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Synergistic inhibition of human immunodeficiency virus type 1 (HIV-1) replication in vitro by 1-[(2-hydroxyethoxy)methyl]-6-phenylthiothymine (HEPT) and recombinant alpha interferon

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Summary

1-[(2-Hydroxyethoxy)methyl]-6-(phenylthio)thymine (HEPT) has recently proved to be a potent and selective inhibitor of human immunodeficiency virus type 1 (HIV-1) in vitro. Combinations of HEPT and recombinant alpha interferon (IFN-α) synergistically inhibit the replication of HIV-1 in MT-4 cells at non-toxic concentrations. Synergistic inhibition of HIV-1 replication has also been observed in peripheral blood lymphocytes. These results indicate that the combination of HEPT with IFN-α should be further pursued in the treatment of retrovirus infections [i e. acquired immune deficiency syndrome (AIDS)].

IFN-α; AIDS; Synergistic inhibition

Introduction

Since the identification of human immunodeficiency virus type 1 (HIV-1) as the etiologic agent of the acquired immune deficiency syndrome (AIDS), various com-

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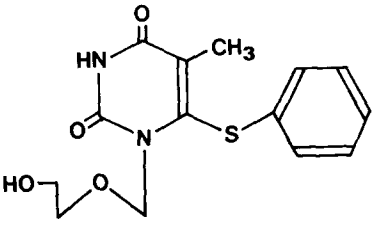

Fig 1 Structural formula of HEPT

pounds have been reported to inhibit the replication of HIV-1 in vitro, as recently reviewed by De Clercq (1987, 1988, 1989, 1990) 3-Azido-3′-deoxythymidine (AZT) is the only drug that has so far been licensed for clinical use in patients with AIDS or AIDS-related complex (ARC). A double-blind placebo-controlled clinical trial has demonstrated that AZT treatment leads to a significant reduction in the mortality rate at short term (Fischl et al., 1987) Although the long-term use of AZT is often limited by serious side effects (i e myelosuppression) (Richman et al., 1987), recent clinical trials have shown that reduced daily doses of AZT are as effective as the standard dose (1500 mg per day) but are less toxic (Collier et al , 1990; Fischl et al., 1990). Another factor that may hamper the long-term use of AZT is the appearance of AZT-resistant HIV-1 isolates (Larder et al , 1989) Combination chemotherapy is an attractive approach in the control of infectious diseases, as it may lead to a synergistic effect between the compounds without increasing their toxicity In addition, the emergence of drug-resistant virus strains may also be reduced.

Combinations of antiviral agents with interferons have been evaluated for their inhibitory effects on the replication of herpesviruses and found to be synergistic (Baba et al., 1984; Moran et al , 1985). Hartshorn et al. (1986, 1987) have shown that the combinations of recombinant alpha interferon (IFN-α) with either phosphonoformate or AZT give synergistic inhibition of HIV-1 replication in peripheral blood lymphocytes (PBLs). We have recently demonstrated that 1-[(2-hydroxyethoxy)methyl]-6-(phenylthio)thymine (HEPT) (Fig. 1) is a potent and selective inhibitor of HIV-1 replication in various T4 cell cultures including PBL (Baba et al., 1989; Miyasaka et al , 1989) In this study, we have evaluated the combination of HEPT and IFN-α against HIV-1 replication in vitro

Materials and Methods

Compounds

Human recombinant IFN-α 2a ($2\,6 \times 10^8$ IU/mg) was kindly provided by Takeda Pharmaceutical Co., Osaka, Japan. Human recombinant interleukin 2 (IL-2) (5×10^3 U/μg) was purchased from Genzyme Corp., Boston, MA. HEPT was prepared according to the procedure described previously (Miyasaka et al , 1989) The other

compounds used in this study were purchased from Sigma Chemical Co., St. Louis, MO.

Cells and virus

MT-4 cells and PBLs were used in the anti-HIV-1 assays. MT-4 cells were grown and maintained in RPMI 1640 medium (Nissui Pharmaceutical Co., Tokyo, Japan) supplemented with 10% heat-inactivated fetal calf serum (FCS) (Cell Culture Lab., Cleveland, OH), 100 IU/ml penicillin G, and 100 μg/ml streptomycin. PBLs were obtained from healthy HIV-seronegative donors. PBLs were stimulated with 1% phytohemagglutinin (DIFCO Lab., Detroit, MI) and cultured with RPMI 1640 medium containing 20% FCS, antibiotics, and IL-2 (40 U/ml) HIV-1 (HTLV-III$_B$ strain) was obtained from the culture supernatant of MOLT-4 cells persistently infected with the virus (MOLT-4/HTLV-III$_B$) HIV-1 stocks were titrated in MT-4 cells and stored at −80°C until used

Antiviral assays

The anti-HIV-1 assay in MT-4 cells was based on the inhibition of virus-induced cytopathogenicity. In Expt 1, MT-4 cells were incubated in the presence of various concentrations of IFN-α After a 24-h incubation period, MT-4 cells were infected with cell-free HIV-1 at a multiplicity of infection (MOI) of 0.02 and suspended in culture medium at 1×10^5 cells/ml One hundred μl of the cell suspension was brought into each well of a flat-bottomed microtiter tray containing various concentrations of HEPT. After a 4-day incubation period at 37°C, the number of viable cells was determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) method (Pauwels et al., 1988). In Expt. 2, MT-4 cells were not pretreated with IFN-α but exposed to both IFN-α and HEPT at the same time (i e. immediately after virus infection).

The assay procedure for measuring the anti-HIV-1 activity of the compounds in PBLs was based on the quantitative detection of HIV-1 p24 antigen in the cell culture supernatant by a sandwich ELISA (Abbott Lab., IL). PBLs were infected with the virus at an MOI of 0.2 After a 2-h virus adsorption period, the cells were washed three times with culture medium to remove unadsorbed virus particles and suspended in culture medium at 1×10^5 cells/ml. One hundred μl of the cell suspension was brought into each well of a microtiter tray and incubated in the presence of various concentrations of IFN-α and HEPT. The assay was performed on day 5 after virus infection. In all experiments, serial $10^{0.5}$-fold dilutions of either a fixed combination of IFN-α and HEPT or each compound alone were examined

Cytotoxicity of the compounds was evaluated in parallel with their antiviral activity. It was based on the viability of mock-infected MT-4 cells and PBLs as determined by the MTT method.

Synergy calculations

The multiple-drug effect was evaluated by the median-effect principle and the isobologram method (Chou and Talalay, 1984) The analysis was carried out with a microcomputer using the computer software established by Chou and Chou (1986) The details on this method have been described elsewhere (Hartshorn et al , 1987; Johnson et al , 1989, Hayashi et al , 1989, Smith et al , 1989) The interaction between IFN-α and HEPT was determined by calculating the combination index (CI). CI values of <1, =1, and >1 indicate synergism, summation (additive effect), and antagonism, respectively

Results

When IFN-α was added to the cells 24 h before virus infection (Expt. 1), a concentration-dependent inhibition of HIV-1-induced cytopathogenicity was observed on day 4 after virus infection (Table 1) Its 50% antiviral effective concentration (EC_{50}) was 0.37 U/ml. When IFN-α was added to MT-4 cells immediately after virus infection (Expt. 2), approximately 25-fold higher concentrations of IFN-α were required to achieve an equivalent inhibition of viral cytopathogenicity as in Expt 1 (Table 1). The EC_{50} of IFN-α in Expt 2 was 9 5 U/ml. HEPT alone also proved inhibitory to HIV-1 replication in a concentration-dependent fashion (Table 1). The EC_{50} of HEPT alone was 4.6 μM (Expt. 1) and 8.7 μM (Expt. 2). No reduction of the viability of mock-infected MT-4 cells was detected up to the highest

TABLE 1

Effect of IFN-α and HEPT on HIV-1-induced cytopathogenicity in MT-4 cells

IFN-α (U/ml)	Percent inhibition of HIV–1 cytopathogenicity[a]					
	HEPT (μM)	0	0 316	1	3 16	10	31 6
Experiment 1							
0		0	4	9	25	78	94
0 0316		4	7	10	38	80	100
0 1		7	18	22	42	87	99
0 316		51	74	81	100	100	99
1		83	100	100	100	100	89
3 16		95	100	100	100	100	79[b]
Experiment 2							
0		0	3	6	17	64	81
0 316		3	6	9	25	72	91
1		4	10	21	45	76	87
3 16		21	29	44	64	86	94
10		56	66	81	82	93	88
31 6		80	88	100	97	88	87

[a]Percent inhibition was determined, as previously described (Pauwels et al 1988)

[b]For this combination cell proliferation was slightly suppressed, whereas no virus-induced cytopathogenicity was observed All data represent mean values for triplicate experiments

TABLE 2

Combination index for treatment of HIV-1 replication in MT-4 and PBL cell cultures

Treatment[a]	CI		
	0 50[b]	0 70[b]	0 90[b]
MT-4 (Exp 1)	0 33	0 33	0 33
MT-4 (Exp 2)	0 68	0 68	0 67
PBL	0 93	0 63	0 36

[a]Treatment consisted of combination of HEPT and IFN-α, as described in Materials and Methods
[b]CI (combination index) values giving 50, 70, or 90% reduction of HIV-1-induced cytopathogenicity in MT-4 cells or HIV-1 p24 antigen in culture supernatant of PBLs CI values were determined under mutually nonexclusive assumptions

concentration examined in these experiments (data not shown).

When we looked at the combined inhibitory effects of IFN-α and HEPT on HIV-1 replication in MT-4 cells, the combination exerted synergistic antiviral activity against HIV-1 in both Expts. 1 and 2 (Table 1). Since the median-effect plots for IFN-α, HEPT and their combinations could not be judged as parallel to each other in MT-4 cells (data not shown), the CIs were calculated under mutually nonexclusive assumptions (i.e., when different modes of action are assumed) (Chou and Chou, 1986). The CIs of Expts. 1 and 2 giving a 50% reduction of HIV-1-induced cytopathogenicity (EC_{50}) were 0.33 and 0.68, respectively (Table 2). Furthermore, these CIs were almost constant irrespective of the endpoints used for their calculations (i.e., 50, 70, or 90% reduction of cytopathogenicity) (Table 2). These results indicate that the combination of IFN-α and HEPT produced a synergistic effect. No inhibitory effect on viability or proliferation of mock-infected MT-4 cells was observed with any drug combination. There were no anti-proliferative or cytotoxic effects of IFN-α or HEPT on mock-infected MT-4 cells at the concentrations used in Expts. 1 and 2 (data not shown)

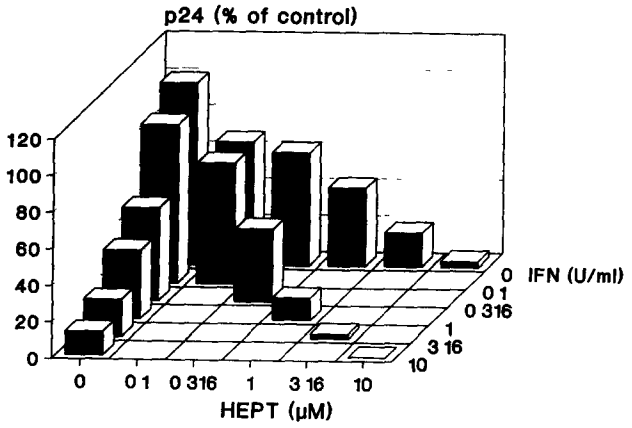

Fig 2 Effect of IFN-α and HEPT, alone and combined, on HIV-1 p24 antigen production in PBL cultures PBLs were infected with HIV-1 and cultured for 5 days The amount of HIV-1 p24 antigen in culture supernatant was determined by a sandwich ELISA technique and expressed as percent of virus-infected control

Fig 2 shows the effect of IFN-α and HEPT on HIV-1 p24 antigen in culture supernatant of PBLs In this experiment, the EC_{50} values of IFN-α alone and HEPT alone were 0 62 U/ml and 0 41 μM, respectively (data not shown) The CIs giving 50, 70, and 90% reduction of HIV-1 p24 antigen were 0.93, 0 63, and 0 36, respectively (Table 2) These results again indicate a synergistic effect of IFN-α and HEPT on HIV-1 replication in PBLs Neither IFN-α alone nor HEPT alone or their combinations affected the viability of PBLs up to the highest concentrations examined (data not shown)

Discussion

HEPT is a 6-substituted acyclouridine derivative which demonstrates marked activity against HIV-1 (Baba et al , 1989; Miyasaka et al., 1989) HEPT is only effective against HIV-1 Other retroviruses including HIV-2 are not susceptible to the compound (Baba et al , 1989) HEPT is a less potent inhibitor of HIV-1 replication in vitro than AZT, however, it is also less toxic to the host cells (Baba et al , 1989) Our recent studies on derivatives of HEPT have indicated that the anti-HIV-1 activity of HEPT in vitro and its pharmacokinetic behavior in vivo can be improved by structural modifications of the parent compound (Baba et al , 1990) Unlike AZT, the HEPT derivatives have little effect on the proliferation of bone-marrow progenitor cells in several animal models (Umezu et al , personal communication) HEPT and its derivatives can thus be considered as promising candidates for treatment of HIV-1 infections

Although the mode of action of HEPT has not been fully elucidated, it appears that HEPT may interfere with HIV-1 reverse transcriptase without being converted to HEPT triphosphate (Baba et al , unpublished data). The mode of action of IFN-α against HIV-1 replication is also obscure, yet it is assumed to affect a late stage of the virus replicative cycle (i e , virus assembly or release) (Hartshorn et al , 1987, Pitha et al , 1982) As mentioned in the Introduction, combinations of antiviral agents with different modes of action can lead to increased activity and reduced toxicity, and diminish the emergence of drug-resistant virus mutants In this perspective we evaluated the combined inhibitory effects of HEPT and IFN-α on HIV-1 replication Our results clearly indicate that the combination of IFN-α and HEPT confers a synergistic inhibitory effect on HIV-1 replication in MT-4 and PBL cell cultures

In terms of clinical efficacy, IFN-α treatment has proven to decrease tumor size and tumor number in patients with AIDS-related Kaposi sarcoma (de Wit et al , 1988, Lane et al , 1988). More recently, a randomized, placebo-controlled trial with IFN-α in asymptomatic HIV-1 carriers has demonstrated that IFN-α treatment may decrease the frequency of virus isolation, although the use of IFN-α alone is often accompanied by dose-dependent drug toxicity, i e. flu-like symptoms (Lane et al , 1990) Combined therapy of IFN-α with AZT seems effective in AIDS patients with Kaposi sarcoma (Krown et al., 1990)

IFN-α, but not AZT, is able to suppress HIV-1 expression in chronically

infected cell lines (Poli et al., 1989) This mechanism of action is clearly different from that of HEPT which is assumed to interfere with the reverse transcription process in acutely infected cells. HEPT and IFN-α thus interact at entirely different levels of the HIV-1 replicative cycle. Our present observations suggest that the combination of IFN-α and HEPT should be further pursued in the treatment of HIV-1 infections.

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