0026-895X/92/030441-05$03.00/0

MOLECULAR PHARMACOLOGY, 41:441–445

ACCELERATED COMMUNICATION

Membrane-Permeable Dideoxyuridine 5′-Monophosphate Analogue Inhibits Human Immunodeficiency Virus Infection

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Received August 13, 1991; Accepted December 2, 1991

SUMMARY

2′,3′-Dideoxyuridine (ddU) is ineffective at controlling human immunodeficiency virus type 1 (HIV-1) infection in human T cells, because it is not biotransformed to the active 5′-triphosphate. The metabolic block resides in the poor substrate affinity of ddU for cellular nucleoside kinases. This problem cannot be overcome by supplying the preformed nucleotides, because such compounds are unable to penetrate cells. To circumvent the requirement of ddU for enzymic phosphorylation, we have prepared bis(pivaloyloxymethyl) 2′,3′-dideoxyuridine 5′-monophosphate (piv_2 ddUMP), as a potential membrane-permeable prodrug of ddUMP, and investigated its metabolism and anti-HIV activity in two human T cell lines, one with wild-type thymidine kinase activity (MT-4) and the other deficient in thymidine kinase activity (CEM-tk$^-$). The 5′-mono-, di-, and triphosphates of ddU were formed in both cell lines after exposure to piv_2-ddUMP. In contrast, phosphorylated metabolites were not observed in cells treated with ddU or ddUMP alone. piv_2-ddUMP also reduced the cytopathic effects of HIV-1 in MT-4 cells (ED_{50}, 4.75 μM) and inhibited virus production in culture fluid (ED_{50}, 20 μM). In addition, piv_2-ddUMP protected CEM-tk$^-$ cells from HIV-1 infection, as demonstrated by inhibition of intracellular p24 antigen levels (ED_{50}, 3 μM) and reverse transcriptase activity in culture medium (ED_{50}, 2.5 μM). Based on these findings, we propose that the "masked nucleotide" strategy may make available for development nucleoside analogues hitherto considered inactive because of failure to undergo biotransformation to the corresponding 5′-monophosphates. Moreover, by circumventing metabolic dependency on nucleoside kinases, the strategy may overcome acquired resistance to nucleoside analogues caused by the loss or depletion of nucleoside kinases.

Among the various structural classes of antiviral agents investigated for the treatment of HIV-1 infections, 2′,3′-deoxynucleosides are among the most effective. One such compound, 3′-azido-2′,3′-deoxythymidine (azidothymidine), prolongs the life and reduces the morbidity of patients with AIDS and AIDS-related complex (1–3). Anti-HIV dideoxynucleosides are not independently active. To exert biological activity, they require biotransformation by cellular kinases, first to the 5′-monophosphates and then to the corresponding diphosphates and triphosphates (4, 5). The 5′-triphosphates are potent and specific inhibitors of HIV reverse transcriptase and interfere with the incorporation of endogenous 2′-deoxynucleotides into viral DNA. In addition, the 5′-triphosphates can be incorporated into viral DNA, resulting in termination of nascent DNA chain growth.

Although dideoxynucleosides offer great promise for the treatment of patients with HIV infection, several shortcomings limit realization of the full therapeutic potential of these agents. One problem is the failure or limited ability of certain dideoxynucleosides to undergo biotransformation to the active 5′-triphosphates in target cells. For example, ddUTP is a powerful and selective inhibitor of HIV reverse transcriptase (K_i = 0.05 μM) (6–8). Yet the parent nucleoside, ddU, is completely ineffective at blocking HIV infection of cells in culture (8). Biochemical pharmacological studies in three different human T cell lines (CEM, ATH8, and MOLT-4) showed that ddU was not anabolized to the 5′-monophosphate, apparently because it was a poor substrate for cellular nucleoside kinases (8). This finding is not unexpected, because uridine kinase and thymidine kinase, the enzymes that normally phosphorylate uridine analogues, show considerable substrate specificity. Thus, uri-

This investigation was supported in part by National Institutes of Health Grants AI 28213 (to D.F.) and AI 29308 (to R.B.A.) and by seed funds from the University Cancer Foundation at The University of Texas M. D. Anderson Cancer Center (to D.F. and R.B.A.).

ABBREVIATIONS: HIV(−1), human immunodeficiency virus (type 1); ddU, 2′,3′-dideoxyuridine; ddUMP, 2′,3′-dideoxyuridine 5′-monophosphate; ddUDP, 2′,3′-dideoxyuridine 5′-diphosphate; ddUTP, 2′,3′-dideoxyuridine 5′-triphosphate; piv_2, bis(pivaloyloxymethyl); MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolinium bromide; AIDS, acquired immunodeficiency syndrome.

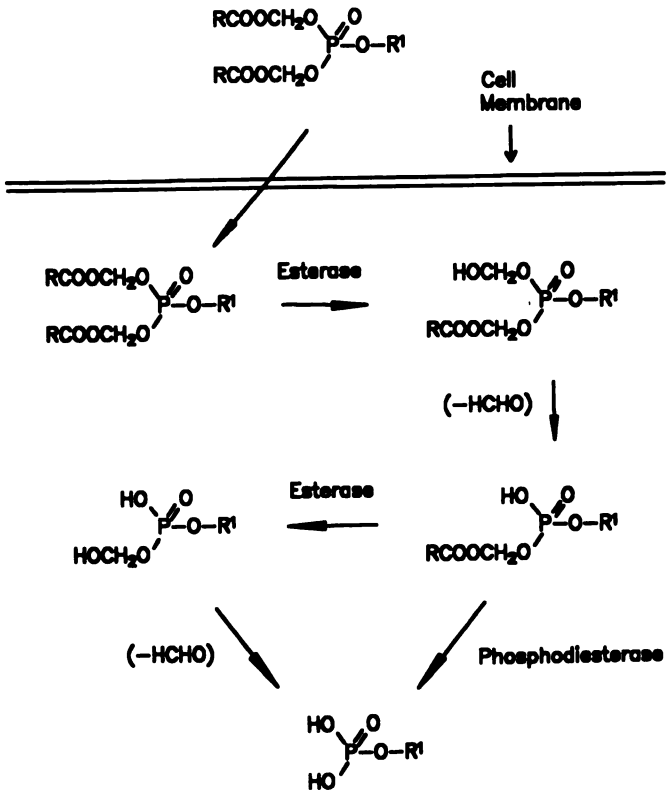

Fig. 1. Schematic representation of the transport of piv$_2$-dideoxynucleoside 5′-monophosphates into cells and their subsequent biotransformation to the corresponding dideoxynucleoside 5′-monophosphates.

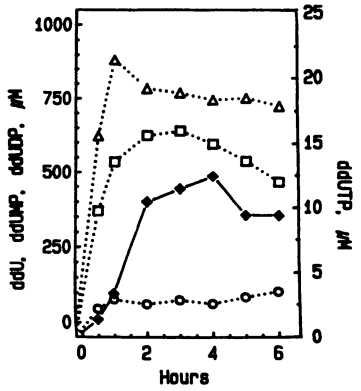

Fig. 2. piv$_2$ ddUMP is metabolized to ddUMP, ddUDP, and ddUTP in MT-4 cells. Exponentially growing cells were incubated with 20 μM [2′,3′-^{3}H]piv$_2$ ddUMP (specific activity, 2.1 mCi/mmol) for the indicated times, and intracellular pools of ddU metabolites were determined as described in Materials and Methods. The concentration of each metabolite was calculated by reference to drug specific activity, the number of cell equivalents analyzed, and the mean cell volume of the cells in each sample. ○, ddU; △, ddUMP; □, ddUDP; ♦, ddUTP.

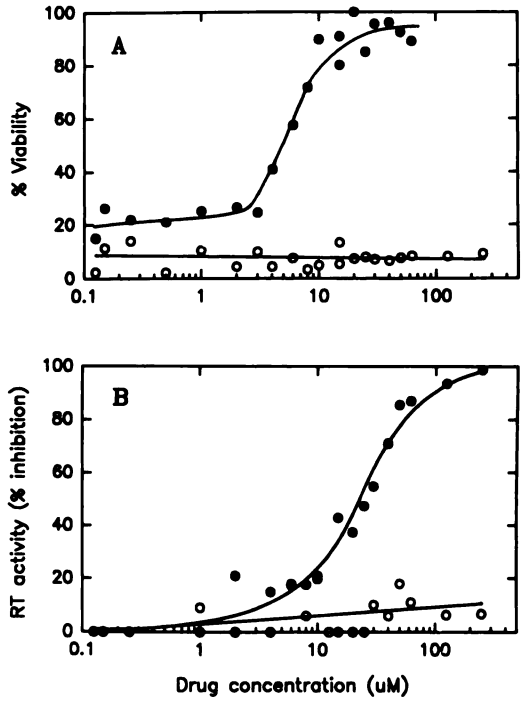

Fig. 3. piv$_2$ ddUMP (●) but not ddU (○) inhibits the cytopathic effect of HIV-1 in MT-4 cells and blocks virus production. A, Inhibition of cytopathic effects. MT-4 cells (5×10^4/100 μl) were preincubated in microtiter plates with various drug concentrations, for 2 hr at 37°, and then challenged with HIV-1 (600 cpm of reverse transcriptase activity/well). Controls included cells alone and cells infected with HIV-1 without added drugs. On day 7 after infection, the total number of viable cells was determined using the MTT dye reduction assay, as described by Larder *et al.* (20). B, Inhibition of reverse transcriptase (*RT*) activity. The experimental conditions were the same as described for A. On day 7 after infection, reverse transcriptase activity was determined in the culture medium by the method of Popovic *et al. (23).*

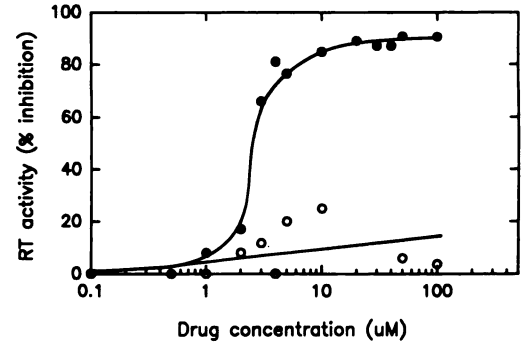

Fig. 4. piv$_2$ ddUMP (●) but not ddU (○) inhibits virus replication in HIV-1-infected CEM-tk$^-$ cells. CEM-tk$^-$ cells (1×10^5 cells/well) were exposed in 48-well plates to HIV-1 (600 cpm of reverse transcriptase activity/well), in the presence or absence of drugs, as described in Fig. 3. On days 2 and 4 after infection complete medium (with appropriate concentration of drug) was replenished, and on day 7 after infection reverse transcriptase (*RT*) activity was measured in the culture fluid by the method of Popovic *et al.* (23).

dine kinase is specific for ribofuranosyl nucleosides (9, 10), whereas thymidine kinase efficiently phosphorylates only nucleoside analogues that closely resemble the natural substrate (11–13).

The inability of ddU to undergo phosphorylation by cellular kinases cannot be overcome by administering the corresponding 5′-mononucleotide (ddUMP). Nucleotides are unable to permeate lipid cell membranes (14) because of their ionic character and low lipophilicity. Moreover, they are susceptible to rapid degradation in tissues by nucleotidases and nonspecific phosphatases.

To promote the entry of nucleoside 5′-monophosphates into cells, we have developed a "masked nucleotide" strategy (15–

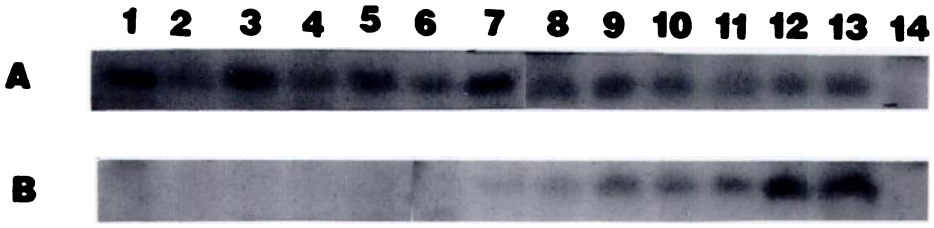

Fig. 5. piv_2 ddUMP but not ddU inhibits p24 gag antigen expression in HIV-1-infected CEM-tk$^-$ cells. Western blot analysis was performed on extracts from CEM-tk$^-$ cells treated as described in the legend to Fig. 4. Both ddU (A) and piv_2 ddUMP (B) were used at concentrations of 100, 50, 40, 30, 20, 10, 5, 4, 3, 2, 1, and 0.5 μg/ml (*lanes 1–12*). *Lanes 13* and *14*, HIV-1-infected and control CEM-tk$^-$ cells, representing positive and negative controls for p24 antigen, respectively. Cell extracts (25-μl aliquots) from control and drug-treated cultures were electrophoresed on a 10% polyacrylamide gel, transferred to nitrocellulose, and reacted with a 1/1000 dilution of serum derived from a patient with AIDS.

17). In our approach, the nucleoside 5′-monophosphates are converted into neutral lipophilic phosphotriesters, using phosphate-masking groups. The phosphotriesters are designed to penetrate cells by passive diffusion and then revert to the nucleoside 5′-monophosphates after cleavage of the masking groups by cellular carboxylate esterases (Fig. 1). Once liberated, the nucleoside 5′-monophosphates can be anabolized to the corresponding di- and triphosphates by cellular kinases.

To assess the feasibility of this approach for the development of anti-HIV dideoxynucleotides, we prepared piv_2 ddUMP, as a potential "masked" precursor of ddUMP, and determined its ability to inhibit HIV-1 replication in two different human T cell lines.

Materials and Methods

Cells and virus. MT-4 cells are human T cells that are infected by human T cell leukemia virus type 1 and undergo lytic infection with HIV (18–20). CEM-tk$^-$ cells are human T cells that are deficient in thymidine kinase activity. MT-4 and CEM-tk$^-$ cells were obtained through the AIDS Research and Reference Reagent Program of the National Institute of Allergic and Infectious Diseases, National Institutes of Health. Both cell lines were grown in RPMI 1640 medium supplemented with glutamine and 10% fetal calf serum. The HIV-1 studied was the IIIB strain, obtained from the culture supernatant of the H9 human T cell line infected with the virus. Persistently HIV-1-infected H9 cells were obtained from the Frederick Cancer Research Facility of the National Cancer Institute (Frederick, MD).

Drugs. ddU was purchased from Sigma Chemical Co. (St. Louis, MO). piv_2 ddUMP was synthesized by condensing ddU with piv_2 phosphate, in the presence of triphenylphosphine and diethyl azadicarboxylate (21).[1] ddUMP was prepared from ddU by the general method of Yoshikawa *et al.* (22). The radiolabeled analogues [2′,3′-^{3}H]piv_2-ddUMP and [2′,3′-^{3}H]ddUMP were prepared similarly, starting with [2′,3′-^{3}H]ddU (Moravek Biochemicals, Inc., Brea, CA). For antiviral and cytotoxicity assays, the drugs were serially diluted in serum-free RPMI 1640 medium, and 50 μl of each concentration were added to each well of a 96-well microculture plate.

Cellular metabolism of piv_2 ddUMP. Exponentially growing MT-4 cells were incubated with 20 μM [2′,3′-^{3}H]piv_2 ddUMP (specific activity, 2.1 mCi/mmol) for various time periods, and then 2×10^7 cells were removed, washed, and extracted overnight with 60% methanol at −40°. After centrifugation, the supernatant was dried under vacuum and reconstituted in phosphate-buffered saline. The metabolites were separated by anion exchange high performance liquid chromatography. The eluate was collected as 2-min fractions, into scintillation vials containing 10 ml of Safety-Solve (Research Products International Corp., Mount Prospect, IL), and radioactivity was quantitated in a Tricarb model 1900CA liquid scintillation analyzer (Packard Instrument Co., Downers Grove, IL). The concentration of each metabolite was calculated from the drug specific activity, the number of cell equivalents analyzed, and the mean cell volume of the cells in each sample. Similar studies were conducted with [2′,3′-^{3}H]ddU (10 mCi/mmol) and [2′,3′-^{3}H]ddUMP (10.7 mCi/mmol) as controls.

Antiviral assay. The anti-HIV activities of piv_2-ddUMP and the parent compound ddU were assessed by measuring their inhibitory effects on virus-induced cytopathogenicity in MT-4 cells, monitored by the MTT dye reduction method, as described previously (20). The cytotoxicity of the compounds was also monitored by the MTT assay (20).

Reverse transcriptase assay. The reverse transcriptase activity in culture supernatants of HIV-1-infected cells was assayed according to the method described by Popovic *et al.* (23). Briefly, the culture supernatant from each well of triplicate wells was pooled and clarified at 4000 rpm for 10 min in a GP-R centrifuge (Beckman Instruments, Fullerton, CA), using a GH-3.7 horizontal rotor. Virus particles were precipitated from 2 ml of the culture supernatant with 30% (w/v) polyethylene glycol (Carbowax 8000) and 4 M NaCl, at 4° overnight. The following day, the virus precipitate was centrifuged at 3000 rpm for 30 min at 4°, and the pellet was resuspended in 100 μl of 50% (by volume) glycerol containing 25 mM Tris·HCl, pH 7.5, 5 mM dithiothreitol, 150 mM KCl, and 0.5% Triton X-100. A 10-μl aliquot of the enzyme preparation was assayed for reverse transcriptase activity, using poly(rA)-poly$(dT)_{12-18}$ (0.05 unit/ml final concentration) as the substrate and [^{3}H]TTP (10 μCi). The reverse transcriptase activity was expressed as cpm/ml of culture medium.

Western blot analysis. Intracellular extracts from CEM-tk$^-$ cells infected with HIV-1 and treated with various concentrations of drugs for 7 days were collected by lysis with 0.125 M Tris·HCl, pH 6.8, containing 2% sodium dodecyl sulfate, 10% 2-mercaptoethanol, 10% glycerol, and 0.02% bromophenol blue. Control extracts consisted of cells infected with HIV-1 but not treated with drugs and cells not infected with HIV-1. A 25-μl aliquot of the cell extract from each sample was subjected to electrophoresis on a 10% polyacrylamide gel, and the proteins were transferred to nitrocellulose paper. HIV-1-specific proteins were detected using serum from a patient with AIDS as the antibody source.

Results

Metabolism of piv_2 ddUMP in MT-4 cells. To assess the feasibility of the masked nucleotide approach for the development of anti-HIV dideoxynucleotides, we determined the ability of piv_2 ddUMP to give rise to ddU nucleotides in MT-4 cells (Fig. 2). After a 1-hr incubation with 20 μM piv_2-ddUMP, intracellular levels of ddUMP and ddUDP were in excess of 500 μM, a concentrative effect typical of therapeutic nucleosides. The concentration of ddUTP exceeded 10 μM, more than

[1] D. Farquhar *et al.* Manuscript in preparation.

200-fold greater than the K_i for HIV reverse transcriptase *in vitro* (8). In contrast, MT-4 cells exposed to ddU or ddUMP failed to accumulate detectable levels of nucleotide (<0.05 μM) (data not shown). These findings demonstrate that the masked nucleotide strategy is capable of delivering potentially inhibitory concentrations of ddUTP into cells that otherwise would not accumulate the active nucleotide.

Anti-HIV activity of piv$_2$ ddUMP in MT-4 cells. The ability of ddU and piv$_2$ ddUMP to protect MT-4 cells from the cytopathic effects of HIV-1 was investigated by the MTT dye reduction assay (Fig. 3A). ddU was ineffective at all concentrations tested. However, piv$_2$ ddUMP afforded 50% protection (ED_{50}) at 4.75 μM and 90% protection at 10 μM with no observable cytotoxicity. The effects of ddU and piv$_2$ ddUMP on live virus production were determined by measuring reverse transcriptase activity in the culture media from HIV-infected MT-4 cells grown in the presence and absence of the drugs (Fig. 3B). Consistent with the cytopathogenicity data, piv$_2$ ddUMP inhibited reverse transcriptase by 50% at 20 μM, whereas ddU was without effect. These findings are in accord with the report of Hao *et al.* (8) that ddU exhibited poor, if any, anti-HIV activity in CEM, ATH8, and MOLT-4 cells but that ddUTP was a potent and selective inhibitor of HIV reverse transcriptase *in vitro*. The toxic effects of ddU and piv$_2$ ddUMP on MT-4 cells were also determined using the MTT drug reduction assay. piv$_2$ ddUMP was toxic to the cells at concentrations of 100 μM and above (data not shown). ddU, on the other hand, was not toxic at concentrations up to 500 μM, consistent with its failure to undergo metabolic activation.

Anti-HIV activity of piv$_2$-ddUMP in CEM-tk$^-$ cells. We also studied the metabolism of piv$_2$ ddUMP in CEM cells deficient in thymidine kinase (CEM-tk$^-$). piv$_2$ ddUMP was readily taken up by these cells and converted to ddUMP, ddUDP, and ddUTP (data not shown). In addition, piv$_2$-ddUMP protected CEM-tk$^-$ cells from HIV-1 infection, as evidenced by the inhibition of reverse transcriptase activity in culture supernatants (Fig. 4) (ED_{50}, 2.5 μM) and the suppression of intracellular p24 antigen levels (Fig. 5) (ED_{50}, 3 μM).

Discussion

ddU is ineffective at controlling HIV-1 infection because it is unable to undergo biotransformation to the active triphosphate within target cells (8). In the present investigation, we have prepared piv$_2$ ddUMP, a masked nucleotide, and demonstrated its anti-HIV activity in two human T cell lines, one with wild-type thymidine kinase activity (MT-4) and the other deficient in thymidine kinase activity (CEM-tk$^-$). Moreover, we have shown that piv$_2$ ddUMP, unlike ddU and ddUMP, gives rise to ddUMP, ddUDP, and ddUTP in both cell lines.

These findings have several implications for anti-HIV drug development. In particular, a mechanism is now at hand to overcome resistance to dideoxynucleosides arising from the deletion of activating kinases in cells permissive for virus replication. Although this resistance mechanism has not yet been reported for anti-HIV dideoxynucleosides, experience with anticancer nucleosides suggests that it may become a significant problem in the therapy of patients with AIDS. Moreover, because most anti-HIV dideoxynucleosides are phosphorylated by either thymidine kinase or deoxycytidine kinase, resistance to one agent will likely lead to cross-resistance to many others (24). The masked nucleotide strategy should circumvent this problem.

Another potential application of the strategy is to alter favorably the ratio of dideoxynucleotides to endogenous nucleotides in infected cells; this balance is a critical determinant of the effectiveness of anti-HIV dideoxynucleosides. Thus, many dideoxynucleosides are comparatively poor substrates for thymidine kinase and deoxycytidine kinase, and their phosphorylation is strongly antagonized by the natural enzyme substrates. These same kinases are also subject to potent feedback inhibition by endogenous nucleotides. The masked nucleotide approach for the direct delivery of dideoxynucleotide into target cells is not susceptible to such regulatory constraints and should lead to a more favorable ratio of dideoxynucleotides to nucleotides.

Finally, the masked nucleotide approach should facilitate the development of anti-HIV dideoxynucleosides hitherto considered inactive because of inherently poor substrate affinity for nucleoside kinases.

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