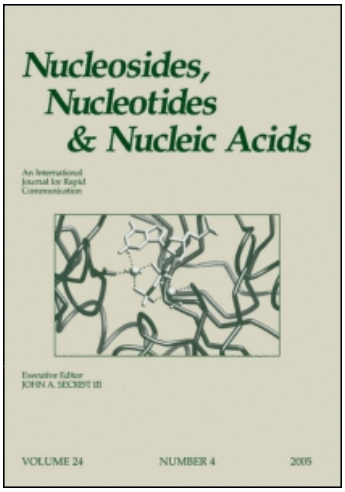

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Phosphorothioate Oligonucleotides as Potential Antiviral Compounds Against Human Immunodeficiency Virus and Herpes Viruses

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PHOSPHOROTHIOATE OLIGONUCLEOTIDES AS POTENTIAL ANTIVIRAL COMPOUNDS AGAINST HUMAN IMMUNODEFICIENCY VIRUS AND HERPES VIRUSES.

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Abstract

Phosphorothioate oligonucleotides (S-oligos) with and without sequence specificity complementary to viral genes were found to have activities against human immunodeficiency virus (HIV) and herpes simplex virus type 2. The potential usage of S-oligo as an antisense molecule and as antiviral compound is discussed.

Phosphorothioate oligonucleotides (S-oligos) have been shown to be relatively resistant to serum nuclease in comparison with regular oligonucleotides (1) and more water soluble than methyl phosphonate oligonucleotides (2). They are able to get into the cell and hybridize preferentially with nucleic acids having a sequence complementary to their sequence (3,4). When hybridized with RNA, the RNA of hybridized molecules is susceptible to the action of RNase H (5). For those reasons, many groups around the world are interested in exploring types of chemical entities which control gene expression intracellularly. Matsukura et al. synthesized S-oligos with the sequence complementary to human immunodeficiency virus (HIV) *rev* gene transcript as well as phosphorothioate oligodeoxycytidylate (S-dC_n) and oligodeoxyadenylate (S-dA_n) (6). Those compounds were examined against HIV-1 (HTLV-III_B) replication in the ATH8 cell line. It was found that all phosphorothioate oligonucleotides with chain lengths above 14 are capable of inhibiting HIV replication. The longer

the chain length of S-oligo, the more potent is its antiviral activity. There was no sequence specificity of S-oligos in exerting their action. Subsequently Majumdar et al. demonstrated that 28-mer of phosphorothioate deoxycytidinate ($S\text{-}dC_{28}$) could inhibit the reverse transcriptase (RT) activity of HIV RT by competing with template (7). The K_I value was estimated to be 2.8 nM. It was also shown that $S\text{-}dC_{28}$ could inhibit DNA polymerase γ from pig liver, DNA polymerase α from calf thymus and DNA polymerase β from human sources to a different degree.

HIV RT has three enzyme activities: RT, DNA polymerase (DP) as well as RNase H. It was not clear whether $S\text{-}dC_{28}$ could inhibit all three enzyme activities to the same degree, and what the role of the chain length of $S\text{-}dC_n$ was in terms of inhibitory potency. This issue was addressed by using HIV-1 RT preparation purified from virions (8).

The extent of $S\text{-}dC_{28}$ inhibition on the three enzyme activities associated with HIV RT was found to be different and the results are shown in Figure 1. The degree of the susceptibility of the activities to S-oligo was in the order of RNase H > RT > DP. This implied the active site of RT and DP activity of HIV RT is not identical. $S\text{-}dC_n$ with longer chain lengths was more inhibitory than those with shorter ones (Fig. 1). This is consistent with their anti-HIV-1 activity in ATH8 acute viral infection system. It should be noted that the anti-HIV activity of S-oligo could still be due to other mechanisms such as the inhibition of HIV adsorption as suggested by Stein et al. (9). This is not clear at the present time. Recently, Matsukura et al. (10) were able to demonstrate the sequence specific suppressor of viral expression in T cells (H9) chronically infected with HIV-1 using antisense S-oligos against *rev*. The action appeared to be sustained even after the drugs were removed. This suggested that S-oligo could get into cells in sufficient quantity to inhibit the viral gene in a selective manner. It has also raised the question of whether $S\text{-}dC_n$ could interfere with human DP or RNase H in a nonselective manner in view of the observation by Majamdar et al. (7).

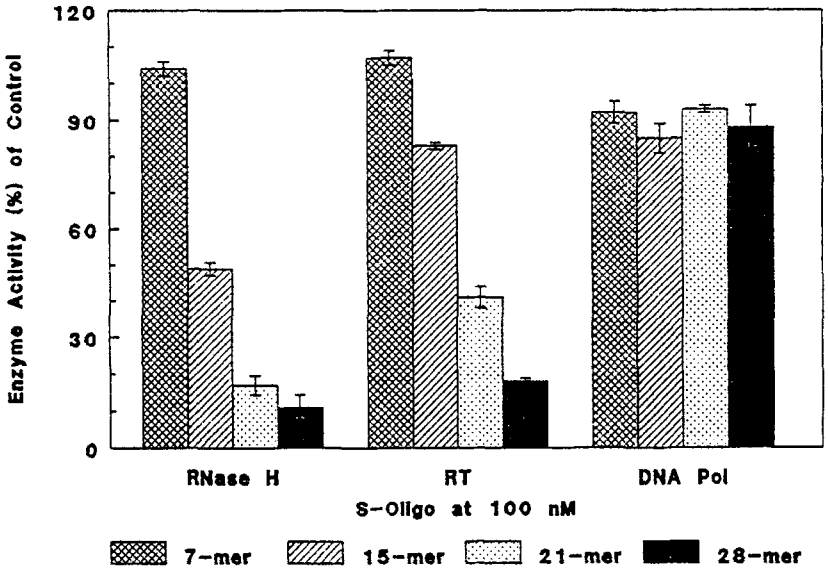

FIG. 1 Effects of S-dC_{28} on HIV reverse transcriptase associated enzyme activities. All enzyme assays were performed in the presence of 100 nM S-dC_n, 50 mM Tris-HCl (pH 8.0), 8 mM $MgCl_2$, and 50 mM KCl, except that the substrate or the templates were different. The substrate, $(dC)_n.(rG)_n$, was used in the RNase H assays. The templates, $(rC)_n.(dG)_{12}$ and $(dC)_n.(dG)_{12}$, were used in the DNA polymerase assays and the RT assays, respectively. Part of these results have been submitted for publication.

Four human DNA polymerases and two RNase H activities which were identified in human cells, were highly purified and examined against S-dC_{28}. The assay conditions for different types of DP were identical except for the concentration of salt. The results are shown in Figure 2. S-dC_{28} could inhibit the different human DPs to a different degree. DNA polymerase δ and γ were most sensitive whereas DNA polymerase β was quite resistant. When the activity of S-dC_{28} against both types of RNase H was examined, RNase H 1 was found to be more sensitive than RNase H_2. The inhibition of both DP and RNase H activities by S-oligos did not appear to be sequence specific but dependent on the chain length of the oligomer. This nonspecific inhibition of

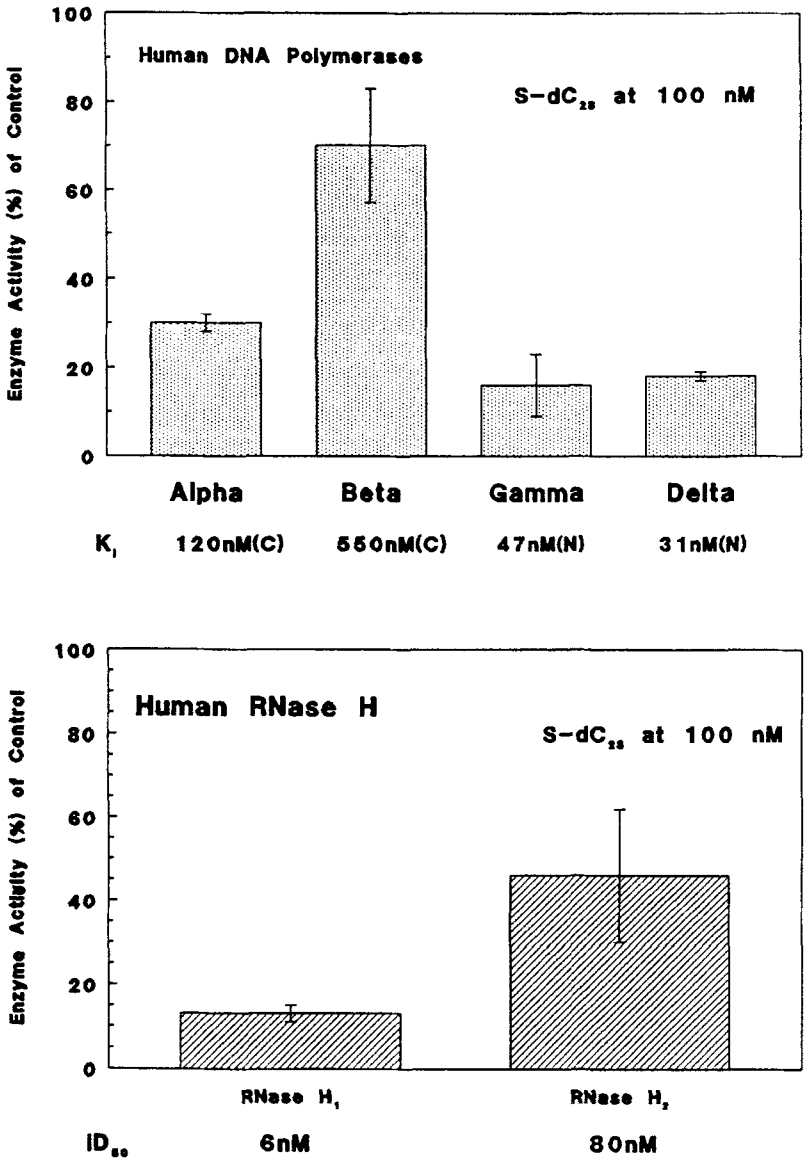

Fig. 2 Effects of S-dC_{28} on human DNA polymerase and RNase H activities. Enzyme assays were performed under an indentical condition except for the salt concentration. No KCl was used in the human alpha and delta assays, 100 mM KCl was used in human beta and gamma assays, 50 mM KCl was used in human RNase H_1 assays and 120 mM KCl was used in human RNase H_2 assays. K_I values were determined by Lineweaver-Burk plots. The mode of inhibition is competitive (C) or noncompetitive (N). Part of these results was published previously (13).

DNA polymerase and RNase H could present a major concern for utilization of phosphorotioate oligonucleotides as antisense molecules intracellularly.

Herpes viruses as a group are capable of inducing their type specific DP activity in cells upon infection. The properties of herpes virus induced polymerases are quite different from that of human enzymes (11,12) and the presence of viral DP activity is critical for viral replication. Since S-oligos could inhibit HIV RT and human DPs, it was of interest to examine the action of S-oligos in inhibiting DP induced by HSV-1, HSV-2, EBV and HHV-6. The results indicate that S-oligos inhibited herpes virus DP in the order of HSV-2 > EBV = HSV-6 > HSV-1 as shown in Figure 3. All the assay conditions were the same as Figure 2 except for the concentration of salt employed. The mechanism of the inhibition of HSV-2 DNA polymerase by S-oligo was further explored; S-oligo could inhibit both DP as well as its associated exonuclease by competing with DNA. The K_I was estimated to be around 7 nM for S-dC_{28}. The longer the chain of S-oligo is, the greater is the inhibitory activity.

In view of S-dC_n's potent activity against HSV DP *in vitro*, the potential antiviral activity of S-dC_n against HSV-1 and HSV-2 in HeLa cells was examined. The results are shown in Figure 4. It was found that the compounds are more inhibitory against HSV-2 than against HSV-1, and that the longer the chain lengths the more potent the antiviral activity (14). HSV-2 strains which are resistant to phosphonoformate as a result of altered DNA polymerases are more sensitive to S-dC_{28} than the parental virus. When the susceptibility of their DP to PFA and SdC_{28} were examined, the DP from PFA^r was found to be more resistant to PFA than that from the parental strain with ID_{50} 0.5 and 5 μM, respectively. Whereas, the DP from PFA^r was found more susceptible to SdC_{28} than that from its parental strain with ID_{50} 14 $\pm$ 1 nM and 20 $\pm$ 1 nM, respectively. HSV-2 viruses which could not induce viral thymidine kinase and become resistant to acyclovir (ACV) and ganciclovir (DHPG) were still sensitive to S-dC_{28}. The lack of cross

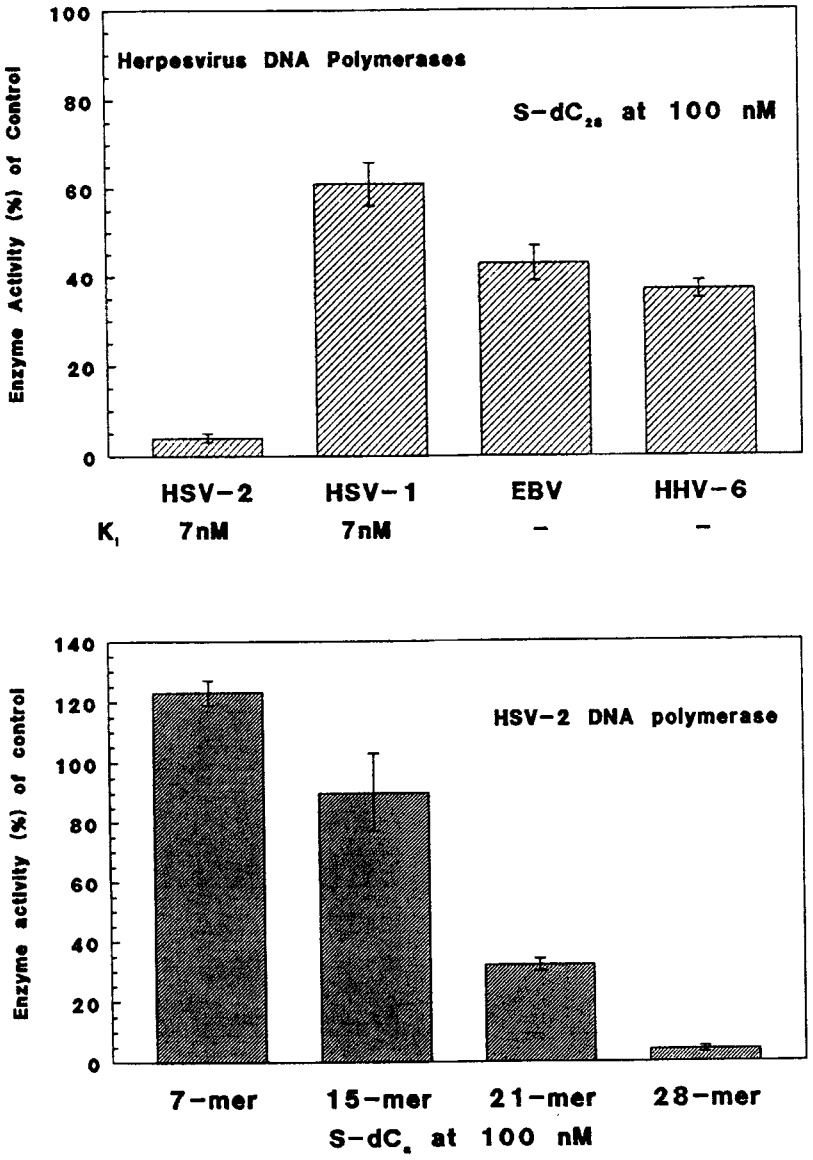

Fig. 3 Effects of S-oligos on herpesvirus-induced DNA polymerases. The DNA poly merase activities were determined in a identical condition in the presence of 200 mM KCl and 100 nM S-dC_n. The chain length effects of S-dC_n were examined on HSV-2 DNA polymerase. Part of the results was published previouly (13).

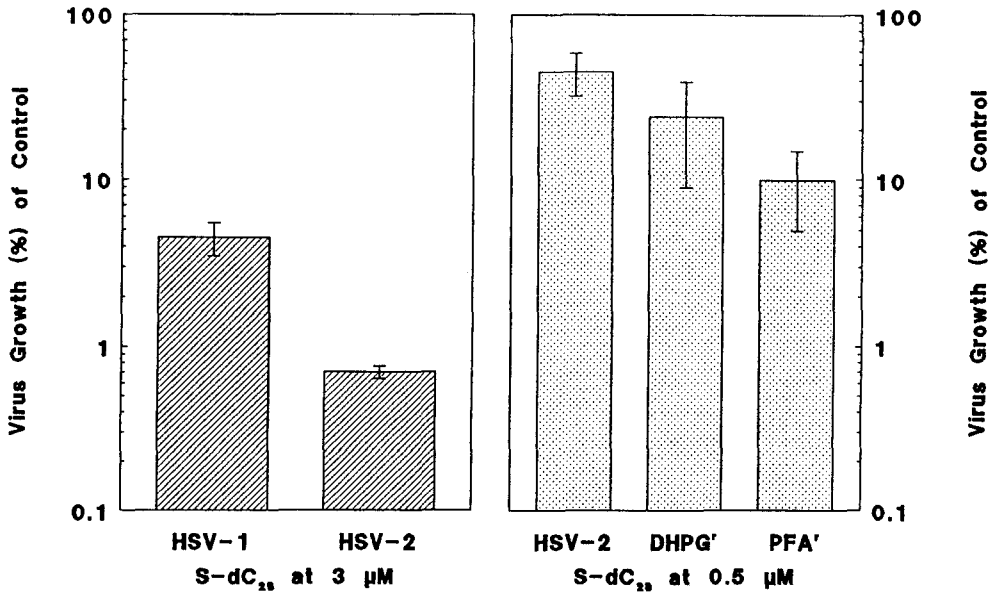

FIG. 4 Effects of SdC_{28} on HSV-1, HSV-2 and HSV-2 drug resistant mutants. HeLa cells were infected with HSV-1 or HSV-2 at 3 PFU per cell in the presence of 3 μM SdC_{28} and virus growth was examined by using the virus yield assays (left). The thymidine kinase mutant of HSV-2 that is resistant to DHPG and the DNA polymerase mutant of HSV-2 that is resistant to PFA were susceptible to SdC_{28} at 0.5 μM (right). Part of these results was published previously (14).

resistance between S-oligos and other antivirals such as phosphonoformate, ACV, and DHPG was indicated. Furthermore, $S\text{-}dC_{28}$ was found to potentiate the action of phosphonoformate and to have an additive effect with DHPG on the inhibition of HSV-2 replication in culture.

The mechanism of $S\text{-}dC_{28}$ against HSV-2 in culture was explored. The inhibition of HSV-2 growth by $S\text{-}dC_{28}$ was found to be schedule dependent. The presence of $S\text{-}dC_{28}$ during the viral adsorption period is critical for its antiviral activity as shown in Figure 5. Preincubation of cells with $S\text{-}dC_{28}$ before viral infection or the addition of compounds to culture after viral infection did not show potent antiviral activity. This schedule dependence of

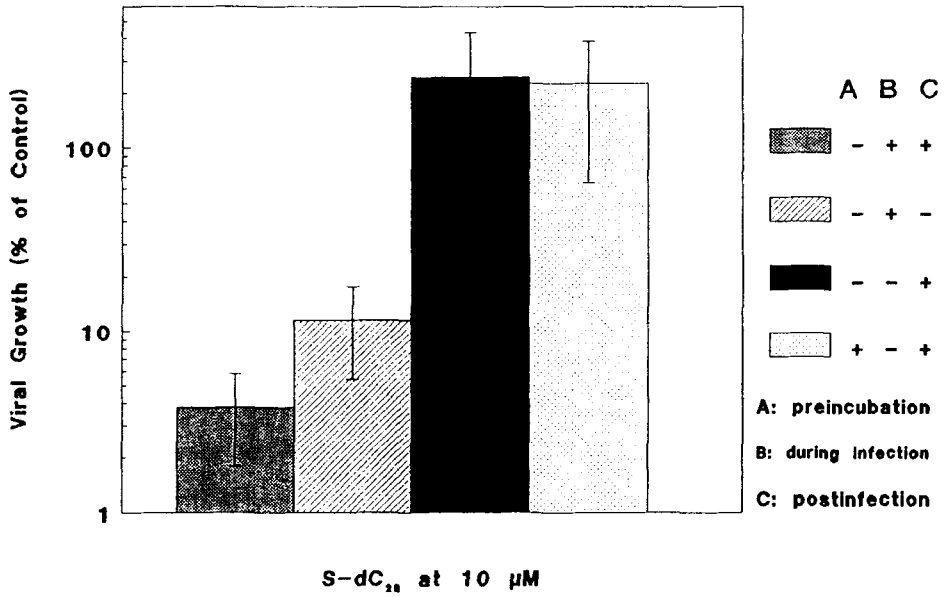

Fig. 5 Effects of different exposure protocols of S-dC_{28} on HSV-2 growth. HeLa S3 cells were infected at 3 plaque forming units (PFU) per cell and S-dC_{28} were examined by using the virus yield assays. A, drug added 24 h prior to viral infection; B, drug added during 1 h infection period; C, drug added after the 1 h infection period; C, drug added after the 1 h infection for 24 h. +, S-dC_{28} added at 10 μM; -, no drug added. Part of these results was published previously (14).

antiviral action could be due to the inhibition of viral internalization by S-dC_{28}, or the possibility that viruses facilitate S-dC_{28} entry into cells. This was addressed, and S-dC_{28} was found to inhibit HSV-2 internalization to some extent. Since there is a lack of correlation between the inhibition of HSV-2 uptake and the concentration of S-dC_{28}, this inhibition did not appear to be sufficient to explain its antiviral activity against HSV-2. Scatchard analysis of the interaction between HSV-2 viron and S-dC_{28} revealed the dissociation constant could be as low as 9 nM. When the uptake of S-dC_{28} in HSV-2 infected cells was examined in comparison with that in HSV-1 or mock infected cells the internalized S-dC_{28} in HSV-2 infected cells was at least 10

times greater than that in HSV-1 or mock infected cells (unpublished data). The enhancement of the uptake in HSV-2 cells could be the result of the tight interaction between HSV-2 virons and S-dC_{28}. The biochemical components of HSV-2 virons responsible for this tight interaction are currently under investigation.

Once S-dC_{28} was taken up in the cells, the majority of S-dC_{28} was present in the cytoplasm initially. There was a time dependent accumulation of the infected cells. The majority of intracellular S-dC_{28} stayed intact even 24 hr post viral infection. The impact of S-dC_{28} on viral enzyme (thymidine, kinase and DNase) induction and viral DNA synthesis in HSV-2 infected cells was examined. Viral DNA synthesis was inhibited and the extent of inhibition was correlated to the antiviral activity of S-dC_{28}. Viral enzyme induction was only inhibited to a small extent, which could be due to the inhibition of HSV-2 uptake by S-dC_{28}.

In summary, S-oligo with defined sequences are entertained as attractive candidates for developing antisense drugs which target on selective genes or their product. The premise of this approach is S-oligo once inside the cells could hybridize with its complementary RNA and the hybridized RNA could be degraded by RNase H. The cellular functions unrelated to the target gene product will not be affected by S-oligo in a direct fashion. In this report, we demonstrated that S-oligo, in a sequence nonspecific manner, could potently inhibit human DNA polymerase δ and α which are responsible for nuclear DNA synthesis, DNA polymerase γ which is responsible for mitochondrial DNA synthesis, and RNase H_1 which could play an important role in degrading S-oligo complementary RNA and nucleic acid synthesis. Thus if sufficient quantities of S-oligo with a defined sequence are accumulated in the cells, S-oligos will not only exert an anti-sense effect in a sequence specific manner but also inhibit critical cellular enzyme functions which retard the cell growth in a sequence nonspecific manner. The intracellular concentration of S-oligo should play a critical role in terms of its

selectivity. Whether other antisense chemical entities such as methylphosphonate oligo will also have potential activity against those host enzymes in a sequence nonspecific manner should be explored.

The antiviral effects of S-oligo in a sequence nonspecific manner should deserve attention. In the case of its anti-HSV-2 activity, the mechanism of its action is due to: 1) interference with the uptake of HSV-2, 2) enhancement of the uptake of S-oligo in HSV-2 infected cells, and 3) inhibition of HSV-2 synthesis intracellularly as a result of the inhibition of viral DP. S-oligo could interact with other antivirals at least additively and active against HSV-2 strains which are resistant to other antivirals. Topical usage for the treatment of localized infection should be tried if the systemic usage proves to be costly and troublesome. In the case of HIV, it is not clear what the mechanism of S-oligo antiviral action is. It appears due to: 1) inhibition of HIV uptake, 2) inhibition of RT, RT and RNase H function of HIV RT, 3) inhibition of host proteins which are critical for viral replication but not so critical for cell growth, and 4) selective interference with the target RNA. The first three mechanisms are sequence nonspecific and the last mechanism is sequence specific. The observation that the anti-HIV action of S-oligo targets on HIV *rev* sequence could raise the possibility of utilizing such type of compounds for the treatment of AIDS patients by loading the bone marrow cells or blood cells with nontoxic concentration of defined sequence S-oligo targets on HIV critical genes for protective or consolidation purposes. Its effectiveness will depend on the efficiency of uptake and half-life of S-oligo in T cells or macrophages. This will make the use of such types of compounds less costly and more effective. It may also be useful for blood banks in ensuring the absence of HIV in blood for transfusions.

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