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INHIBITION OF HUMAN IMMUNODEFICIENCY VIRUS TYPE 1 REPLICATION BY PHOSPHONOFORMATE ESTERS OF 3'-AZIDO-3'-DEOXYTHYMIDINE

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SUMMARY: The water-soluble ammonium salt of 3'-azido-5'-(O-ethoxycarbonylphosphinyl)-3'-deoxythymidine (ECP-AZT), the prototype of a novel class of compounds incorporating two active antiretroviral agents, in this case 3'-azido-3'-deoxythymidine (AZT) and phosphonoformic acid (PFA), within the same structure, was synthesized and tested as an inhibitor of the replication of human immunodeficiency virus type 1 (HIV-1) in Jurkat cells, a CD4+ human T-lymphocyte cell line. The corresponding 5'-(O-methoxycarbonylphosphinyl) derivative (MCP-AZT) was also prepared. The rationale for the synthesis of ECP-AZT and MCP-AZT was that they may be cleaved intracellularly to AZT and PFA via hydrolysis of the phosphate ester bond or to AZT 5'-monophosphate by oxidative cleavage of the carbon-phosphorus bond. ECP-AZT was found to block viral replication at a 50% inhibitory concentration (IC_{50}) of ca. 10^{-6} M as measured by reverse transcriptase (RT) activity in supernatants from cultures of infected cells. Little or no inhibition of cell growth was observed at this concentration, and there was <20% inhibition of cell growth at 10^{-4} M. AZT itself was a more potent inhibitor of HIV-1 replication than ECP-AZT, but was also more cytotoxic. The antiviral selectivity of ECP-AZT, defined as the ratio IC_{50}(virus inhibition)/IC_{50}(cell growth inhibition), was in the range considered to be therapeutic for anti-AIDS nucleosides.

Despite intensive and continuing efforts in many laboratories, the most effective drugs presently available against the human immunodeficiency virus type 1 (HIV-1) remain those directed against reverse transcriptase (RT) (1). The enzyme, carried into susceptible host T-lymphocytes and monocyte/macrophages by the virus itself, is responsible for the production of DNA copies of the viral genome, a required first step in the infectious lifecycle of all retroviruses, including HIV-1. The most prominent class of RT inhibitors are 2',3'-deoxynucleosides, of which the first to show promising anti-HIV-1 activity was 3'-azido-3'-deoxythymidine (**1**, AZT) (2). This compound is metabolized in cells to AZT 5'-triphosphate (AZTTP) (3), a potent inhibitor of RT. The therapeutic selectivity of AZT resides in the fact that AZTTP has a lower K_i for the viral enzyme than for host DNA polymerase α, which is also inhibited to some degree (4,5). Since AZT lacks a 3'-OH group, it cannot be incorporated into DNA except as a chain terminator. While AZTTP is a substrate analogue, another agent with demonstrated anti-HIV-1 activity (6,7), the sodium salt of phosphonoformic acid (**2**, PFA, foscarnet), is considered a *product analogue* because of its resemblance to pyrophosphate (PP_i) (8). Since substrate and PP_i analogues are likely to occupy adjacent but non-identical binding sites on the RT-template complex, as indicated by site-directed mutagenesis data (9), there is a potential for synergistic interaction between AZT (or other nucleosides) and PFA even though they share a common enzyme target.

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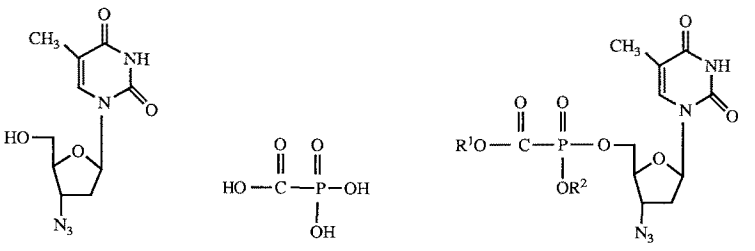

1 (AZT) **2** (PFA) **3**

Figure 1. Structures of 3'-azido-3'-dideoxythymidine (AZT, **1**), phosphonoformic acid (PFA, **2**), and phosphonoformate esters of AZT (**3**).

Two groups of workers recently investigated the action of combinations of AZT and PFA in human peripheral blood monocyte/macrophages (PBMs) (10) and T-lymphocytes (H9 cells) (11) infected with HIV-1. Excess PFA (as much as 4000-fold with PBMs and 160-fold with H9 lymphocytes) was used in the viral replication assays because of the slower uptake into cells of PFA versus AZT and the weaker binding to RT of PFA versus AZTTP (10). Multiple drug median dose effect analysis (12) showed that inhibition of HIV-1 replication in PBMs (10) as well as T-lymphocytes (11) by combinations of AZT and an excess of PFA was synergistic, whereas the toxicity of these combinations to host cells was not. Inhibition of partially purified HIV-1 RT in a cell-free assay by combinations of AZTTP and PFA ranging from 1:20 to 1:200 was reported to be only additive (10). However, our own data using purified recombinant HIV-1 RT demonstrated synergism between AZTTP and PFA when the two inhibitors were used at ratios of 1:25 or 1:13, respectively (R.M. Ruprecht et al., unpublished observations).

Though we were aware that AZT and excess PFA were synergistic in intact cells when given in combination (10,11), it occurred to us that a single molecule consisting of *covalently linked AZT and PFA* might also be active. An AZT-PFA conjugate consisting of a molecule of PFA stably linked to the 5'-OH group of AZT might inhibit RT as effectively, or perhaps more effectively, than separate molecules of AZTTP and PFA. On the other hand, depending on the nature of the linkage, an AZT-PFA conjugate could perhaps also undergo cleavage to AZT and PFA inside the cell, thereby serving as a prodrug. Moreover, regardless of whether the PFA were

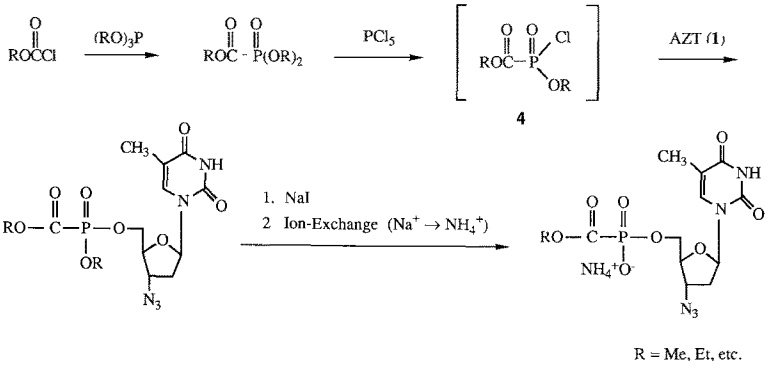

Figure 2. Possible intracellular cleavage pathways for PFA esters of AZT.

linked to AZT through the COOH or $PO(OH)_2$ group, decreased negative charge of this molecule relative to PFA ought to facilitate passage across the cell membrane. Finally, if the conjugate consisted of PFA linked directly to AZT through the $PO(OH)_2$ group, it could serve as an AZT 5'-monophosphate (as opposed to AZT) prodrug via an oxidative decarboxylation reaction as shown in Figure 1. According to this mechanism, the role of the PFA would simply be that of masked phosphate group. On the basis of these considerations, we began a program of synthesis and biological evaluation of AZT-PFA conjugates of general structure **3** (13), in which it would be possible in principle to modulate lipophilicity by varying R^1 and R^2. A synthetic route permitting access to a large variety of derivatives of this type is presented in Figure 2.

MATERIALS AND METHODS

Synthesis of 5'-(O-Alkoxycarbonylphosphinyl) Derivatives of AZT. Treatment of trimethyl phosphonoformate with phosphorous pentachloride at 50°C (17), followed by direct addition of AZT at low temperature, without isolation of the putative intermediate **4**, afforded the triester **3** ($R^1 = R^2 =$ Me) in 35% overall yield. Reaction of the triester with sodium iodide in tetrahydrofuran (18) at room temperature for 3 h resulted in selective loss of the methyl group on the phosphonate oxygen, giving the monosodium salt **3** (R^1 = Me, R^2 = Na). Purification of this product on DEAE-cellulose column using 0.05 M ammonium bicarbonate as the eluent led to isolation of the ammonium salt **3** (R^1 = Me, $R^2 = NH_4$) (MCP-AZT). The structure of **3** ($R^1 = R^2$ = Me) was evident from its ^1H NMR spectrum, which contained a three-proton singlet at δ 3.8 (C-OCH_3) and a six-proton doublet at δ 3.9 (J = 10 Hz, P-OCH_3) in addition to the usual resonances for the sugar and base of the AZT moiety. The selectivity of the sodium iodide reaction for the phosphonate methyl was revealed by the spectrum of the demethylated product, which contained only a three-proton singlet at δ 3.7 (C-OCH_3). The triester was much more lipophilic than the sodium salt of the diester. The triester could be readily chromatographed on silica gel with 98:2 CH_2Cl_2-MeOH as the eluent, whereas purification of the diester salt required a C_{18} reversed phase column and acetonitrile as the eluent. A large difference in mobility between the triester and diester was likewise observed by thin-layer chromatography. The diethyl ester **3** ($R^1 = R^2$ = Et) and monoethyl ester **3** (R^1 = Et, $R^2 = NH_4$) were prepared by the same methods as **3** ($R^1 = R^2$ = Me) and **3** (R^1 = Me, $R^2 = NH_4$), except that (a) a higher temperature and longer time had to be used for the reaction of carbon tetrachloride with triethyl phosphonoformate (85°C, 3 h) than trimethyl phosphonoformate (50°C, 1.5 h), and (b) a longer time had to be used to cleave the diethyl ester (20 h) than the dimethyl ester (3 h) with sodium iodide. The ^1H-NMR spectrum (D_2O, 300 MHz) of **3** (R^1 = Et, $R^2 = NH_4$) was consistent with retention of the ethyl group on the phosphorus oxygen: δ 1.2 (t, 3H, C-OCH_2CH_3), 1.8 (s, 3H, 5-CH_3), 2.4 (m, 1H, $C_{2'}$-H), 3.95 (m, 1H, $C_{3'}$-H), 4.2 (m, 4H, $C_{5'}$-H), C-OCH_2CH_3), 4.4 (m, 1H, $C_{4'}$-H), 6.2 (t, 1H, $C_{1'}$-H), 7.6 (s, 1H, 6-H). The ammonium salt of the monoethyl ester could be converted to the acid by passage through an ion-exchange column.

Cells and Virus. Human Jurkat cells (CD4+ T-lymphocyte cell line) were grown in RPMI-1640 medium supplemented with 10% heat inactivated fetal calf serum, penicillin-streptomycin, and L-glutamine. HIV-1 was used as a cell-free supernatant from persistently infected Jurkat cells originally transfected with the molecular HIV-1 clone pHXBc2 (19). Stocks were standardized by RT activity using poly$(A)_n$·oligo(dT) as previously described (20).

Assay for Anti-HIV-1 Activity. Non-infected Jurkat cells were plated at a density of $1x10^5$ cells per well in six-well culture dishes in 2 mL of media. One hundred μL of HIV-1 stock solution (20,000 cpm of RT units) was then added. Drug was added 4 h after the virus, at the same time as the virus, or 4 h before the virus. After several days of incubation in the continuous presence of drug, 1 mL of supernatant from each well was harvested and assayed for RT activity. Cell growth was determined by staining parallel uninfected cultures with trypan blue.

RESULTS AND DISCUSSION

ECP-AZT was of particular interest because the length of the side chain in this compound is approximately the same as that of the triphosphate moiety in AZTTP. However, since a large

variety of methyl alkoxycarbonylphosphonates can be prepared from alkyl chloroformates and trimethyl phosphite via an Arbuzov reaction, the route given in Figure 3 provides access to a series of carboxylate esters of different chain length and lipophilicity. Moreover, since methyl chloroformate can be condensed with trialkyl phosphites other than trimethyl, selective deblocking of the carboxyl moiety potentially leads to a series of products with alkyl groups on the phosphonyl rather than the carboxyl oxygen. Lastly, it should be noted that these methods have been found to be applicable with antiretroviral 2',3'-dideoxynucleosides other than AZT (J. Saha and A. Rosowsky, unpublished results).

ECP-AZT was tested first for its ability to inhibit HIV-1 replication in human $CD4^+$ T-lymphocytes (Jurkat cells) (21). The RT activity present in supernatants from infected cells was used as a measure of viral replication, and drug was added to the cultures at the same time as the virus or 4 h post-infection. Parallel cytotoxicity assays were performed in non-infected Jurkat cells to assess therapeutic selectivity. As shown in Figure 4, cell growth was decreased by less than 10% at drug concentrations of up to almost 10^{-3} M. In contrast, 50% inhibition of RT activity relative to non-treated HIV-infected cells was observed at a concentration in the 10^{-7}-10^{-6} M range. This showed the compound to be both active and selective when added to cells at the same time as the virus or 4 h later.

The ability of ECP-AZT to inhibit viral replication was also assessed in Jurkat cells with drug being added 4 h prior to infection. Again, the IC_{50} for viral replication was approximately 10^{-6} M, with essentially no effect on the growth of non-infected cells at concentrations of up to 10^{-4} M (Fig. 5A). Virus replication in the presence of 10^{-4} M ECP-AZT was virtually complete. By comparison, AZT under identical conditions led to ca. 90% inhibition of viral replication at 10^{-7} M, but there was 20% inhibition of cell growth at 10^{-6} M and 40% inhibition at 10^{-4} M (Fig. 5B). ECP-AZT was therefore less potent than AZT, but was also less toxic to the host cells. Viral replication also appears to be inhibited by ECP-AZT to a similar degree when the drug is given 4 h prior to viral infection (Fig. 5A) as it is when the drug is added to cultures at the same time as the virus or 4 h after the virus (Fig. 4). Since the IC_{50} of ECP-AZT for cell growth inhibition is very likely to be $>10^{-3}$ M (Figs. 4 and 5A), the selectivity index for this compound, as defined by the ratio IC_{50}(virus inhibition)/IC_{50}(cell growth inhibition), must be at least 1000. This value is in the range considered appropriate for candidate anti-HIV-1 nucleoside analogues.

To examine whether RT inhibition by ECP-AZT could by itself account for the antiretroviral effect in intact cells, we tested the effect of the compound on RT activity in a cell-free assay and

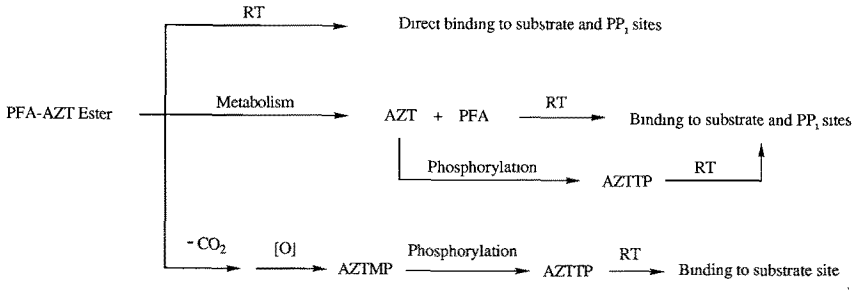

Figure 3. Synthesis of PFA esters of AZT.

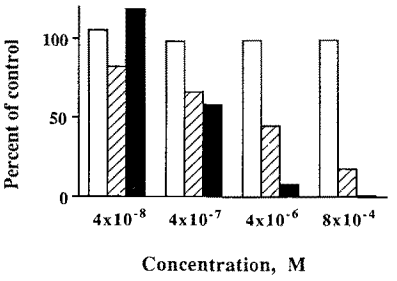

Figure 4. HIV-1 replication in Jurkat cells cells treated with ECP-AZT at the same time as the addition of virus (shaded bars) or 4 h after the addition of virus (solid bars). Growth of uninfected cells (open bars) in the presence of the indicated concentrations of ECP-AZT is also shown. The RT assays were performed after culturing the cells for 11 days in the presence of drug. Results are expressed as a percent of control (no drug).

obtained an IC_{50} value (>10^{-4} M) at least 100-fold greater than the IC_{50} for viral replication. Therefore, it appears that metabolism is required in the action of ECP-AZT or that the drug has one or more targets other than RT in the cell. Additional biochemical and pharmacological studies are planned to address these issues.

The reason for the lower potency of ECP-AZT as both an antiretroviral agent and as a cell growth inhibitory agent may reflect slower uptake into cells, as well as slow metabolism via the various possible routes shown in Figure 3. It has been reported that AZT differs from natural ribonucleosides and deoxyribonucleosides in that it enters cells by non-facilitated diffusion (22). Compounds such as ECP-AZT, in which a negative charge resides on the phosphorus oxygen, might be taken up less efficiently than AZT by this route. To evaluate the possibility that our initial choice of an ethyl group was less than optimal for uptake and metabolism, other 5'-O-(alkoxy-carbonylphosphinyl) derivatives of AZT, as well as those of related dideoxynucleosides, are being synthesized and tested as inhibitors of the replication of HIV-1 and other retroviruses in $CD4^+$ cells (23). Since it is also possible that the uptake of compounds such as ECP-AZT is dependent on the

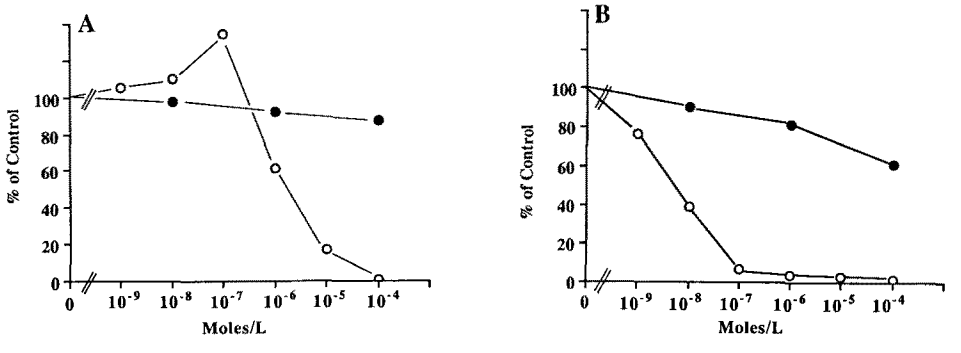

Figure 5. HIV-1 replication in Jurkat cells treated with ECP-AZT (Panel A) or AZT (Panel B) 4 h prior to the addition of virus as measured by RT activity in the supernatant. RT assays were performed after culturing the cells in the continuous presence of drug for 7 days. Results are expressed as a percent of control (no drug). Open circles: RT activity; solid circles: growth of non-infected cells.

type of cell infected with virus, the activity of these compounds is also being assessed in freshly isolated blood leukocytes and in purified monocyte/macrophages.

In summary, we have developed a straightforward three-step synthesis of ECP-AZT, a novel compound that may be viewed as the prototype of a new class of 5'-blocked antiviral dideoxynucleosides in which the blocking group itself has antiviral activity. ECP-AZT inhibits HIV-1 replication in cultured human T-lymphocytes, and is less cytotoxic than AZT. In view of the side effects observed in AIDS patients on prolonged treatment with AZT (24), PFA-dideoxynucleoside conjugates related to ECP-AZT are attractive candidates for evaluation of *in vivo* antiretroviral activity.

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