 and the antisense-2, which possess much higher efficacy as compared to that of the antisense-3, are targeted to unpaired bases of the target. A comparison of the antisense-2 and the antisense-3 clearly revealed that the unpaired bases of the target at the termini of the antisense are of importance for the efficacy of the latter. The antisense-3 is a modification of the antisense-2, which is shortened by three nucleotides from the 3′-terminus. This terminus of the shorter sequence is localized in the region of the G—C hairpin of the target, which may, in principle, hinder base pairing of the oligonucleotide to this mRNA region. Since such base pairing is a necessary event for the specific action of an antisense, an oligonucleotide possessing low ability to form a DNA—RNA duplex, apparently, must exhibit lower biological efficacy.

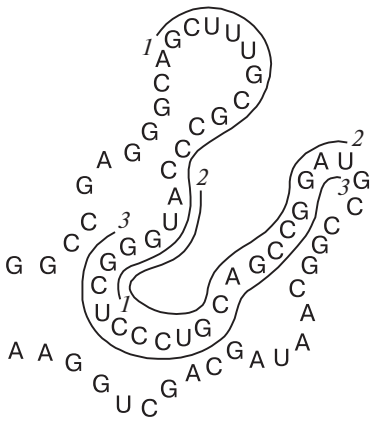

Fig. 1. Region of rat α_{2A}-adrenoceptor mRNA from the (—22 — G) to (+41 — A) positions with respect to the start codon in the conformation corresponding to the free energy minimum of this transcript: *1—1*, the region overlapped by the antisense-1 (—11—+7); *2—2*, the region overlapped by the antisense-2 (+1—+21); *3—3*, the region overlapped by the antisense-3 (+4—+21).

The structure of the target is presently difficult if not impossible to unambiguously establish by theoretical methods because neither the approach used by us nor other available algorithms of calculations take into account the factors occurring in the cell, which can influence the secondary RNA structure. At the same time, full-sized transcripts of most of clinically important targets are not always readily accessible in amounts sufficient for the experimental examination of their structures, which are carried out *in vitro* and, hence, have the above-mentioned limitations. In spite of the evident problems associated with calculations of the target structure, the results of such calculations may be of practical usefulness. Thus, the results of our calculations and the differences in biological efficacy of the antisenses under consideration agree with the known and assumed characteristic features of the mechanisms of action of antisense oligonucleotides.

On the whole, the results of the analysis indicated that the highest biological efficacy is shown by the antisense oligonucleotide targeted to the target region, which contains the largest number of unpaired bases in the theoretically calculated conformation corresponding to the free energy minimum of the mRNA molecule. Targeting of both ends of the antisense on the unpaired bases of the target is also favorable for the enhancement of its biological efficacy.

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