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Synergistic Inhibition of Human Immunodeficiency Virus Type 1 Replication by 5-Ethyl-1-Ethoxymethyl-6-(Phenylthio)Uracil (E-EPU) and Azidothymidine In Vitro

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A novel 6-substituted acyclouridine derivative, 5-ethyl-1-ethoxymethyl-6-(phenylthio)uracil (E-EPU), has recently proved to be a highly potent and selective inhibitor of human immunodeficiency virus type 1 (HIV-1) in vitro. Combinations of 3′-azido-2′,3′-dideoxythymidine (AZT) and E-EPU synergistically inhibit the replication of HIV-1 in MT-4 cells, whereas the cytotoxic effects of AZT and E-EPU on mock-infected MT-4 cells are not enhanced by the drug combination. Synergistic inhibition of HIV-1 replication has also been observed in peripheral blood lymphocytes. These results indicate that the combination of AZT and E-EPU should be further pursued in the treatment of AIDS.

A large number of compounds have been reported as promising candidates for the treatment of AIDS (7, 17). Treatment with 3-azido-2′,3′-dideoxythymidine (AZT), a potent and selective inhibitor of the replication of human immunodeficiency virus type 1 (HIV-1) in vitro (16), leads to an improvement in the clinical symptoms and prolongation of life of AIDS patients (8, 9), but long-term treatment with AZT is often limited by its serious side effects, such as bone marrow suppression (21) or the emergence of drug-resistant variants of HIV-1 (14, 15).

Combination chemotherapy is an attractive approach, since it may lead to synergistic activity without increasing toxicity. Moreover, the emergence of drug-resistant virus strains may also be suppressed by combination chemotherapy. In fact, combinations of AZT with various anti-HIV-1 agents have been examined for their inhibitory effects on HIV-1 replication in vitro. These include, among others, human alpha interferon (11), granulocyte macrophage colony-stimulating factor (10), dextran sulfate (26), and recombinant soluble CD4 (13). In this study, we have evaluated the combination of AZT and a novel 6-substituted nucleoside derivative, 5-ethyl-1-ethoxymethyl-6-(phenylthio)uracil (E-EPU) (Fig. 1), for its inhibitory effect on HIV-1 replication in vitro. E-EPU has recently been reported to be a highly potent and specific inhibitor of HIV-1 (2).

MATERIALS AND METHODS

Compounds. E-EPU was prepared as described previously (2, 24). AZT was synthesized at the Rega Institute.

Cells and viruses. MT-4 (19) and peripheral blood lymphocyte (PBL) cells were used for the anti-HIV-1 assays. MT-4 cells were grown and maintained in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS), 100 U of penicillin G per ml, and 100 μg of streptomycin per ml. PBL cells were obtained from healthy donors. PBL cells were stimulated with phytohemagglutinin and cultured with RPMI 1640 medium containing 20% FBS, antibiotics, and interleukin 2. Two HIV-1 strains were used for the anti-HIV-1 assays. HTLV-III_B was obtained from the culture supernatant of MOLT-4 cells persistently infected with virus. HIV-1_{HE} is a clinical isolate from a Belgian AIDS patient. HIV-1_{HE} was propagated in MT-4 cells. Titers of HIV-1 stocks were determined in MT-4 cells, and virus stocks were stored at −80°C until use.

Antiviral assays. Anti-HIV-1 activity measurements were based on the inhibition of virus-induced cytopathogenicity in MT-4 cells. Briefly, MT-4 cells were suspended in a culture medium at 10^5 cells per ml and infected with HIV-1 at a multiplicity of infection of 0.02. Immediately after virus infection, the cell suspension (100 μl) was brought into each well of a flat-bottom microtiter tray containing various concentrations of the test compounds. After a 4-day incubation at 37°C, the number of viable MT-4 cells was determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide method (20).

The assay procedure for measuring the anti-HIV-1 activity of the compounds in PBL cells was based on the quantitative detection of HIV-1 p24 antigen in the cell culture supernatant by using a sandwich enzyme-linked immunosorbent assay kit (Abbott), as previously described (2). Phytohemagglutinin-stimulated PBL cells (10^6/ml) were infected with HIV-1 at a multiplicity of infection of 0.2. After virus adsorption for 2 h, the cells were washed three times with culture medium to remove unadsorbed virus particles and incubated at 37°C in the presence of various concentrations of the test compounds. On day 4 after virus infection, the cells were subcultured at a ratio of 1:5 with fresh culture medium containing appropriate concentrations of the test compounds. The assay (HIV-1 p24 antigen detection) was performed on day 7 after virus infection.

Cytotoxicity of the compounds was evaluated in parallel with their antiviral effects. Cytotoxicity measurements were based on the viability of mock-infected cells as determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide method.

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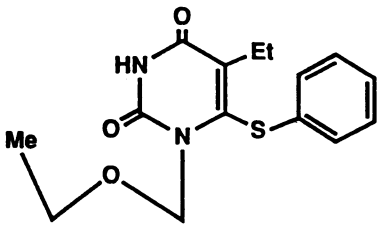

FIG. 1. Formula of E-EPU.

Synergy calculations. The multiple-drug effect was evaluated by the median-effect principle and the isobologram method (6). The analysis was carried out with a microcomputer using computer software established by Chou and Chou (5). The details of this method have been described elsewhere (10–13, 22). The interaction between AZT and E-EPU was determined by calculating the combination index (CI). CIs of <1, 1, and >1 indicate synergism, summation (additive effect), and antagonism, respectively.

RESULTS

With both AZT alone and E-EPU alone, a concentration-dependent inhibition of HIV-1 (HTLV-III_B) cytopathogenicity in MT-4 cells was observed on day 4 after virus infection (Table 1). The 50% antivirally effective concentration (EC_{50}) of AZT was 2.0 nM, and the EC_{50} of E-EPU was 22 nM. When the combined inhibitory effects of AZT and E-EPU were analyzed by the isobologram method, it was clear that the two compounds exerted a synergistic activity against HIV-1 (Fig. 2A). Table 2 lists the CIs of different mixtures of AZT and E-EPU. Since the median-effect plots indicated that AZT and E-EPU gave parallel lines but the mixtures of the compounds did not (data not shown), the CIs were calculated under mutually nonexclusive assumptions (5). Synergy was more evident (smaller CIs) when EC_{70}s or EC_{90}s were used as the calculation end points (Table 2). Furthermore, synergistic inhibition was also detected for the replication of a HIV-1 clinical isolate (HIV-1_{HE}) in MT-4 cells. The EC_{50} of AZT alone, E-EPU alone, and the 1:32 mixture of AZT and E-EPU were 5.0, 141, and 37 nM, respectively (Table 3). When EC_{50} was used as the calculation end point, the CI was 0.54 (Table 3).

No inhibitory effect on viability or proliferation of mock-infected MT-4 cells was observed with any drug combination used in the anti-HIV-1 assays (data not shown). When the combined effects of AZT and E-EPU on the viability of mock-infected MT-4 cells was evaluated at higher compound concentrations, the combination resulted in antagonism of cytotoxicity (Fig. 2B). The CIs based on the 50% cytotoxic concentrations ranged from 1.3 to 1.8. Again, these results indicate that cytotoxicity is diminished rather than enhanced following combination of the compounds.

When the inhibitory effects of AZT alone, E-EPU alone, and the combination of AZT and E-EPU at a ratio of 1:16 on HIV-1 (HTLV-III_B) replication in PBL cells were examined, the EC_{50}s of AZT alone and E-EPU alone were 1.2 and 41 nM, respectively (Table 4). The EC_{50} of the 1:16 mixture of

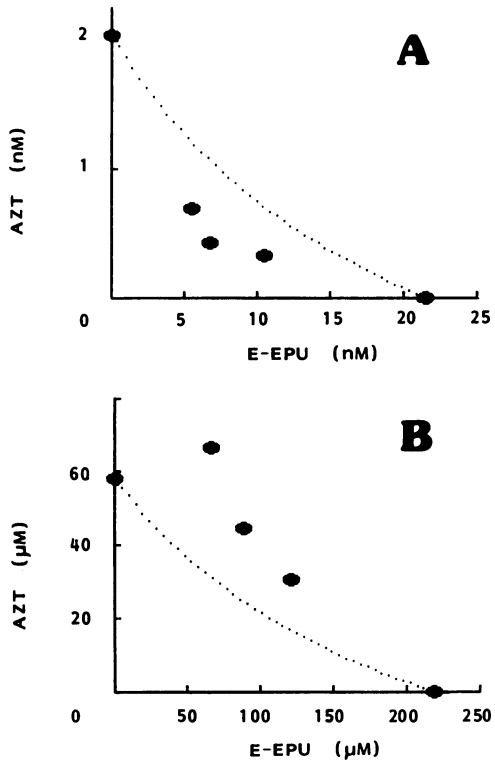

FIG. 2. Computer-generated isobolograms of the combined inhibitory effects of AZT and E-EPU on HIV-1 (HTLV-III_B) replication (A) and cell viability (B) in MT-4 cells. HIV-1-infected (A) and mock-infected (B) MT-4 cells were cultured in the presence of various concentrations of the test compounds. After a 4-day incubation, the number of viable cells was determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide method. The data were analyzed with a microcomputer, as described in Materials and Methods. EC_{50}s (A) and 50% cytotoxic concentrations (B) were used as the end points for calculations. Dotted lines represent the unity lines for CIs equal to 1 under mutually nonexclusive assumptions.

TABLE 1. Effects of AZT and E-EPU on HIV-1 (HTLV-III_B) cytopathogenicity in MT-4 cells

AZT (nM)	% Inhibition[a] with the following concn of E-EPU (nM):					
	0	4	8	16	32	64
0	0	7 ± 1	14 ± 4	24 ± 5	42 ± 8	77 ± 7
0.25	9 ± 3	24 ± 4	28 ± 3	48 ± 4	74 ± 2	100
0.5	20 ± 7	33 ± 5	49 ± 5	68 ± 3	97 ± 4	100
1	39 ± 11	56 ± 10	71 ± 6	93 ± 7	100	100
2	64 ± 10	83 ± 7	90 ± 12	100	100	100
4	82 ± 9	100	100	100	100	100

[a] Percent inhibition was determined as previously described (20). Data represent mean values ± standard deviations for four separate experiments.

TABLE 2. CIs for AZT and E-EPU in MT-4 cells infected with HIV-1 (HTLV-III_B)

AZT-E-EPU[a]	CI[b]		
	50	70	90
1:8	0.68	0.43	0.21
1:16	0.58	0.41	0.23
1:32	0.73	0.47	0.24

[a] Concentration ratio in mixture.

[b] CIs giving 50, 70, or 90% inhibition of HIV-1 (HTLV-III_B) cytopathogenicity in MT-4 cells. CIs were determined under mutually nonexclusive assumptions.

TABLE 3. Effects of AZT and E-EPU on HIV-1 (HIV-1_{HE}) cytopathogenicity in MT-4 cells

Concn (nM)		% Inhibition of HIV-1 cytopathogenicity (CI)[a]		
AZT	E-EPU	AZT alone	E-EPU alone	Mixture
0	0	0	0	0
0.25	8	2	2	8
0.5	16	8	4	25
1	32	11	10	53
2	64	21	23	69
4	128	44	51	85
EC_{50} (nM)		5.0	141	37 (0.54)
EC_{70} (nM)		9.9	255	66 (0.50)
EC_{90} (nM)		29	656	162 (0.45)

[a] Data represent mean values for two separate experiments. CIs were determined under mutually nonexclusive assumptions.

AZT and E-EPU was 9.8 nM (Table 4). When EC_{50}, EC_{70}, and EC_{90} were used as the calculation end points, the CIs were 0.80, 0.63, and 0.45, respectively (Table 4). These results again indicate a synergistic activity of AZT and E-EPU against HIV-1 replication in PBL cells. None of the drug combinations affected the viability of PBL cells up to the highest concentrations examined (data not shown).

DISCUSSION

After the discovery of 1-[(2-hydroxyethoxy)methyl]-6-(phenylthio)thymine (HEPT) as a novel HIV-1 inhibitor (4, 18), we have synthesized and examined a variety of HEPT derivatives for their inhibitory effects on HIV-1 replication in vitro (1, 2, 23–25). Among these derivatives, E-EPU proved to be a highly potent and selective inhibitor of HIV-1 (2). The EC_{50} of E-EPU for HIV-1 in MT-4 cells (22 nM) is approximately 300-fold lower than that of HEPT (6.5 μM) (2). Furthermore, we have found that E-EPU interacts specifically with HIV-1 reverse transcriptase according to a mechanism which is different from that of AZT and other 2′,3′-dideoxynucleosides (2).

Although the combination of AZT with ribavirin results in an antagonistic effect on HIV-1 replication (3, 27), the combinations of antiviral agents with different modes of action generally lead to increased activity. Therefore, we considered the combination of AZT and E-EPU worthy of evaluation. The present study clearly indicates that this combination results in a synergistic inhibition of HIV-1 replication in both MT-4 and PBL cell cultures. A potential advantage of combination chemotherapy over single-agent chemotherapy is that it may reduce the emergence of drug-resistant virus mutants. This possibility has not been examined in the present study; however, E-EPU is equally inhibitory to AZT-resistant and AZT-susceptible variants of HIV-1 (2).

TABLE 4. Effects of AZT and E-EPU on HIV-1 (HTLV-III_B) p24 antigen in the supernatant of PBL cell cultures

Concn (nM)		% Inhibition of p24 (CI)[a]		
AZT	E-EPU	AZT alone	E-EPU alone	Mixture
0	0	0	0	0
0.25	4	2	11	31
0.5	8	8	16	36
1	16	45	26	57
2	32	68	44	85
4	64	85	63	99
EC_{50} (nM)		1.2	41	9.8 (0.80)
EC_{70} (nM)		2.1	99	15 (0.63)
EC_{90} (nM)		5.2	400	31 (0.45)

[a] Data represent mean values for two separate experiments. CIs were determined under mutually nonexclusive assumptions.

It is also noteworthy that E-EPU, unlike AZT, has little effect on the proliferation of bone marrow progenitor cells in several animal models (unpublished data). No inhibition of the in vitro proliferation of murine bone marrow cells was observed with E-EPU at concentrations of up to 10 μM, whereas AZT inhibited 95% of the proliferation at this concentration (data not shown). The bone marrow suppression is the most serious side effect of AZT during the treatment of AIDS patients (21). Combination chemotherapy of AZT with E-EPU would allow the use of lower doses of both compounds, thereby minimizing their toxic side effects. In fact, from our in vitro data it appears that the cytotoxicities of AZT and E-EPU are reduced when combined.

In conclusion, in view of the severity of the AIDS pandemic and the urgent need for an effective and nontoxic therapy, the combination of AZT and E-EPU should be further pursued in the treatment of HIV-1 infections.

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