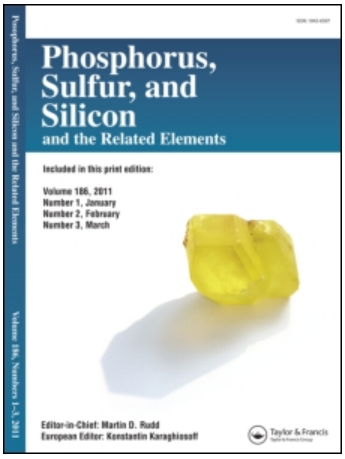

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Oligo(nucleoside Phosphorothioate)s: The Quest of P-Chirality

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OLIGO(NUCLEOSIDE PHOSPHOROTHIOATE)S: THE QUEST OF P-CHIRALITY

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The influence of the sense of chirality at phosphorus of internucleotide phosphorothioate groups in oligo(nucleoside phosphorothioate)s is discussed in terms of stability of stereodefined PS-Oligos in the intra- and intercellular media and newly discovered exclusive ability of R_P-PS-Oligos containing homopurine tracts towards the formation of triple-stranded species of one phosphorothioate and two complementary RNA strands stechiometry.

Keywords: 3′-Exonuclease degradation; antisense strategy; polyadenylated PS-Oligos; PS-Oligos; stereodependent triplex formation

Oligo(nucleoside phosphorothioates (PS-Oligo), analogues of natural oligonucleotides that possessed at each internucleotide linkage one of two non-bridging bound to those phosphorus oxygen atoms replaced by sulfur, appeared in the focus of numerous research establishments as the *first generation* of antisense therapeutics.[1] First antisense PS-Oligo for curing retinitis in AIDS-infected patients was approved by FDA under the trade name *Vitravene*, and numerous other PS-Oligos are in the process of phase I, II, or III clinical evaluations as the drugs to fight against viral, cardiovascular, cancer, and other diseases. Among the so-called *second generation* antisense oligonucleotide therapeutics containing modified base-, sugar- or sugar-phosphate backbone, phosphoro-thioates still play a major role as components stable for enzyme assisted degradations and otherwise are able to elicit RNase H activity towards the complementary target RNA. It has to

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be emphasized that the advantage of PS-Oligos over any other generation of antisense agents relies upon the simplicity and economics of their production via phosphoramidite or *H*-phosphonate methods.[2] Since biotechnology companies involved in the process of developing the new generation of gene-targeted medicines must regard the market potential and capacity of the future drugs, the combination of deoxyribonucleoside phosphorothioates and phosphates, limiting the side-effects of "pure" phosphorothioates,[3] must be appreciated. For the same reason of economics and market potential, the problem of P-chirality of PS-Oligos[4] is unappreciated, generally neglected, or purposely silenced. All used or evaluated as potential drugs, PS-Oligos consist of the mixture of 2^n diastereomers, where n signifies the number of internucleotide phosphorothioate linkages. Developed in this laboratory, the stereo-controlled method of synthesis of PS-Oligos allowed for the synthesis of any oligonucleotide 2–22 mers long[5] with a predetermined sense of P-chirality at each phosphorus atom of internucleotide phosphorothioate linkage and a comparison of physicochemical and biological properties of such stereo-defined PS-Oligos *vs* Mix-PS-Oligos, or *All* R_P-PS-Oligos otherwise prepared by enzymatic methods. The major advantage of PS-Oligos, as compared with isosequential PO-Oligos, is their enhanced stability in intra- and intercellular media. It has been proved that oligonucleotides of S_P-phosphorothioate internucleotide linkages at 3′-end are not degraded by 3′-exonuclease.[6] Is that observation important in the context of the use of PS-Oligos for human treatment? Besides integrity of the antisense constructs that are considered to have a catalytic function for the degradation of target RNA, products of their degradation, namely nucleoside 5′-*O*-phosphorothioates, are not neutral species towards host cells. In our recent studies, we have proved that in HeLa, HL-60, K562, and HUVEC cell cultures, the nucleoside 5′-*O*-phosphorothioates, albeit undergoing dephosphorothioylation with ecto-nucleotidases at a much lower rate than harmful nucleoside 5′-phosphates, interfere with the growth of cells under studies and, surprisingly in the case of HUVEC and HL60 cells, enhance their growth and viability.[7] Although the physiological consequences of such observation are still obscure, from the point of selection of "ideal" antisense agents *All*-S_P-PS-Oligos seem to be advantageous.

Results of preliminary studies on stereodependent immunostimulatory effects of PS-Oligos, measured by the induction of spleen cell proliferation with octa(nucleoside phosphorothioate) containing the ACGT motif, unambiguously proved an 80-fold (at concentration 24 micrograms/ml) difference between *All*-R_P and *All*-S_P diastereomers. More active was the *All*-S_P isomer, most probably due to the increased stability under physiological conditions.[8]

Another pre-condition for the selection of "ideal" antisense drug is the avidity of an oligonucleotide construct towards complementary RNA. That avidity is usually quantified in terms of the T_m parameter, which means the temperature when statistically 50% of PS-Oligo is still bound to target RNA. From numerous studies performed with the use of 8–28 mers of PS-Oligos with diversified base composition, we could conclude that T_m parameter for hybrids PO-Oligo/RNA is always higher than that of corresponding isosequential R_P-PS-Oligo/RNA, *Mix*-PS-Oligo/RNA, and S_P-PS-Oligo/RNA (in descending order). In that respect, the use of PS-Oligos of R_P-configuration would be advantageous but with 3′-end internucleotide phosphorothioate of S_P-configuration. Such "*All*-R_P–but-one" PS-Oligos appeared to be stable in 50% human plasma, and their complexation with complementary RNA is still manifested in higher T_m parameters. That seems to be important, since the efficacy of RNase H towards *All*-R_P-PS-Oligo/RNA heteroduplexes has been shown to be higher than those measured for *Mix*-PS-Oligos/RNA and *All*-S_P-PS-Oligos/RNA.[9] However, recent studies by Chattopadhyaya[10] have shown that efficacy of RNase H cleavage of RNA complexes with complementary DNA is not strictly T_m-dependent. Moreover, it has been found that certain PS-Oligos of *All*-R_P-configuration are able to form higher order structures with complementary RNA. On the basis of the results of titration studies, Boczkowska et al.[11] found that dodecamer *All*-R_P-PS-d5′(GAGAAAAAAGAG)3′ forms the triplex with two molecules of complementary 5′-r(CUCUUUUUUCUC). One antiparallel strand interacts via Watson-Crick hydrogen bonds while the second RNA strand is attached to phosphorothioate strand in parallel manner, most probably via Hoogstein type hydrogen bonds. T_m for this triplex was 54°C, while isosequential deoxyribonucleotide forms with the same complementary RNA (1:1, titration studies) heteroduplex of T_m 26°C. Of particular interest is the observation that T_m for *All*-S_P-diastereomer is 16°C, while the *Mix*-PS-counterpart forms, like the PO-construct, a heterodimer with T_m 23°C. The nature of contacts stabilizing the triplex structure is not clear until the detailed structure is solved. The data presented here, with other collected sequence- and stereochemistry-dependent examples, uniformly indicating the function of homopurine sequences and R_P-stereochemistry of internucleotide phosphorothioates and influencing the formation of higher order structures, argue for the use as antisense constructs PS-Oligos of S_P-configuration. Stability in physiological fluids resulting from the resistance against inter- and intracellular nucleases and the formation of heteroduplexes with complementary RNA eliciting the activity of RNase H are the strong assets of *All*-S_P-PS-Oligos. *All*-R_P-constructs are hydrolyzed with the rate

comparable to PO-Oligos by 3′-exonucleases with the formation of deoxyribonucleoside 5′-*O*-phosphorothioates; their cellular function is not yet well defined.

Besides interest in the implementation of observed stereodependent properties of PS-oligonucleotides into therapeutic practice, the observed ability of homopurine R_P-PS-Oligos towards the formation of higher order structures with complementary RNA constitutes the novel example of structural polymorphism of DNA/RNA hybrids with (thus far) obscure structural motif of RNA strand interaction with pro-R-oxygen of internucleotide phosphate bond. Its replacement with sulfur increases dramatically attractive forces that stabilize the triplex. The function of 2′-OH groups at RNA strands as the proton-donor stabilizing site has been eliminated, since 2′-*O*-methylated RNA components form even more stable triple-stranded structures. Studies on the elucidation of the structural motif involving the sulfur atom of R_P-PS-polyadenylated Oligos are in progress.

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