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Newly Designed Six-membered Azasugar-Containing Phosphorothioate Oligonucleotide as a Potent AIDS Therapeutic Drug

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ABSTRACT

A series of modified oligonucleotides containing a phosphorothioate (P=S) backbone and a six-membered azasugar (6-AZS) as a sugar substitute in a nucleotide were synthesized and tested for their ability to inhibit the human immunodeficiency virus type 1(HIV-1) *in vitro* without the aid of any transfecting agents. While P=S oligonucleotides with natural nucleotides had little anti-HIV-1 activity, the six-membered azasugar nucleotide (6-AZN)-containing P=S oligonucleotides (AZPSONs) potently inhibited the HIV-1/SHIV replication and syncytium formation (EC_{50} = 0.02~0.2 μM) without cytotoxicity up to 100 μM. DBM-2198, the most effective in anti-HIV-1 activity among the AZPSONs, consists of random sequence and five 6-AZNs evenly distributed in 18 nucleotides. DBM-2198 showed strong antiviral activity against, not only laboratory strains, but also primary isolates and even drug-resistant strains of HIV-1. DBM-2198 was much more effective than ddI or ddC in its anti-HIV-1 activity in vitro. Particularly noteworthy is that the anti-HIV-1 activity of DBM-2198 was better than that of AZT with respect to its long-lasting efficacy after a single treatment. Nevertheless, the antiviral activity of the AZPSONs was very specific to HIV-1. Poliovirus, or even simian immunodeficiency virus (SIV), was not inhibited by the AZPSONs. Taken together, our results strongly suggest that AZPSON can be used as a safe and effective AIDS-therapeutic drug against a broad spectrum of HIV-1 strains.

RESULTS AND DISCUSSION

In this study, we demonstrated that novel 6-membered azarsugar-containing P=S ONs (AZPSONs) have a strong antiviral activity against HIV-1 infection only by simple treatment of

infected culture without the aid of transfecting reagents. The substitution of sugar moiety with 6-membered azarsugar (6-AZS) in P=S ONs seems to be essential for the potent anti-HIV-1 activity of the AZPSONs, since only weak or no antiviral activity was observed with P=S ONs such as DBM-2136 or DBM-2241 alone, even though they have the same nucleotide sequence as the AZPSONs DBM-2134 and DBM-2198, respectively. The AZPSONs showed HIV-1 inhibition in a dose-dependent manner, implying that the antiviral activity of the AZPSONs was not due to non-specific side effects. Actually, the P=S ONs themselves have been widely studied for their improved stability and anti-HIV-1 activity (2-17). In the present study, however, we found that the P=S ONs themselves were not as effective in the inhibition of HIV-1 as reported previously, when treated *in vitro* without transfection. Whereas, AZPSONs containing specific substitution of sugar moiety of adenosine in their P=S ONs were much more potent in their anti-HIV-1 activity than the P=S ONs alone. Nevertheless, the P=O backbone only or the mixed backbone (P=O and P=S) oligonucleotides did not exhibit any anti-HIV-1 activity, even though these ONs contained 6-AZNs. These results suggest that the P=S backbone and the 6-AZS substitutions of sugar moiety worked synergically for the anti-HIV-1 activity of the AZPSONs. The anti-HIV capacity of the AZPSONs seems to depend on the number and/or distribution patterns of the 6-AZNs in the ONs.

Recently, various tetrameric G-quartet oligonucleotides have drawn attentions as promising anti-HIV-1 compounds by blocking the HIV-1 attachment/adsorption (5-9) or inhibition of HIV-1-specific enzymatic activity (10-17). It has been reported that the antiviral activity of these analogues originated in the biological function of the G-quartet structure, even though the exact mechanism is still unclear. In contrast to the previous reports however, we did not find any such remarkable antiviral activity in these G-quartet-forming oligonucletides in our repeated experiments. Whereas, many of the AZPSONs, even those with no G-quartet composition, showed strong anti-HIV-1 activity. Among these, DBM-2193, 2196 and 2198 in particular, were more effective than AZT for their long-lasting inhibition of HIV-1 replication after a single treatment of the HIV-1-infected cells. In particular, these were found much more potent than ddI or ddC in our efficacy test.

Among the AZPSONs, DBM-2198 was the most effective in its anti-HIV-1 activity (0.05 ± 0.01 μM$<EC_{50}<0.1 \pm 0.02$ μM) and showed little toxicity to most of the cell lines ($CC_{50}>100$ μM) tested *in vitro*. In addition, DBM-2198 was little attenuated in its anti-HIV-1

activity in the presence of serum. These results strongly suggest that DBM-2198 would also be an effective candidate *in vivo* as an AIDS therapeutic agent (its therapeutic index being at least over 1000). Other AZPSONs were slightly affected by the sera, but the degree of inhibition was neither proportional to the concentration of the sera, nor affected by the kind of sera. These results imply i) that only small amounts of sera would be required to saturate the serum-reactive sites of the AZPSONs, and ii) that the reactive sites of the inhibitory components in the sera may have nothing to do with the direct active sites of the anti-HIV-1 activity of the AZPSONs.

The AZPSONs were as stable as the P=S ONs in their nuclease conditions. Interestingly, while the P=O ONs themselves, such as DBM-2180, was very unstable in the culture supernatant, the P=O ONs were resistant in the same condition when they contained even a few sporadic AZNs and the resistance was almost equivalent to that of the P=S ONs, suggesting that the AZNs may have the capacity to promote the stability of ONs, even with a P=O backbone. However, the DBM-2137 was not as stable as DBM-2134 or -2198 in a nuclease-containing media (data not shown), indicating that the incorporation of a few AZNs themselves is not enough to protect the P=O ONs from strong nuclease-attacking conditions.

In order to identify the anti-HIV-1 mechanisms, we have tested the AZPSONs for their effects on LTR-mediated CAT expressions in the LTR-CAT-transformed cells. While CAT activity was markedly reduced by the transfection of TAR-specific P=S antisense ONs (DBM-2136), simple treatment of this ON in the medium did not have any influence on the CAT activity. Whereas, TAR/LTR sequence-specific AZPSON, DBM-2134 and 2177, did not show any inhibition of the LTR-mediated CAT expression, either by simple treatment or by liposome-mediated transfection. This could be explained as follows: i) AZPSONs might not have an affinity to the TAR/LTR sequence, even when introduced into the cytoplasm, or ii) they may not get into the cells, even in the cases of transfection experiments. However, our finding that FITC-labeled AZPSONs were nicely detected in the cytoplasm when transfected (data not shown), suggests that no effects of DBM-2134 and 2177 on the LTR-mediated CAT expression, even in the transfection experiments, is likely due to the AZPSONs' lack of sequence-specific affinity to the mRNA of TAR/LTR in the cytoplasm. In other words, in contrast to the previous results of AZN function in P=O ONs with its affinity to sequence-specific mRNA (29), AZNs in P=S ON are probably to reduce its affinity to the target mRNA, and the degree of inhibition depends on the number and distribution patterns of the AZNs. DBM-2175, a TAR-specific antisense

AZPSON, which has only 2 AZNs at both ends of the ON, showed partial inhibition of the LTR-mediated CAT expression when transfected. This was probably due to the affinity of the internal P=S sequence between the 2 AZNs at both ends of DBM-2175. Partial suppression by DBM-2180 seemed to be associated with its instability in the cytoplasm. The result that the anti-HIV-1 activity of the AZPSONs has nothing to do with the intracellular sequence-specific antisense mechanisms was also confirmed in the transfection experiments with Magi cells (HeLa-CD4-LTR-β-gal). Dose-dependent inhibition of the LTR-mediated β-gal expression was shown only in P=S ON (DBM-2136), but was not detected at all in other AZPSONs tested. Taken as a whole, the anti-HIV-1 activity of AZPSONs in a simple treatment was very likely due to their extracellular non-specific reaction, rather than their intracellular sequence-specific antisense or other inhibitory mechanisms.

Our finding that AZPSONs work extracellularly by non-specific interaction with proteins raises the question whether they may block the cell surface molecules, resulting in the inhibition of other viral infections. However, neither poliovirus nor SIV was inhibited in its replication in the presence of AZPSONs at the concentration for sterile inhibition of HIV-1 replication, indicating that the antiviral activity of AZPSONs is highly specific to HIV-1. In particular, DBM-2198, among the AZPSONs, showed potent antiviral activity against a broad spectrum of HIV-1, not only primary isolates, but also drug-resistant HIV-1 variants, in cultures of T cell lines, and in primary PBMCs as well. Monotropic virus, Ba-L strain, was also markedly inhibited by DBM-2198 (data not shown). It was reported recently that dG3T4G3-s, a kind of P=S ON, was effective only on the T-cell tropic HIV-1 (11). While SIV was not affected by DBM-2198, $SHIV_{89.6}$, a chimeric virus consisting of SIV-originated *gag-pol* gene and HIV-1-derived *env* gene (39-41), was very sensitive to DBM-2198, strongly suggesting that the anti-HIV-1 activity of DBM-2198 is associated with the HIV-1 envelope rather than the other viral proteins or the cell surface molecules. Actually, many of the P=S ONs have been reported for their anti-HIV-1 mechanisms in association with the V3 loop of HIV-1 gp120 (6-11). However, some other reports have shown that the P=S ONs were bound to the cell surface receptors (27, 28) or were associated with cytoplasmic or nuclear proteins (26).

Acute HIV-1 infections with high MOIs were not completely blocked by DBM-2198 at a final concentration of 0.5 μM. However, we could not detect any syncytium formation in the culture, even though some amounts of HIV-1 were measured in the supernatant by RT assay.

Interestingly, the RT activity in the culture supernatant decreased as time passed, after 6 days p.i., and then was finally undetectable at day 15. This means that AZPSONs do not affect the intracellular HIV-1 replication once cells are infected. Rather, they are likely to inhibit the further spreading of HIV-1 infections by blocking virus attachment to the nearby-uninfected cells, finally resulting in the eradication of the infectious virus particles. This has also been confirmed in experiments to find cures. Numerous severe syncytia in cultures infected with HIV-1 disappeared within 2 days following treatment with DBM-2198, with no recurrence of the syncytium formation in further cultures, indicating that DBM-2198 inhibited further infection of progeny viruses even at the high titer produced by the culture before treatment.

In conclusion, our results strongly suggest that AZPSONs can be used as safe and effective AIDS-therapeutic drugs against a broad spectrum of HIV-1 strains in a sequence-selective nonantisense manner. They would be much more effective if used with other target-specific AIDS therapeutic drugs, such as RT-inhibitors or protease-inhibitors. The details of the AZPSONs' anti-HIV-1 mechanisms are under investigation. Further experiments with other 6-AZS-containing nucleosides (cytidine, guanosine and thymidine) will give us some further insight into the structural requirements of this series of ONs.

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