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Effect of the extent of thiolation and introduction of phosphorothioate internucleotide linkages on the anti-HIV activity of Suligovir [$(s^4dU)_{35}$]

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Abstract—Suligovir is a 35-mer homo-oligonucleotide, containing exclusively 4-thio deoxyuridylate, proved to be a potent inhibitor of HIV entry. In this paper, we described the effect of extent of thiolation and the introduction of nuclease-resistant phosphorothioate linkages on the anti-HIV activity of Suligovir. We found that the decreased thiolated nucleotide content decreases the anti-HIV potency of the compound and the introduction of phosphorothioate linkages does not improve its antiviral activity.

A versatile and promising class of potential anti-HIV agents is oligonucleotides affecting the life cycle of HIV at various sites. Oligonucleotides may inhibit the expression of viral genes,[1,2] the reverse transcriptase,[3] and/or viral entry.[4] Recently we have published the activity of a new potent and nontoxic oligonucleotide inhibitor of HIV entry (3 ng/ml) designated as Suligovir.[5] It is a 35-mer, composed exclusively of 4-thiodeoxyuridylates; its chemical formula is $(s^4dU)_{35}$. Suligovir inhibits the replication of wild type and some drug-resistant viral strains. It does not penetrate into the cells, but colocalizes on the cell surface with thioredoxin and CD4.

The promising activity of Suligovir prompted us to study the antiviral activity of the less thiolated congeners, which may be formed during the synthesis of Suligovir. The Suligovir is prepared by H_2S treatment of $(dC)_{35}$.[5,6] This treatment is highly effective producing near complete thiolation. However, a minute amount of unmodified deoxycytidylate or deoxyuridylate (due to incomplete thiolation and/or sulfur loss during isolation) residues may be present in the oligonucleotide affecting its antiviral activity. Another issue which may significantly affect the biological activity of oligonucleotides, including Suligovir, is the stability of the molecules. Although we showed that the Suligovir is highly stable, a further increase of its anti-HIV activity may be expected by introducing nuclease-resistant phosphorothioate internucleotide linkages to both the 3′ and 5′ end[7] (Fig. 1). In this note, we describe how the extent of thiolation and the introduction of nuclease-resistant linkages affect the antiviral activity of the molecule.

All of the methods applied in this paper were described earlier;[5,6,8] therefore we give here only a brief description of them. The antiviral assay was performed in MT-4 cells measuring the RT activity in the culture fluid after 96 h of infection.[5] The partially thiolated oligonucleotides were prepared by incomplete thiolation of $(dC)_{35}$ and their composition were determined by HPLC analysis after degradation to nucleosides with phosphodiesterase and phosphomonoesterase.[6] To prepare Suligovir [$(s^4dU)_{35}$] with nuclease-resistant linkages, we thiolated $(dC)_{35}$, containing one, two or three phosp-

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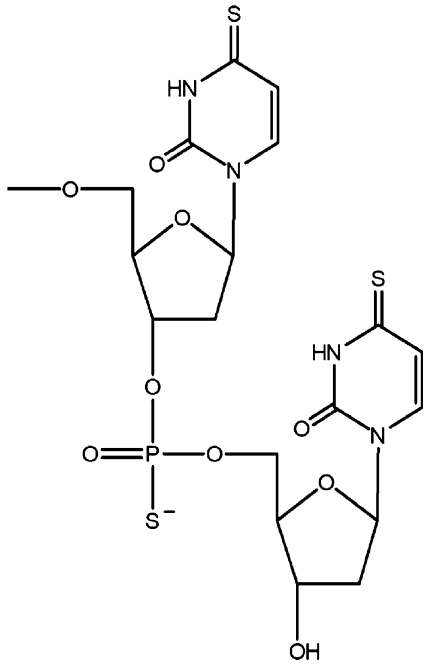

Figure 1. 3′ end of Suligovir molecule with phosphorothioate internucleotide linkage.

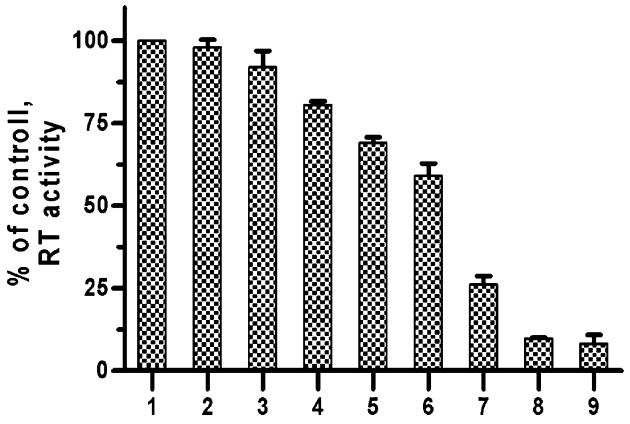

Figure 2. Effect of the thiolated nucleotide content on the anti-HIV activity of oligonucleotides with the structure of $(dC_X,s^4dU_Y)_{35}$. The anti-HIV activity was measured by the determination of the RT activity in the supernatant of MT4 cells, 4 days after infection. The inhibitors were added 30 min before infection (1 μg/ml, 88 nM). The experiments were performed in triplicate; error bars represent standard deviation. 1: control, no oligonucleotide was added. The average of three experiments was: 78212 dpm; 100%; 2: $(dC)_{35}$; 3: $(dC_{0.73},s^4dU_{0.27})_{35}$; 4: $(dC_{0.66},s^4dU_{0.34})_{35}$; 5: $(dC_{0.58},s^4dU_{0.42})_{35}$; 6: $(dC_{0.50},s^4dU_{0.50})_{35}$; 7: $(dC_{0.26},s^4dU_{0.74})_{35}$; 8: $(dC_{0.11},s^4dU_{0.89})_{35}$; 9: $(s^4dU)_{35}$.

horothioate internucleotide[9] linkages, using the standard thiolation procedure.[5,6] All of the oligonucleotides were purified as we described.[8]

Suligovir is a homo-oligonucleotide and exerts its antiviral action by a sequence-independent manner. It is important to know from both theoretical and practical aspects how the antiviral activity is related to the extent of thiolation; therefore we studied partially thiolated congeners of Suligovir composed of 4-thio-deoxyuridylates and deoxycytidylates in random distribution (Fig. 2). The results indicated that the extent of thiolation has a strong effect on the antiviral activity. The oligonucleotide with only 51% of thiolated base (no. 6) inhibits the HIV replication by 41%, while Suligovir, essentially thiolated completely, inhibits by 92% under our experimental conditions. As we reported, the Suligovir is colocalized with cell surface thioredoxin and CD4 receptor. The interaction with these proteins may be weakened by the decreased rate of thiolation. We have also reported that the stability of Suligovir is about 40 times higher against nucleases than the stability of the parent oligonucleotide, $(dC)_{35}$, indicating the role of the thiolated base in the stability of Suligovir. Consequently, the lower degree of thiolation is yielding a less stable oligonucleotide inhibitor, which may be degraded during the 4-day long antiviral assay. However, it should be noted that the no. 8 oligonucleotide, with 89% thiolation, is not significantly weaker inhibitor of HIV replication than Suligovir (in the same molarity). This observation is considerable in respect to large-scale preparation of Suligovir and is in good agreement with its nonsequence-specific mode of action.[5]

In the next experiment we studied the Suligovir derivatives with increased nuclease-stability carrying one, two or three phosphorothioate internucleotide linkages at both ends. Since it was found that the predominant nuclease activity, responsible for the degradation of oligonucleotides, are exonucleases,[10] we introduced the nuclease-resistant linkages[11] to the 3′ and 5′ end of the molecule expecting more stable and more active Suligovir analogues. Surprisingly, these derivatives were not better inhibitors of HIV replication (Fig. 3), even a slight decrease of antiviral efficacy was observed for

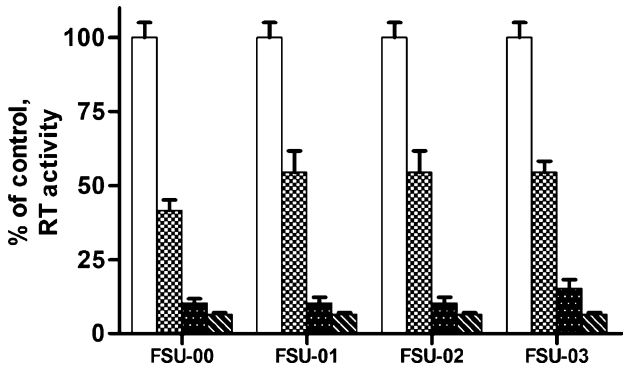

Figure 3. Effect of phosphorothioate internucleotide linkages on the anti-HIV activity of $(s^4dU)_{35}$. FSU-00 represents $(s^4dU)_{35}$. FSU-01 represents $(s^4dU)_{35}$ with one phosphorothioate internucleotide linkage at its 3′ and one at its 5′ ends. FSU-02 represents $(s^4dU)_{35}$ with two phosphorothioate internucleotide linkages at its 3′ and two at its 5′ ends. FSU-03 represents $(s^4dU)_{35}$ with three phosphorothioate internucleotide linkages at its 3′ and three at its 5′ ends. The open bar represents the controls without inhibitors (the average of three experiments was: 67820 dpm; 100%, for all determinations only one set of control was used). The gray bars indicate the RT activity in the supernatant of treated and infected MT4 cells at the following concentrations: 0.25, 0.50 and 1.00 μg/ml (from left to right). The experiments were performed in triplicate; error bars represent standard deviation.

the thio-phosphate containing derivatives. The result indicated that the base modification (4-thiono-group) alone converted the natural, nuclease-sensitive oligonucleotide to a highly nuclease-resistant derivative and introduction of nuclease-resistant linkages did not improve the antiviral activity of Suligovir. It must be emphasized that oligodeoxycytidylates with phosphorothioate internucleotide linkages, without any base modification, are potent inhibitors of HIV replication in vitro.[7]

Summarizing the above observations, we found that the degree of base-thiolation strongly affects the antiviral activity of the Suligovir. However, 100% thiolation is not required for its full anti-HIV activity. The introduction of phosphorothioate internucleotide linkages did not increase the antiviral activity of Suligovir indicating that further effort should not be made for stabilization of the molecule against nucleases.

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