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Short communication

Antiviral activities of nucleotide heterodimers against human immunodeficiency virus type 1 in vitro

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Abstract

Nucleotide heterodimers were synthesized and examined for their inhibitory effects on the replication of human immunodeficiency virus type 1 (HIV-1), including HIV-1 reverse transcriptase (RT) inhibitor-resistant mutants. 3′-Azido-3′-deoxythymidilyl-(5′)-phospho-(5′)-6-[(3′,5′-dimethylphenyl)thio]-5-ethyl-1-[(2-hydroxyethoxy)methyl]uracil (AZT-*P*-E-HEPU-dM) and 3′-azido-3′-deoxythymidilyl-(5′)-phospho-(5′)-2′,3′-dideoxyinosine (AZT-*P*-ddI) proved to be highly potent and selective inhibitors of HIV-1 (III_B strain) in MT-4 cells. The mechanism of inhibition by these heterodimers may be attributed to their degradation and the formation of each constituent. AZT-*P*-E-HEPU-dM was also markedly inhibitory to an AZT-resistant mutant (HIV-1-$III_{B/AZT}$) and an E-HEPU-dM-resistant mutant (HIV-1-III_{B-R}). However, AZT-*P*-ddI was found to have a less inhibitory effect on HIV-1-$III_{B/AZT}$ than on HIV-1-III_B. The heterodimers of (5′,5′) AZT and ribavirin (AZT-*P*-Ribavirin) and (5′,5′) ddI and ribavirin (ddI-*P*-Ribavirin) were also synthesized: AZT-P-Ribavirin inhibited HIV-1 replication, but ddI-*P*-Ribarvirin did not.

Keywords: Heterodimer; HIV-1; Reverse transcriptase inhibitor-resistant mutants

It has been reported that drug-resistant mutants of human immunodeficiency virus type 1 (HIV-1) are frequently isolated from patients suffering from acquired immune deficiency syndrome (AIDS) during long-term treatment with HIV-I reverse transcriptase (RT) inhibitors, such as 3′-azido-3′-deoxythymidine (AZT) (Land et al., 1990; Larder et al., 1989), 2′,3′-dideoxyinosine (ddI) (St. Clair et al., 1991), and nevirapine (Rich-

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man et al., 1994). Combination chemotherapy of AZT with other nucleoside analogues or with other classes of anti-HIV-1 agents is thought to circumvent or delay the emergence of drug-resistant mutants. Several nucleotide heterodimers composed of dideoxynucleoside derivatives were shown to selectively inhibit HIV-I replication in cell cultures (Schinazi et al., 1990). Furthermore, drug-resistant mutants have not been generated in cell cultures in the presence of increasing concentrations of such heterodimers (Gao et al., 1993a). Combination of a nucleoside and a non-nucleoside reverse transcriptase inhibitor (NNRTI) was advocated by Nanni et al. (1993). Recently, Velázquez et al. (1995) reported that heterodimers of AZT and NNRTIs linked through an alkyl spacer inhibited HIV-1 replication in vitro. We have now synthesized novel heterodimers that consist of one of the dideoxynucleosides (AZT or ddI) and the NNRTI 6-[3,5-(dimethyl-phenyl)thio]-5-ethyl-1-[(2-hydroxyethoxy)methyl]uracil (E-HEPU-dM), or the inosine-5′-monophosphate dehydrogenase inhibitor ribavirin, and examined them for their inhibitory effects on HIV-1 replication in vitro.

All compounds used in this study were synthesized at the chemistry laboratory of Yamasa Corporation. Their formulae are shown in Fig. 1. 3′-Azido-3′-deoxythymidilyl)-(5′)-phospho-(2′)-1-[(2′ - hydroxyethoxy)methyl] - 6 - [(3, 5 - dimethyl-phenyl)thio]-5-ethyluracil (AZT-*P*-E-HEPU-dM) was prepared by reacting E-HEPU-dM (Tanaka et al., 1992) with AZT cyanoethylphosphate (Busso et al., 1988), as previously described. 3′-Azido-3′-deoxythymidilyl-(5′)-phospho-(5′)-2′,3′-dideoxyinosine (AZT-*P*-ddI) was prepared according to the established method (Busso et al., 1988). 3′-Azido-3′-deoxythymidilyl-(5′)-phospho-(5′)-1-β-D-ribofuranosyl-1,2,4-triazole-3-carboxyamide (AZT-*P*-Ribavirin) and 2′,3′,-dideoxyinosine-(5′)-phospho-(5′)-1-β-D-ribofuranosyl-1,2,4-triazole-3-carboxamide (ddI-*P*-Ribavirin) were also prepared by reacting AZT cyanoethylphosphate or ddI cyanoethylphosphate (Tener, 1961) with 2′,3′-*O*-diacetyl ribavirin, which was synthesized according to the general procedure by deprotection of 5′-*O*-(*tert*-buthyldimethylsilyl)-2′,3′,-*O*-diacetyl protected nucleoside with tetrabutylammonium fluoride in tetrahydrofuran. The deprotection of cyanoethylphosphate acetate ester with 1 N sodium hydroxide solution afforded the sodium salts of the heterodimers. Nuclear magnetic resonance and mass spectrometry data were satisfactory for all compounds. The sodium salts of the heterodimers were purified by reversed-phase high-performance liquid chromatography(HPLC), and their purity was more than 99% by HPLC analysis.

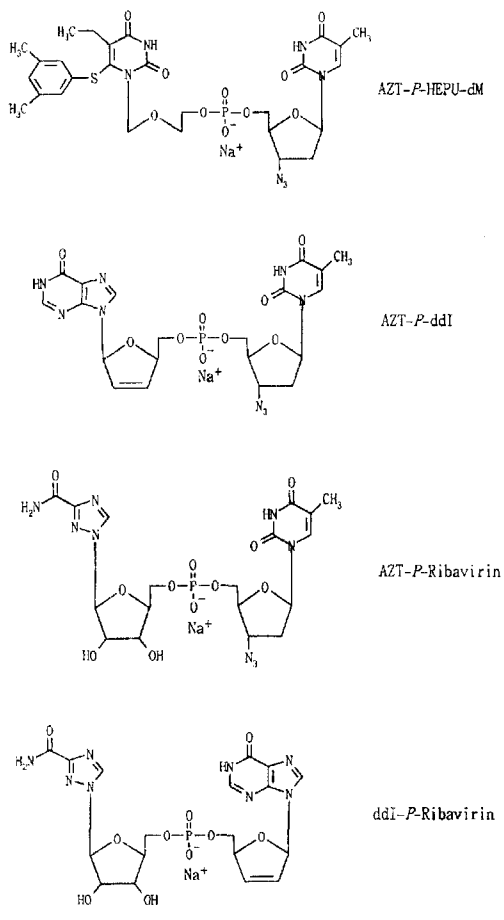

Fig. 1. Structural formulae of AZT-*P*-E-HEPU-dM, AZT-*P*-ddI, AZT-*P*-Ribavirin and ddI-*P*-Ribavirin.

MT-4 cells were used in the anti-HIV-1 assay. The cells were grown and maintained in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS), 100 units of penicillin G per ml, and 20 μg of gentamicin per ml. Three HIV-1 strains (III_B, $III_{B/AZT}$ and III_{B-R}) were used in the assay. HIV-1-III_B was obtained from R.C. Gallo (National Cancer Institute, Bethesda, MD). HIV-1-$III_{B/AZT}$ is an AZT-resistant mutant that was established by a serial passage of HIV-1-III_B in cell culture in the presence of increasing concentrations of AZT. This mutant has three amino acid changes, namely $Asp^{67} \rightarrow$ Asn, $Lys^{70} \rightarrow$ Arg, and $Lys^{172} \rightarrow$ Arg, in its RT (unpublished data). HIV-1-III_{B-R} is a NNRTI-resistant mutant isolated by a serial passage of HIV-1-III_B in cell culture in the presence of increasing concentrations of the HEPT derivative 6-benzyl-1-ethoxymethyl-5-isopropyluracil (MKC-442). RT sequence analysis has revealed that it has a single amino acid change $Tyr^{181} \rightarrow$ Cys (Baba et al., 1994). The antiviral activity of the test compounds against the replication of HIV-1 strains was based on the inhibition of the virus-induced cytopathic effect (CPE) in MT-4 cells, as described previously (Baba et al., 1994). The number of viable cells was determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) method on day 4 or 5 after virus infection (Pauwels et al., 1988).

AZT-*P*-E-HEPU-dM completely inhibited HIV-1-III_B-induced CPE in MT-4 cells at a concentration of 0.02 μM (Fig. 2). However, the compound did not affect the viability of mock-infected MT-4 cells at concentrations up to 2.5 μM. The 50% effective concentration (EC_{50}) and 50% cytotoxic concentration (CC_{50}) of AZT-*P*-E-HEPU-dM were 0.002 and 15.5 μM, respectively. The selectivity index, based on the ratio of CC_{50} to EC_{50}, was 7750. AZT-*P*-E-HEPU-dM was equally effective against both HIV-1-III_B and HIV-1-III_{B-R} (an NNRTI-resistant mutant), yet it was slightly less effective against the AZT-resistant mutant HIV-1-$III_{B/AZT}$. The EC_{50} values for HIV-1-III_{B-R} and HIV-1-$III_{B/AZT}$ were 0.002 and 0.01 μM, respectively.

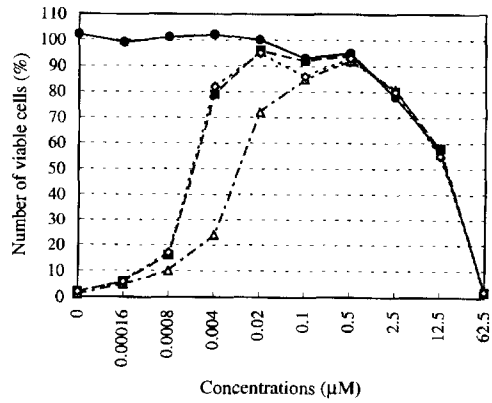

Fig. 2. Inhibitory effect of AZT-*P*-E-HEPU-dM on the replication of HIV-1 in MT-4 cells. MT-4 cells were infected with the III_B (■), $III_{B/AZT}$ (△), or III_{B-R} (◇) strain, and incubated in the presence of various concentrations of the compound. After a 4-day incubation, the viability of virus-infected cells and mock-infected cells (●) was determined by the MTT method. The number of viable cells was expressed as a percentage of the mock-infected control.

Table 1 summarizes the inhibitory effects of several nucleotide heterodimers and their constituents on HIV-1 replication in MT-4 cells. Like AZT-*P*-E-HEPU-dM, AZT-*P*-ddI and AZT-*P*-Ribavirin were found to be potent and selective inhibitors of HIV-1 replication. In contrast, ddI-*P*-Ribavirin did not show any inhibition at concentrations that were not toxic to the host cells. AZT-*P*-E-HEPU-dM was highly effective against all of the three HIV-1 strains, but the EC_{50} values were identical to those obtained by the the combination treatment with AZT and E-HEPU-dM. For AZT-*P*-ddI, the EC_{50} values were quite similar to those obtained for AZT alone or for the combination of AZT with ddI. Since the anti-HIV-1 activity of AZT was much higher than that of ddI the in MT-4 cells, the inhibitory effects of the heterodimers seem to be reflected by the activity of the more active constituent. Although AZT-*P*-ddI does not seem to have a more inhibitory effect on HIV-1-$III_{B/AZT}$ than AZT itself in MT-4 cells, it may be possible that the compound is more effective than AZT in resting cells. It has been reported that ddI exerts more potent anti-

Table 1
Inhibitory effects of nucleotide heterodimers on HIV-1 replication in MT-4 cells [a]

Compound	EC_{50} [b] (μM)			CC_{50} [c] (μM)
	HIV-1-III_B	HIV-1-$III_{B/AZT}$	HIV-1-III_{B-R}	
AZT-*P*-E-HEPU-dM	0.002 ± 0.001	0.010 ± 0.001	0.002 ± 0.001	15.5 ± 2.9
AZT-*P*-ddI	0.002 ± 0.001	0.045 ± 0.002	0.002 ± 0.001	18.2 ± 2.8
AZT-*P*-Ribavirin	0.004 ± 0.001	N.D. [d]	N.D.	N.D.
ddI-*P*-Ribavirin	> 17.5	> 17.5	> 17.5	17.5 ± 2.2
AZT + E-HEPU-dM	0.001 ± 0.001	0.006 ± 0.001	0.002 ± 0.001	11.5 ± 1.0
AZT + ddI	0.002 ± 0.001	0.041 ± 0.001	0.001 ± 0.001	12.2 ± 0.9
AZT	0.002 ± 0.001	0.051 ± 0.001	0.001 ± 0.001	10.7 ± 1.9
E-HEPU-dM	0.007 ± 0.001	0.014 ± 0.003	2.4 ± 0.2	155 ± 27
ddI	13.3 ± 3.4	10.8 ± 0.3	6.5 ± 0.3	226 ± 17
Ribavirin	> 3.3	> 3.3	> 3.3	3.3 ± 0.1

[a] All data represent means ± standard deviations for at least three separate experiments.
[b] 50% Effective concentration based on HIV-1-induced CPE in MT-4 cells.
[c] 50% Cytotoxic concentration based on the reduction of viability of mock-infected cells.
[d] N.D., not determined.

HIV-1 activity in resting cells than in activated cells, while AZT preferentially protects activated cells against HIV-1 infection (Gao et al., 1993b). The same group reported the effect of dideoxynucleoside analogues on HIV-1 replication in resting cells that were infected with HIV-1 before or after phytohemagglutinin (PHA) activation (Shirasaka et al., 1995).

A plausible explanation for the mechanism of inhibition by heterodimers is their degradation and the formation of each constituent in cell cultures. To examine their stability in culture medium, each heterodimer (100 μM) was incubated at 37°C for 72 h in culture medium containing 10% heat-inactivated FBS. They were then analyzed by HPLC as follows: 10 μl of each sample were injected onto the column and eluted with various concentrations of acetonitrile in 50 μM triethylamine acetate (pH 7.0), while being monitored with a UV-detector at a wavelength of 270 nm. When AZT-*P*-E-HEPU-dM was incubated in the culture medium, 94% of the heterodimer was degraded, and AZT and E-HEPU-dM became detectable within 24 h (data not shown). The heterodimer was not detectable any more after 72 h of incubation. On the other hand, AZT-*P*-ddI, AZT-*P*-Ribavirin, and ddI-*P*-Ribavirin were still detected for 57, 36, and 44%, respectively, when the heterodimers were incubated at 37°C for 72 h. The order of their stability in the culture medium containing 10% heat-inacvitated FBS was as follows: AZT-*P*-ddI > ddI-*P*-Ribavirin > AZT-*P*-Ribavirin ≫ AZT-*P*-E-HEPU-dM. Puech et al. (1992) reported that dideoxycytidine phosphodiester dimer was decomposed for 60% in the culture medium containing 10% heat-inactivated FBS, and this decomposition was escalated in the medium containing 10% non-heat-inactivated FBS. It has also been demonstrated that a heterodimer of AZT and 2′,3′-dideoxyadenosine is metabolized rapidly at a rate of approximately 10% per hour in human plasma (Hahn et al., 1989). Thus, the stability of heterodimers in serum/plasma may, as expected, be an important determinant of their anti-HIV-1 actvity in vitro and in vivo.

Inosine-5′-monophosphate (IMP) dehydrogenase inhibitors, such as ribavirin and tiazofurin, have been shown to decrease the phosphorylation of 2′,3′-dideoxyribopyrimidines (including AZT) and to reduce their anti-HIV-1 activity, while they enhanced the phosphorylation of purine 2′,3′-dideoxynucleosides and potentiated their anti-HIV-1 activity (Vogt et al., 1987; Baba et al.,

1987; Hartman et al., 1991). Based on these findings, we examined the anti-HIV-1 activities of AZT-*P*-Ribavirin and ddI-*P*-Ribavirin in MT-4 cells. AZT-*P*-Ribavirin inhibited the replication of HIV-1-III_B by 50% at a concentration of 0.004 μM, similar to the EC_{50} of AZT itself for HIV-1-III_B (Table 1). On the other hand, ddI-*P*-Ribavirin did not show any inhibition of HIV-1 replication at concentrations below the CC_{50} (Table 1). These results may not have been expected from the previous reports. However, this discrepancy could be explained by the fact that the heterodimers were degraded to the equimolar amounts of each constituent. The concentration of ribavirin that reduced the anti-HIV-1 activity of AZT was 200-fold higher than that of AZT (Vogt et al., 1987). Even for ddI, a 2-fold higher concentration of ribavirin was required to enhance the inhibitory effect of ddI (Hartman et al., 1991). These results suggest that, although some interaction may exist between each component, the anti-HIV-1 activity of heterodimers is primarily attributed to the activity of the more potent component.

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