ELSEVIER

Antiviral Research 50 (2001) 129–137

Antiviral Research

www.elsevier.com/locate/antiviral

Cellular metabolism in lymphocytes of a novel thioether–phospholipid–AZT conjugate with anti-HIV-1 activity

Gregory L. Kucera [a,*], Christy L. Goff [a], Nathan Iyer [a], Susan Morris-Natschke [b], Khalid S. Ishaq [b], Steven D. Wyrick [b], Ronald A. Fleming [a,1], Louis S. Kucera [a]

[a] *Wake Forest University School of Medicine, Medical Center Boulevard, Winston-Salem, NC 27157, USA*
[b] *School of Pharmacy, University of North Carolina, Chapel Hill, NC 27599, USA*

Received 19 December 2000; accepted 19 February 2001

Abstract

We previously synthesized a thioetherphospholipid–AZT conjugate (3′-azido-3′-deoxy-5′-(1-hexadecylthio-2-methoxypropyl)-phosphothymidine, CP-102) with potent anti-HIV-1 activity and significant reduction in cell cytotoxicity compared to AZT alone. To study the cellular metabolism of the conjugate compound we synthesized a double-tritium-labeled thioetherphospholipid-AZT conjugate (3′-azido-3′-deoxy-5′-(1-[9, 10-^{3}H]-*S*-octadecylthio-2-*O*-methoxypropyl)-phosphothymidine-[methyl-^{3}H], [^{3}H]CP-102). The intracellular radioactive metabolic products of [^{3}H]CP-102 treated human lymphoblastoid CEM-SS cells were analyzed by HPLC and thin-layer chromatography. Results of this investigation provide evidence that a putative intracellular lipid cleavage enzyme metabolizes [^{3}H]CP-102 to form a thioetherdiglyceride compound that migrates with an authentic 1-*S*-octadecyl-2-*O*-methyl-thioglycerol standard on TLC. The thioetherdiglyceride metabolite did not react with the ninhydrin reagent indicating it did not contain a primary amine such as that found on serine or ethanolamine containing phospholipids. Also, the product did not contain a phosphatidic acid group based on migration characteristics in the TLC plate. The other major hydrophilic metabolite was 3′-azido-3′-deoxythymidine-[methyl-^{3}H]-monophosphate (AZT–MP) with lesser amounts of AZT, AZT–DP and AZT–TP. In summary, the best interpretation of these data is that the thioether-phospholipid–AZT conjugate, [^{3}H]CP-102, is cleaved by a putative intracellular lipid cleavage enzyme to release a thioetherdiglyceride compound and AZT–MP. The resulting AZT–MP was either dephosphorylated to AZT or sequentially phosphorylated to AZT–DP and, ultimately, to AZT–TP, the known inhibitory metabolite against

Abbreviations: AIDS, acquired immunodeficiency syndrome; AZT, 3′-azido-3′-deoxythymidine; AZT-DP, 3′-azido-3′-deoxythymidine diphosphate; AZT-MP, 3′-azido-3′-deoxythymidine monophosphate; AZT-TP, 3′-azido-3′-deoxythymidine triphosphate; HIV, human immunodeficiency virus; HPLC, high pressure liquid chromatography; PBS, phosphate buffered saline; TBAP, tetrabutylammonium dihydrogen phosphate.

* Corresponding author. Fax: +336-7160255.
E-mail address: gkucera@wfubmc.edu (G.L. Kucera).
[1] Present address: GlaxoSmithKline, Inc., Research Triangle Park, NC 27109, USA.

0166-3542/01/$ - see front matter © 2001 Elsevier Science B.V. All rights reserved.
PII: S0166-3542(01)00137-1

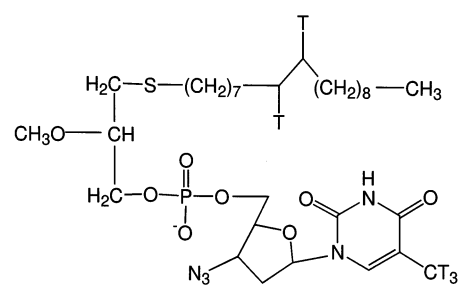
HIV-1 reverse transcriptase. Phospholipid–nucleoside conjugates may provide a unique approach for developing anti-HIV-1 prodrugs that do not have a strict requirement for a nucleoside kinase for initial activation of the prodrug to an antiviral form. © 2001 Elsevier Science B.V. All rights reserved.

Keywords: Cellular metabolism; Lymphocytes of a novel thioether–phospholipid–AZT conjugate; Anti-HIV-1 activity

1. Introduction

Acquired immunodeficiency syndrome (AIDS[1]) is a worldwide illness with epidemic proportions. Many of the compounds approved for use in the treatment of HIV-1 infections belong to a class of compounds known as 2′,3′-dideoxynucleoside analogs e.g. zidovudine, AZT, (Mitsuya et al., 1985), didanosine, ddI (Dahlberg et al., 1987), zalcitabine, ddC (Mitsuya and Broder, 1986) 2′,3′-didehydro-3′-deoxythymidine, stavudine, D4T (Hamamoto et al., 1987; Lin et al., 1987), and lamivudine, 3TC (Coates et al., 1992; Schinazi et al., 1992). Other RT inhibitors include the guanosine analog (1*R*, 4*S*,)-4-[2-amino-6-(cyclopropyl amino)-9H-purin-9-yl]-2-cyclo-pentene-1-methanol, abacavir (Daluge et al., 1997), and non-nucleoside RT inhibitors include Nevirapine, Loviride, Efavirenz and TIBO-compounds (Crowe, 1999; De Clercq, 1997). Although these inhibitors offer some degree of protection against HIV replication, they all share undesirable side effects and induce the emergence of drug resistant mutants (Arts and Wainberg, 1996).

We have developed a series of novel etherphospholipid–nucleoside analog conjugates with markedly reduced cellular cytotoxicity and improved selectivity for anti-HIV-1 activity compared to the nucleoside analog alone (Herrmann et al., 1996; Piantadosi et al., 1991). CP-102, a thioetherphospholipid–AZT conjugate, has been shown to markedly inhibit HIV replication (Piantadosi et al., 1991). In addition, alkylphospholipid and alkylphospholipid–AZT conjugates were also shown to inhibit HIV-1 induced pathogenesis measured by inhibition of cell fusion between treated and untreated infected CEM-SS cells, and inhibition of virus induced gp160/gp120 reactivity on the surface of infected cells as detected with monoclonal antibodies (Krugner-Higby et al., 1995). So far, the metabolism of the thioetherphospholipid conjugate at the biochemical level has not been reported. Accordingly, we synthesized a double-tritium-labeled thioetherphospholipid–AZT conjugate, 3′-azido-3′-deoxy-5′-(1-[9, 10-^{3}H]-octadecylthio-2-methoxypropyl)-phosphothymidine-[methyl-^{3}H] ([^{3}H]CP-102, Fig. 1) and investigated the metabolic products following treatment of human T-lymphoblasts (CEM-SS cells). The best interpretation of the results suggested that the thioetherphospholipid–AZT conjugate, [^{3}H]CP-102, is metabolized by a putative lipid cleavage enzyme to release a thioetherdiglyceride compound and AZT–MP. The AZT–MP is further metabolized to AZT or AZT–DP and, ultimately, to AZT–TP, the known inhibitor of reverse transcriptase activity.



[^{3}H]CP-102

Fig. 1. The chemical structure of [^{3}H]CP-102, a thioetherphospholipid–AZT conjugate. T indicates the presence of the tritium.

2. Materials and methods

2.1. Chemicals

Unless otherwise noted all chemicals were of reagent grade or better from Sigma Chemical Co. (St. Louis, MO). HPLC solvents were HPLC quality and were obtained from Fisher Scientific (Fair Lawn, NJ). AZT–MP, AZT–DP, and AZT–TP were from Glaxo SmithKline, Inc. (Formerly Burroughs Wellcome Inc., Research Triangle Park, NC).

2.2. Cells

Human T lymphoblasts (CEM-SS cells) were maintained in logarithmic growth using RPMI 1640 medium and 20% fetal bovine serum (growth medium) as previously described (Kucera et al., 1990; Piantadosi et al., 1991). The cells were grown at 37°C in T-flasks, spinner flasks, or roller bottles. The doubling time of the cells was between 18 and 24 h. The cells were free of mycoplasma contamination as determined by the PCR test. The growth medium used for the spinner flask and roller bottles was supplemented with 10 mM HEPES to maintain the medium at pH 7.0 while the cells were cultured outside a CO_2 incubator.

2.3. Synthesis of tritiated thioetherphospholipid–AZT conjugate

2.3.1. Synthesis of [9,10-^{3}H]-1-S-octadecyl-2-O-methyl-thioglycero-3-phosphatidic acid (**1**) (Piantadosi et al., 1991)

Δ9,10-1-*S*-octadecyl-2-*O*-methyl-thioglycero-3-phosphatidic acid was prepared by reaction of Δ9,10-1-*S*-octadecyl-2-*O*-methyl-thioglycerol with $POCl_3/Et_3N$ in THF (NMR data were consistent with those previously reported by Piantadosi et al., 1991). A solution of 12.0 g (0.026 mmol) of the phosphatidic acid in absolute ethanol (1.0 ml) was stirred at room temperature over 10 mg of 10% Pd/C under 5.0 Ci (0.086 mmol) of carrier-free tritium gas for 4 h. The catalyst was filtered off through a Celite pipet column and the filtrate blown down nearly to dryness under a stream of nitrogen to afford 956 mCi of crude product. A portion (143 mCi) was removed and purified by silica gel G TLC using CH_2Cl_2–MeOH–H_20 (70:30:4 v/v/v) to afford 32 mCi of approximately 95% radiochemically pure product.

2.3.2. Synthesis of 3′-azido-3′-deoxy-5′-([9,10-^{3}H]-3-octadecylthio-2-methoxypropyl)-phosphothymidine-[methyl-^{3}H]

An aliquot of **1** was diluted to a specific activity of 15 mCi/mmol with cold phosphatidic acid. Likewise, an aliquot of tritiated AZT was diluted to a specific activity of 15 mCi/mmol with cold AZT. The two solutions were mixed and evaporated to complete dryness. Dicyclohexylcarbodiimide (230 mg, 1.12 mmol) was added and the mixture dissolved in 4 ml of dry pyridine and stirred at room temperature for 3 days. The volatiles were removed in vacuo and the residue was chromatographed on a silica gel column (3 g) using CH_2C1_2–MeOH (15:1, 9:1, 2:1 v/v) as mobile phase to afford 0.86 mCi of $>95\%$ radiochemically pure product. The structural formula of [^{3}H]CP-102 is shown in Fig. 1.

2.4. Metabolism of [^{3}H]CP-102 in CEM-SS cells

2.4.1. Short term incubation

For short term incubations ($\leq$ 24 h), 3×10^8 CEM-SS cells from an exponentially growing culture were harvested and incubated at 37°C for 6–24 h in 100 ml of growth medium with 70–167 nM of labeled [^{3}H]CP-102 conjugate (specific activity 30 mCi/mmol). This concentration of CP-102 resulted in an approximate EC_{90} value for inhibition of virus replication (Piantadosi et al., 1991). The cells were harvested by centrifugation ($450 \times g$, 10 min) and washed three times with 50 ml PBS. The washed cells were resuspended in 1 ml PBS for subsequent extraction. Approximately 50% of the added radiolabel was associated with the cells.

2.4.2. Long term incubation

For long term incubations CEM-SS cells were kept at exponentially growing densities in roller

bottle cultures (between 5×10^5 and 2×10^6 cells/ml) at 37°C for 2–11 days. The concentration of [^{3}H]CP-102 was maintained at 0.7 μM (specific activity 30 mCi/mmol). At selected times, 3×10^8 cells were harvested by centrifugation (10 min, $450 \times g$) and washed three times with 50 ml PBS. The washed cells were then resuspended in 1 ml PBS for subsequent extraction.

2.4.3. Cell extraction

The cell suspension was extracted with 3.75 ml $CHCl_3$:MeOH:1 N HCl (1:2.5:0.25 v/v/v), vortexed, then 0.5 ml 1 N HCl and 1 ml $CHCl_3$ were added and the mixture was vortexed again. The extract was centrifuged ($450 \times g$, 10 min) and the bottom $CHCl_3$ layer was carefully removed using a glass Pasteur pipet and saved in a second vial. To the top aqueous layer was added 2 ml $CHCl_3$, and the mixture was vortexed and centrifuged ($450 \times g$, 10 min). The bottom $CHCl_3$ layer was carefully removed and combined with the $CHCl_3$ layer in the second vial. The combined $CHCl_3$ layers were dried under a stream of nitrogen to prepare the lipid extract for TLC. The aqueous top layer was removed from the tube, leaving behind a thick, milky protein layer at the interphase that was discarded. In control experiments, recovery of the AZT metabolites in the aqueous layer was determined to be approximately 90% and there was no observable degradation of the AZT metabolites under the extraction conditions used. The removed aqueous top layer was divided into two microcentrifuge tubes, each containing 1.75 ml. The microcentrifuge tubes were placed in a Speed Vac centrifuge (Savant Instruments) (room temperature, 8 h) in order to evaporate the aqueous extract leaving behind the aqueous soluble AZT metabolites for HPLC analysis.

2.5. TLC analysis of lipid extracts

The dried lipid extract was resuspended in 300 μl of $CHCl_3$: MeOH (2:1 v/v). Seventy-five microlitres of this solution was spotted on a Whatman K5 silica gel TLC plate (Whatman International Ltd., Maidstone, England) along with various authentic standards (CP-102, 1-*S*-octadecyl-2-*O*-methyl-thioglycerol, 1-*S*-octadecyl-2-*O*-methyl-thioglycero-3-phosphatidic acid and 1-*S*-octadecyl-2-*O*-ethyl-thioglycero-3-phosphocholine) (Kucera et al., 1990). The TLC plate was developed two-thirds the length of the entire plate with $CHCl_3$: MeOH: NH_4OH (50:35:10 v/v/v) and dried. The dried TLC plate was then developed to the top of the plate in a solvent system of ethyl ether: hexane: 90% formic acid [60:90:4.5 (v/v/v)]. The developed TLC plate was air-dried and exposed to a phosphor screen for 4 h. The exposed screen was scanned using a Phosphorimager (Molecular Dynamics, Sunnyvale, CA) to determine the presence and location of the radiolabeled lipid conjugate metabolites.

2.6. HPLC analysis of aqueous extracts

Each of the dried aqueous extracts in the microcentrifuge tubes were resuspended in 150 μl of an unlabeled authentic AZT standard solution containing 5 μM each of AZT, AZT–MP, AZT–DP and AZT–TP. The HPLC analysis used was a modification of the method used by others (Molema et al., 1992). The buffer used was modified to consist of 150 mM Na_3PO_4 with 4 mM TBAP (Aldrich Chemical Co.) (pH 7.4) and 5% CH_3CN. The buffer was run at a rate of 1.2 ml/min (uv absorbance was measured at 260 nm) through an Ultrasphere C18 IP (4.6×150 mm) column (Alltech Associates, Inc.). The total 300 μl volume of cell extract was injected at once into the system and 0.3 ml fractions were collected. Aliquots of each fraction were assayed for tritium by liquid scintillation spectrometry and the peaks of radioactivity were identified by the elution profile of the different internal AZT metabolite standards that were added to each sample. The retention times of the AZT metabolite standards were 7.83 ± 0.05, 10.45 ± 0.19, 14.65 ± 0.24, and 16.00 ± 0.39 min (mean $\pm$ S.D., $n = 4$ experiments) for AZT–MP, AZT–DP, AZT, and AZT–TP, respectively.

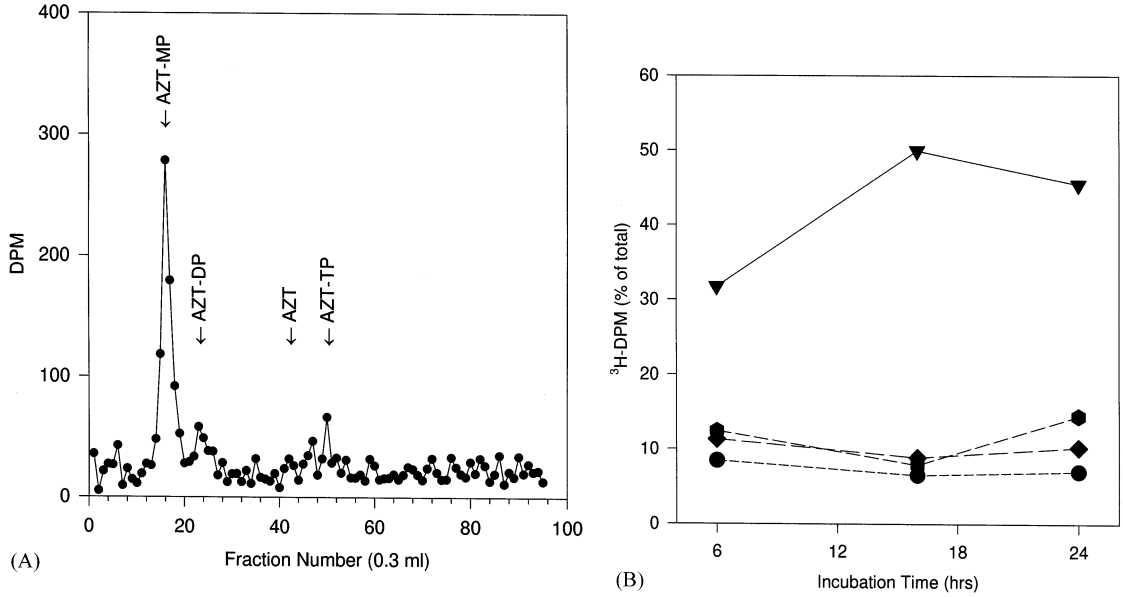


Fig. 2. (A) Profile of aqueous [^{3}H] products collected from HPLC analysis. Note a large AZT–MP peak and significantly smaller AZT, AZT–DP and AZT–TP peaks. Arrows indicate the elution of unlabeled standards detected by uv absorbance. (B) Analysis of radiolabel in each of the four AZT metabolites depicted in (A). Data were obtained by collecting fractions from the HPLC runs and analyzing the radioactivity by liquid scintillation spectrometry. Each data point represents the % of the total [^{3}H] counts from each HPLC run. The data are representative of three independent and reproducible experiments. AZT,⬢ ; AZT-MP, ▼; AZT-DP, ◆; AZT-TP, ●.

3. Results

3.1. HPLC analysis of [^{3}H]CP-102 metabolites

Given the chemical structure (Fig. 1), the initial thioether phospholipid–AZT has a limited number of metabolic products that can be formed. Data from HPLC analysis of metabolites in the soluble aqueous extracts from CEM-SS cells incubated with [^{3}H]CP-102 for 6–24 h (short term incubation) indicated that the major metabolite co-elutes with AZT–MP, together with lesser amounts of metabolites that co-elute with AZT, AZT–DP and AZT–TP (Fig. 2A). The summed values (Fig. 2B) of the integrated areas for each peak depicted in Fig. 2A further suggested that the primary [^{3}H]CP-102 nucleotide metabolite was AZT–MP together with lesser amounts of AZT, AZT–DP and AZT–TP at all time points investigated. Similar results (data not shown) were evident at all time points analyzed from 2 through 11 days (long term incubation). Aqueous products were not observed by HPLC when the unmetabolized [^{3}H]CP-102 was extracted as a control, using the method with 0.5 ml of 1 N HCl described above. Furthermore, upon storage of CP-102 in complete medium containing serum there was no lose of antiviral potency. In summary, these data are interpreted to suggest that the primary nucleotide metabolite of [^{3}H]CP-102 in CEM-SS cells was AZT–MP with considerably lesser amounts of AZT, AZT–DP and AZT–TP formed. Intracellular enzymes metabolize [^{3}H]CP-102 to form the AZT metabolites.

3.2. TLC analysis of CP-102 metabolites

Data from the analysis of intracellular metabolites in lipid extracts from CEM-SS cells incubated with [^{3}H]CP-102 indicated that the primary metabolite migrates with a similar R_f obtained with a known authentic standard, 1-*S*-octadecyl-2-*O*-methyl-thioglycerol, (Fig. 3). Comparison of radioactive spots on the TLC plate using the Phosphorimager resulted in the following density values for the thioetherdiglyceride metabolite area

of the TLC plate: 6 h, 1.1%; 16 h, 1.8%; 24 h, 4.4%.

The thioetherdiglyceride metabolite from the TLC plate did not react with ninhydrin spray suggesting that it did not contain a primary amine such as that found on serine or ethanolamine containing phospholipids. Futhermore, the metabolite was not converted to other detectable metabolites between 1 and 11 days incubation of CEM-SS cells with [^{3}H]CP-102. Alternatively, the addition of a phosphate group at the three position of the thioetherdiglyceride metabolite would be expected to result in the formation of a phosphatidic acid compound. Unlike the migration of an authentic 1-*S*-octadecyl-2-*O*-methyl-thioglycerol-3-phosphatidic acid standard below the [^{3}H]CP-102, the thioetherdiglyceride metabolite migrated above the [^{3}H]CP-102 standard in the TLC system. These data indicate no detectable phosphatidic acid in the thioetherdiglyceride compound (Fig. 3). Unmetabolized [^{3}H]CP-102 appeared as a major band with a closely migrating minor band on the TLC plate. The ratio of the intensity of these two bands did not change as a result of long term incubation (2–11 days) of [^{3}H]CP-102 with cells, suggesting that this minor band may represent an insignificant contaminant of the synthetic process.

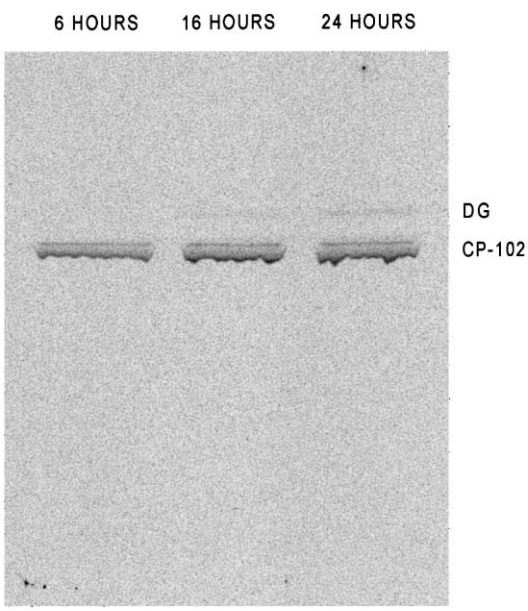


Fig. 3. Phosphorimage of TLC plate showing location of lipid soluble radioactive products after 6, 16, and 24 h (short term incubation) treatment with [^{3}H]CP-102. DG = 1-*S*-octadecyl-2-*O*-methyl-thioglycerol standard.

Since CP-102 has a thioether bond at the 1 position and a methoxyether bond at the 2 position (see Fig. 1), the compound is expected to be resistant to phospholipase A hydrolysis. In point of fact there was no detectable cleavage of the bonds at the 1 or 2 positions of the glycerol backbone as indicated by HPLC or TLC analyses. These data taken together with data from HPLC analysis of cellular aqueous extracts indicating that the major metabolite was AZT–MP, support the conclusion that an intracellular lipid cleavage enzyme similar to phospholipase C is most likely involved in the metabolism of [^{3}H]CP-102.

4. Discussion

Results of this investigation revealed that during incubation of human lymphoblastoid CEM-SS cells with the potent anti-HIV-1 thioetherphospholipid–AZT conjugate ([^{3}H]CP-102), (Piantadosi et al., 1991) the conjugate was metabolized by a putative lipid cleavage enzyme to yield on HPLC a peak identified with AZT–MP as the major nucleotide metabolite with lesser amounts of AZT, AZT–DP, AZT–TP (Fig. 2A) and a thioetherdiglyceride metabolite (Fig. 3). The interpretation made is that AZT–MP was either dephosphorylated to AZT by a phosphatase enzyme and/or phosphorylated to AZT–DP and AZT–TP (Fig. 2A and B), thus bypassing the requirement for a cellular nucleoside kinase activity. The AZT–TP metabolite is the known inhibitor of reverse transcriptase activity required for replication of HIV-1 (Furman et al., 1986; Tornevik et al., 1995).

The intracellular phosphorylation of free AZT to the monophosphate is facile and high concentrations of the monophosphate can be reached in the cell. The metabolite is a very poor substrate for the next kinase in the cascade, thymidylate kinase. Because of this the achievable concentration of the active triphosphates was reported by others to be several orders of magnitude lower

than AZT–MP (Lavie et al., 1997). Also, it has been reported that in other human lymphoblastoid cells (MT-4) AZT alone is readily phosphorylated to AZT–MP while AZT–DP and AZT–TP are generated to a 200–600-fold lower extent than the AZT–MP (Balzarini et al., 1989). Although AZT–MP is the major metabolite and at high concentrations can be responsible for growth inhibitory effects on cells, it is unlikely that the antiviral activity of CP-102 is due to nonspecific cell growth inhibitory activity associated with AZT–MP for the following reasons. It has been reported (Tornevik et al., 1995) that the toxic concentration in CEM cells was 350 μM AZT–MP, a concentration more that 300-fold greater than the starting concentration of 0.7 μM CP-102 used in our experiments. From our HPLC analysis we calculate that the maximum intracellular AZT–MP concentration in our experiments was 0.4 μM. Also, we (Piantadosi et al., 1991) and other investigators (Herrmann et al., 1996) reported that a thiophospholipid AZT conjugate (BM21-1290; FZV) similar in structure to CP-102 was at least ten-fold less toxic on HIV infected cells and uninfected bone marrow cells compared to AZT alone. In addition, FZV was well tolerated by various animal species including nonhuman primates given highest doses of 300 mg/kg/day, p.o. for 6 months and passed pharmacology/toxicological/efficacy and clinical phase I and phase II pharmacokinetics, tolerability and efficacy in human volunteers (Bogner et al., 1997; Girard et al., 2000). Our hypothesis (Krugner-Higby et al., 1995) is that CP-102 and FZV are less toxic than AZT because the lipophilic conjugates (CP-102 and FZV) partition into cell membranes and the lipid cleavage enzyme releases AZT–MP, via hydrolysis slowly over several hours within the cell providing a more continuous supply of AZT–MP and decreasing peak AZT–MP levels.

The targets of the thiophospholipid–AZT conjugate are not entirely clear, however, two targets are proposed: (1) inhibition of infectious viral particle formation by the lipid component; and (2) hydrolysis of the thioetherphospholipid–AZT conjugate yields AZT–MP that is converted to AZT–TP resulting in reverse transcriptase inhibition. We have published evidence that amidoalkyldiglyceride compounds (CP-90, CP-51) alone inhibited HIV-1 induced cell fusion between infected and uninfected cells (Krugner-Higby et al., 1995). Also, amidoalkyldiglycerides alone and amidoalkylphospholipid–AZT conjugates each caused a marked inhibition of HIV-1 induced gp160/gp120 reactivity with specific monoclonal antibody on the surface of infected and treated cells and blocked virus attachment to host cells (Krugner-Higby et al., 1995). Although AZT–TP inhibition of reverse transcriptase activity is most likely the primary mode of action of CP-102 against HIV, it is possible that the lipid (thioetherdiglyceride) metabolite of [^{3}H]CP-102 also contributes some inhibitory activity against HIV-1 infection and pathogenesis. Another possible explanation for the lipid metabolite mode of action could involve inhibition of protein kinase C (Daniel et al., 1987, 1988). Reportedly, protein kinase C induces phosphorylation of the virus receptor complex and allows entry of HIV-1 into CD4+ cells. Inhibitors that block protein kinase C prevent HIV-1 entry and infection of host cells (Fields et al., 1988). Furthermore, there is evidence that phospholipid compounds similar to CP-102 interact with cell membranes, where they can inhibit protein kinases in a dose- and time-dependent manner to bring about inhibition of cell signaling pathways (Ruiter et al., 1999).

In comparison to our metabolic studies, another anti-HIV-1 phospholipid–AZT conjugate (dimyristoylphosphatidylazidothymidine) was reported to undergo a different metabolic pathway (Hostetler et al., 1991). This putative anti-HIV-1 compound was deacylated with subsequent cleavage by a phosphodiesterase to yield AZT or AZT–MP. The difference in the metabolic pathway is explained by the structure of [^{3}H]CP-102 (Fig. 1), which has no acyl esters; therefore [^{3}H]CP-102 is cleaved by a lipid cleavage enzyme at the phosphodiester site yielding the thioetherdiglyceride compound and AZT–MP as the major nucleotide metabolite. Similar to our data, it has been reported (Herrmann et al., 1997) that FZV was hydrolyzed by thymidine-kinase negative cells to yield AZT nucleotides and no free AZT. Finally, cellular metabolism of a

oxyetherphospholipid–acyclovir conjugate yielded acyclovir-MP (Hostetler et al., 2000). In each of these studies, the metabolic activation of the phospholipid conjugate prodrugs bypasses the need for phosphorylation by a nucleoside kinase.

A number of properties make our phospholipid–AZT conjugates attractive as novel agents for the treatment of HIV-1 infections and AIDS. Phospholipid–AZT conjugates have a markedly reduced toxicity against bone marrow progenitor cells and significantly improved selectivity index against HIV-1 infection due to reduced cell cytotoxicity compared to AZT alone (Herrmann et al., 1996; Kucera et al., 1998; Piantadosi et al., 1991). Also, CEM-SS cells tolerate at least 11-days continuous incubation with 0.7 μM of the conjugate without any adverse effects on cell growth. Phospholipids have the potential for being active against AZT resistant HIV-1 due to their unique site of action involving cell membranes and signaling pathways (Ruiter et al., 1999). It is possible to conjugate other small molecular weight antiviral agents to phospholipids (Hostetler et al., 2000) for the treatment of a variety of infectious diseases in addition to HIV-1 infection and AIDS. Phospholipid conjugates are biologically active after oral administration in a variety of animal species including primates. Two independent clinical phase II trials indicated that a thioetherphospholipid–AZT conjugate decreases HIV plasma virus loads in ARC/AIDS patients (Bogner et al., 1997; Girard et al., 2000). Thioetherphospholipid–AZT conjugates are metabolized in both resting and activated human PBL and the anti-HIV-1 activity can be mediated in thymidine kinase negative cells (Herrmann et al., 1997).

In conclusion, the best interpretation of our data is that the thioetherphospholipid–AZT conjugate ([^{3}H]CP-102) is metabolized intracellularly by a putative lipid cleavage enzyme to yield the nucleotide AZT–MP and a thioetherdiglyceride compound. The AZT–MP can be dephosphorylated to AZT by a phosphatase and/or sequentially phosphorylated to AZT–DP and AZT–TP.

Acknowledgements

This research was funded in part by NCI grants; CA 12197 awarded to the Comprehensive Cancer Center of Wake Forest University, the Tissue Culture Core Laboratory, the Experimental Therapeutics Core Laboratory and the Leukemia Cell/Tumor Procurement and Analysis Core Laboratory, CA 58944 to G. Kucera.

References

Arts, E.J., Wainberg, M.A., 1996. Mechanisms of nucleoside analog antiviral activity and resistance during human immunodeficiency virus reverse transcription. Antimicrob. Agents Chemother. 40, 527–540.

Balzarini, J., Herdewign, P., De Clercq, E., 1989. Differential patterns of intracellular metabolism of 2′,3′- didephydro-2′,3′-dideoxythymidine and 3′-azido-2′,3′-dideoxythymidine, two potent anti-human immunodeficiency virus compounds. J. Biol. Chem. 264, 6127–6133.

Bogner, J.R., Roecker, M., Herrmann, D.B.J., Boerner, D., Kaufmann, B., Gurtler, L., Plewig, G., Goebel, F.-D., 1997. Phase I/II trial with fozivudine tidoxil (BM 21.1290): a 7 day randomized, placebo-controlled, dose-escalating trial. Antiviral Ther. 2, 257–264.

Coates, J.A., Cammack, N., Jenkinson, H.J., Jowett, A.J., Jowett, M.I., Pearson, B.A., Penn, C.R., Rouse, P.L., Viner, K.C., Cameron, J.M., 1992. 2′-deoxy-3′-thiacytidine is a potent, highly selective inhibitor of human immunodeficiency virus type 1 and type 2 replication in vitro. Antimicrob. Agents Chemother. 36, 733–739.

Crowe, S., 1999. New reverse transcriptase inhibitors. Adv. Exp. Med. Biol. 458, 183–197.

Dahlberg, J.E., Mitsuya, H., Blam, S.B., Broder, S., Aaronson, S.A., 1987. Broad spectrum antiretroviral activity of 2′,3′-dideoxynucleosides. Proc. Natl. Acad. Sci. USA 84, 2469–2473.

Daluge, S.M., Good, S.S., Faletto, M.B., Miller, W.H., St. Clair, M.H., Boone, L.R., Tisdale, M., Parry, N.R., Reardon, J.E., Dornsife, R.E., Averett, D.R., Krenitsky, T.A., 1997. 1592U89, a novel carbocyclic nucleoside analog with potent, selective anti-human immunodeficiency virus activity. Antimicrob. Agents Chemother. 41, 1082–1093.

Daniel, L.W., Etkin, L.A., Morrison, B.T., Parker, J., Morris-Natschke, S., Surles, J.R., Piantadosi, C., 1987. Ether lipids inhibit the effects of phorbol diester tumor promoters. Lipids 22, 851–855.

Daniel, L.W., Small, G.W., Schmitt, J.D., Marasco, C.J., Ishaq, K., Piantadosi, C., 1988. Alkyl-linked diglycerides inhibit protein kinase C activation by diacylglycerols. Biochem. Biophys. Res. Commun. 151, 291–297.

De Clercq, E., 1997. In search of a selective antiviral chemotherapy. Clin. Microbiol. Rev. 10, 674–693.

Fields, A.P., Bednarik, D.P., Hess, A., May, W.S., 1988. Human immunodeficiency virus induces phosphorylation of its cell surface receptor. Nature 333, 278–280.

Furman, P.A., Fyfe, J.A., St Clair, M.H., Weinhold, K., Rideout, J.L., Freeman, G.A., et al., 1986. Phosphorylation of 3′-azido-3′-deoxythymidine and selective interaction of the 5′-triphosphate with human immunodeficiency virus reverse transcriptase. Proc. Natl. Acad. Sci. USA 83, 8333–8337.

Girard, P.M., Pegram, P.S., Diquet, B., Anderson, R., Raffi, F., Tubiana, R., Sereni, D., Boerner, D., 2000. Phase II placebo-controlled trial of fozivudine tidoxil for HIV infection: pharmacokinetics, tolerability, and efficacy. J. Acquir. Immune Defic. Syndr. 23, 227–235.

Hamamoto, Y., Nakashima, H., Matsui, T., Matsuda, A., Ueda, T., Yamamoto, N., 1987. Inhibitory effect of 2′,3′-didehydro-2′,3′-dideoxynucleosides on infectivity, cytopathic effects, and replication of human immunodeficiency virus. Antimicrob. Agents Chemother. 31, 907–910.

Herrmann, D.B.J., Kucera, L.S., Zilch, H., Mertens, A., Optiz, H.G., 1996. BM21.1290: In-vitro evaluation of a potential new anti-AIDS compound, Abstract A, 1057. In: XI International Conference on AIDS, Vancouver, B.C., Canada July 7–12, p. 64.

Herrmann, D.B.J., Menschik, V., Opitz, H.-G., 1997. Intracellular metabolism of the new anti-AIDS compound Fozivudine in isolated human peripheral blood leukocytes, Abstract 175. Naunyn Schmiedebergs Arch. Pharmacol. 356 (Sept. 14–17), R44.

Hostetler, K.Y., Carson, D.A., Richman, D.D., 1991. Phosphatidylazidothymidine. Mechanism of antiretroviral action in CEM cells. J. Biol. Chem. 266, 11714–11717.

Hostetler, K.Y., Beadle, J.R., Hornbuckle, W.E., Bellezza, C.A., Tochkov, I.A., Cote, P.J., et al., 2000. Antiviral activities of oral 1-*O*-hexadecylpropanediol-3-phosphoacyclovir and acyclovir in woodchucks with chronic woodchuck hepatitis virus infection. Antimicrob. Agents Chemother. 44, 1964–1969.

Krugner-Higby, L., Goff, D., Edwards, T., Iyer, N., Neufeld, J., Kute, T., et al., 1995. Membrane-interactive phospholipids inhibit HIV type 1-induced cell fusion and surface gp160/gp120 binding to monoclonal antibody. AIDS Res. Human Retrovir. 11, 705–712.

Kucera, L.S., Iyer, N., Leake, E., Raben, A., Modest, E.J., Daniel, L.W., Piantadosi, C., 1990. Novel membrane-interactive ether lipid analogs that inhibit infectious HIV-1 production and induce defective virus formation. AIDS Res. Human Retrovir. 6, 491–501.

Kucera, L.S., Iyer, N., Morris-Natschke, S.L., Chen, S.Y., Gumus, F., Ishaq, K., Herrmann, D.B., 1998. In vitro evaluation and characterization of newly designed alkylamidophospholipid analogues as anti-human immunodeficiency virus type 1 agents. Antiviral Chem. Chemother. 9, 157–165.

Lavie, A., Schlichting, I., Vetter, I.R., Konrad, M., Reinstein, J., Goody, R.S., 1997. The bottleneck in AZT activation. Nat. Med. 3, 922–924.

Lin, T.S., Schinazi, R.F., Prusoff, W.H., 1987. Potent and selective in vitro activity of 3′-deoxythymidin-2′-ene (3′-deoxy-2′,3′-didehydrothymidine) against human immunodeficiency virus. Biochem. Pharmacol. 36, 2713–2718.

Mitsuya, H., Broder, S., 1986. Inhibition of the in vitro infectivity and cytopathic effect of human T-lymphotrophic virus type III/lymphadenopathy-associated virus (HTLV-III/LAV) by 2′,3′-dideoxynucleosides. Proc. Natl. Acad. Sci. USA 83, 1911–1915.

Mitsuya, H., Weinhold, K.J., Furman, P.A., St.Clair, M.H., Lehrman, S.N., Gallo, R.C., Bolognesi, D., Barry, D.W., Broder, S., 1985. 3′-Azido-3′-deoxythymidine (BW A509U): an antiviral agent that inhibits the infectivity and cytopathic effect of human T-lymphotropic virus type III/lymphadenopathy-associated virus in vitro. Proc. Natl. Acad. Sci. USA 82, 7096–7100.

Molema, G., Jansen, R.W., Visser, J., Meijer, D.K., 1992. Simultaneous analysis of azidothymidine and its mono-, di- and triphosphate derivatives in biological fluids, tissue and cultured cells by a rapid high-performance liquid chromatographic method. J. Chromatogr. 579, 107–114.

Piantadosi, C., Jr. Marasco, C.J., Morris-Natschke, S.L., Meyer, K.L., Gumus, F., Surles, J.R., Ishaq, K.S., Kucera, L.S., Iyer, N., Wallen, C.A., et al., 1991. Synthesis and evaluation of novel ether lipid nucleoside conjugates for anti-HIV-1 activity. J. Med. Chem. 34, 1408–1414.

Ruiter, G.A., Zerp, S.F., Bartelink, H., van Blitterswijk, W.J., Verheij, M., 1999. Alkyl-lysophospholipids activate the SAPK/JNK pathway and enhance radiation-induced apoptosis. Cancer Res. 59 (10), 2457–2463.

Schinazi, R.F., Chu, C.K., Peck, A., McMillan, A., Mathis, R., Cannon, D., et al., 1992. Activities of the four optical isomers of 2′,3′-dideoxy-3′-thiacytidine (BCH-189) against human immunodeficiency virus type 1 in human lymphocytes. Antimicrob. Agents Chemother. 36, 672–676.

Tornevik, Y., Ullman, B., Balzarini, J., Wahren, B., Eriksson, S., 1995. Cytotoxicity of 3′-azido-3′-deoxythymidine correlates with 3′-azidothymidine-5′-monophosphate (AZTMP) levels, whereas anti-human immunodeficiency virus (HIV) activity correlates with 3′-azidothymidine-5′-triphosphate (AZTTP) levels in cultured CEM T-lymphoblastoid cells. Biochem. Pharmacol. 49, 829–837.