0026-895X/08/7401-92–100$20.00
MOLECULAR PHARMACOLOGY
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Mol Pharmacol 74:92–100, 2008

Vol. 74, No. 1
45526/3356565
Printed in U.S.A.

Activation of 9-[(*R*)-2-[[(*S*)-[[(*S*)-1-(Isopropoxycarbonyl)ethyl]amino] phenoxyphosphinyl]-methoxy]propyl]adenine (GS-7340) and Other Tenofovir Phosphonoamidate Prodrugs by Human Proteases

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Received January 24, 2008; accepted April 22, 2008

ABSTRACT

9-[(*R*)-2-[[(*S*)-[[(*S*)-1-(Isopropoxycarbonyl)ethyl]amino] phenoxyphosphinyl]-methoxy]propyl]adenine (GS-7340) is an isopropylalaninyl phenyl ester prodrug of the nucleotide HIV reverse transcriptase inhibitor tenofovir (TFV; 9-[(2-phosphonomethoxy)propyl]adenine) exhibiting potent anti-HIV activity and enhanced ability to deliver parent TFV into peripheral blood mononuclear cells (PBMCs) and other lymphatic tissues in vivo. The present study focuses on the intracellular metabolism of GS-7340 and its activation by a variety of cellular hydrolytic enzymes. Incubation of human PBMCs in the presence of GS-7340 indicates that the prodrug is hydrolyzed slightly faster to an intermediate TFV-alanine conjugate (TFV-Ala) in quiescent PBMCs compared with activated cells (0.21 versus 0.16 pmol/min/10^6 cells). In contrast, the conversion of TFV-Ala to TFV and subsequent phosphorylation to TFV-diphosphate occur more rapidly in activated PBMCs. The activity of GS-7340 hydrolase producing TFV-Ala in PBMCs is primarily localized in lysosomes and is sensitive to inhibitors of serine hydrolases. Cathepsin A, a lysosomal serine protease has recently been identified as the primary enzyme activating GS-7340 in human PBMCs. Results from the present study indicate that in addition to cathepsin A, a variety of serine and cysteine proteases cleave GS-7340 and other phosphonoamidate prodrugs of TFV. The substrate preferences displayed by these enzymes toward TFV amidate prodrugs are nearly identical to their preferences displayed against oligopeptide substrates, indicating that GS-7340 and other phosphonoamidate derivatives of TFV should be considered peptidomimetic prodrugs of TFV.

Tenofovir (TFV) is an acyclic nucleotide analog active against a variety of retroviruses including human immunodeficiency virus (HIV) and hepatitis B virus (De Clercq, 2003). TFV contains catabolically stable phosphonate moiety; therefore, unlike with nucleoside analogs, its intracellular activation requires only two phosphorylation steps to yield its active form, TFV diphosphate (TFVpp). The phosphorylation of TFV is catalyzed by AMP kinase and nucleoside diphosphate kinase (Robbins et al., 1995). TFVpp is a potent competitive inhibitor of HIV reverse transcriptase, acting as an obligatory DNA chain terminator (Suo and Johnson, 1998). However, the presence of two negative charges on the TFV molecule limits its cellular permeability and precludes oral administration. To overcome these limitations, various TFV prodrugs containing lipophilic groups masking the charged phosphonate moiety have been designed. Among these, tenofovir disoproxil fumarate (TDF; Viread) has been approved for the treatment of HIV infection. Because of its favorable resistance profile and long-term tolerability, TDF therapy is broadly used in treatment of both naive and previously drug-treated HIV-infected patients (for review, see Antoniou et al, 2003; Grim and Romanelli, 2003).

GS-7340 (Fig. 1) is a prototype molecule representing a novel class of TFV mono-phosphonoamidate prodrugs. Unlike TDF, GS-7340 contains phenol and alanine isopropyl ester as the phosphonate masking groups (Fig. 1). Relative to

Article, publication date, and citation information can be found at http://molpharm.aspetjournals.org.
doi:10.1124/mol.108.045526.

ABBREVIATIONS: TFV, tenofovir (9-[(2-phosphonomethoxy)propyl]adenine); HIV, human immunodeficiency virus; TFVpp, TFV diphosphate; TDF, tenofovir disoproxil fumarate; GS-7340, 9-[(*R*)-2-[[(*S*)-[[(*S*)-1-(isopropoxycarbonyl)ethyl]amino] phenoxyphosphinyl]-methoxy]propyl]adenine; PBMC, peripheral blood mononuclear lymphocyte; DTT, dithiothreitol; BSA, bovine serum albumin; Pr3, proteinase 3; LE, leukocyte elastase; PE, pancreatic elastase; PLCE, porcine liver carboxylesterase; DFP, diisopropyl fluorophosphate; α-ABA, α-aminobutyric acid; E64, *trans*-epoxysuccinyl-L-leucylamido(4-guanidino)butane.

parent TFV, GS-7340 exhibits 500- to 1000-fold enhanced activity against HIV-1 in T-cells, activated peripheral blood mononuclear lymphocytes (PBMCs), and macrophages (Lee et al., 2005). It is also a potent inhibitor of hepatitis B virus (Lee et al., 2005) and exhibits markedly enhanced stability in plasma compared with TDF (Eisenberg et al., 2001). As a consequence, in vivo oral administration of GS-7340 results in an increased accumulation of parent TFV and its active diphosphate metabolite in PBMCs relative to TDF (Lee et al., 2005). GS-7340 also increases the accumulation of TFV in lymphatic tissues, which is likely to favorably affect the therapeutic efficacy of this novel prodrug (Lee et al., 2005).

Phosphonoamidate prodrugs of TFV share structural similarity with previously explored aryl phosphoramidate derivatives of antiviral nucleosides (Balzarini et al., 1996; Valette et al., 1996). After penetration into cells, the activation of phosphoramidate prodrugs is initiated by hydrolytic enzymes that cleave the carboxyester bond in the prodrug moiety. This hydrolysis releases a metastable metabolite, from which the phenol group is spontaneously eliminated via intramolecular cyclization and hydrolysis (Balzarini et al., 1996; Valette et al., 1996). In the case of GS-7340, the resulting metabolite is a conjugate of TFV and alanine (TFV-Ala) (Fig. 1). Because GS-7340 is a lipophilic cell-permeant compound, the formation of negatively charged TFV-Ala has important pharmacological implications. As TFV-Ala undergoes further conversion to parent TFV, its efficient formation is crucial for the intracellular accumulation and retention of TFV metabolites delivered via GS-7340. In vitro experiments have demonstrated that the antiviral activity of different TFV phosphonoamidates is greatly affected both by the ester and amino acid moiety present in the prodrug (Lee et al., 2005), which is probably a consequence of differences in the efficiency of hydrolysis of the prodrug carboxy ester bond.

To understand the activation of GS-7340 and other phosphonoamidates of TFV, including the mechanism of prodrug hydrolysis, the metabolism of GS-7340 in quiescent and activated human PBMCs was investigated. In addition, subcellular fractionation was used to demonstrate that GS-7340 undergoes its enzymatic hydrolysis in lysosomes. Finally, the present study shows that in addition to cathepsin A, which was identified as a major hydrolase responsible for the activation of GS-7340 in PBMCs (Birkus et al., 2007) multiple specific serine proteases and hydrolases cleave GS-7340 and other phosphonoamidate prodrugs of TFV. The characterized proteases display substrate preference toward TFV amidate prodrugs that closely resembles their substrate specificity for oligopeptide substrates, indicating that the amidates can be considered peptidomimetic prodrugs of TFV.

Materials and Methods

Reagents. Human cathepsin A was isolated from PBMCs as described previously (Birkus et al., 2007), human leukocyte elastase, human cathepsins B, D, G, and H, bovine cathepsin C, mouse granzymes A and B, porcine liver carboxylesterase, Percoll, 4-methylumbelliferyl-2-actamide-2-deoxy-β-*O*-glucopyranoside, iodonitrotetrazolium chloride, and all common chemicals were purchased from Sigma-Aldrich (St. Louis, MO). Human chymotrypsin and trypsin, human mast cell chymase, and porcine pancreatic elastase were supplied by Calbiochem (San Diego, CA). Human proteinase 3 was from EPC (Owensville, MO). The custom synthesis of [^{14}C]GS-7340 was performed by Moravek Biochemicals (Brea, CA). Amplify Fluorographic Reagent, ECL Western blotting detection kit, and Hyperfilm MP were purchased from GE Healthcare (Chalfont St. Giles, Buckinghamshire, UK). EZ-Link Sulfo-NHS-Biotinylation Kit was from Pierce Biotechnology (Rockford, IL). [^{3}H]Chloroquine was purchased from American Radiolabeled Chemicals (St. Louis, MO). The monoamidate prodrugs of tenofovir were prepared using a modified procedure taken from literature (Lee et al., 2005).

Metabolism of GS-7340 in Human PBMCs. PBMCs were prepared from human buffy coats obtained from Stanford Medical

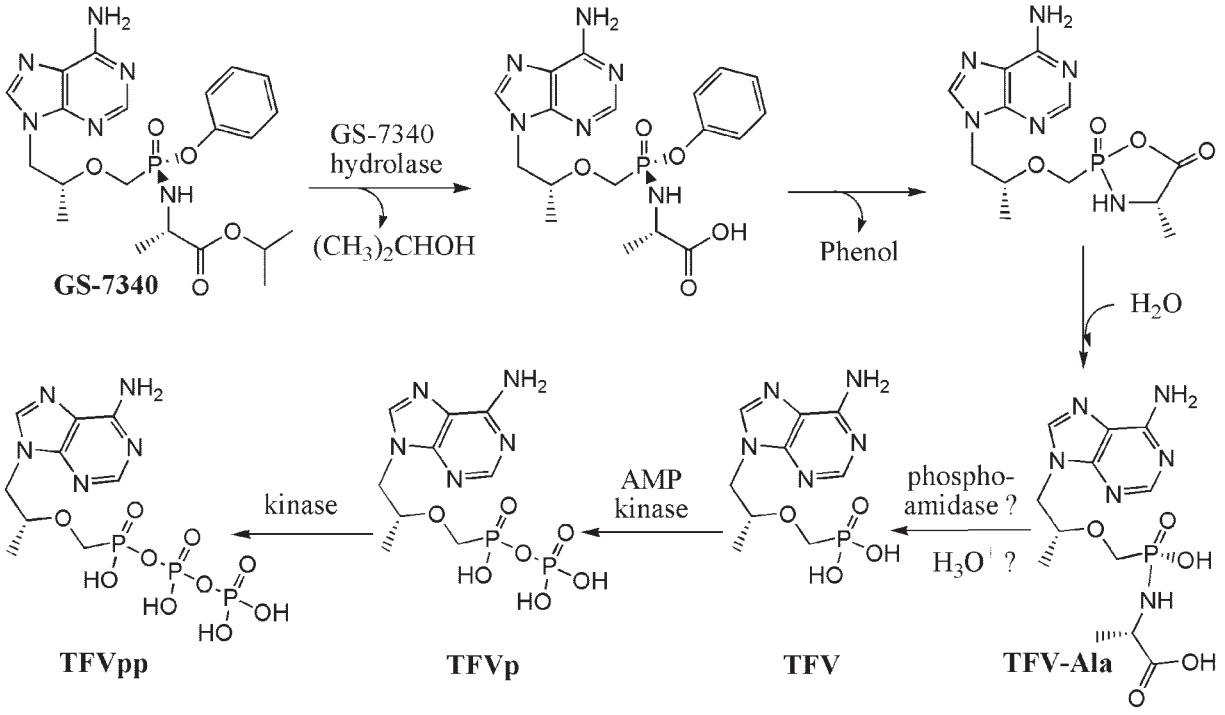

Fig. 1. Activation pathway of GS-7340.

School Blood Center (Stanford, CA). One human buffy coat (~30 ml) was diluted with 10 ml of PBS, and 13 ml of diluted buffy coat was layered over 13 ml of Ficoll Plus (GE Healthcare). Tubes were centrifuged at 500*g* for 15 min at room temperature. The leukocyte layer was removed and mixed with hypotonic red cell lysis buffer (155 mM NH_4Cl, 0.1 mM EDTA, and 10 mM $KHCO_3$). Cells were centrifuged at 500*g* for 5 min at room temperature and washed with red cell lysis buffer. Finally, cells were washed in 50 ml of PBS and pelleted. Quiescent PBMCs were resuspended in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum to a final concentration of 2×10^6 cells per ml. Eight milliliters of cell culture was kept in 15 ml of conical tubes for 1 h at 37°C in a 5% CO_2 air atmosphere before 10 μM [^{14}C] GS-7340 (0.53 μCi/ml) was added. A portion of purified PBMCs were activated for 2 days in RPMI 1640 medium containing 10 units/ml Interleukin-2, 50 ng/ml phorbol 12-myristate 13-acetate, and 1 μg/ml phytohemagglutinin. After the activation, cells were spun at 500*g* and resuspended in fresh RPMI 1640 medium to a final concentration of 2×10^6 cells/ml. One hour later, cells were exposed to 10 μM [^{14}C]GS-7340 for 15 to 180 min. Eight milliliters of the culture were spun (500*g*, 5 min, 4°C) at each time point, and the cell pellets were washed twice with 12 ml of ice-cold PBS. Finally, cells were lysed with 700 μl of ice-cold 70% methanol in 10 mM TrisCl, pH 7.6. The lysates were incubated for 30 min at −20°C and then spun at 13,000*g* for 30 min at 4°C. Supernatants were collected, evaporated, and reconstituted in 200 μl of water. The total amount of all GS-7340 metabolites per 10^6 cells was determined by scintillation counting of an aliquot of the reconstituted cell lysate. A calibration curve of cpm versus pmol of [^{14}C]GS-7340 was constructed to calculate the total intracellular concentration of metabolites.

HPLC Analysis of GS-7340 Metabolites in PBMCs. The analysis was performed using a modified HPLC protocol developed previously (Eisenberg et al., 2001). The metabolites were separated on a Prodigy ODS-3 column (5 mm, 150 × 4.6 mm; Phenomenex, Torrance, CA) using the HP 1090 (series II) HPLC system connected to a Radiomatic Flo-One Beta liquid scintillation detector (Packard Series A-500). A gradient elution from buffer A (5% AcCN in 25 mM phosphate buffer, pH 6, and 5 mM tetrabutylammonium bromide) to 25% buffer A/75% buffer B (60% AcCN in 25 mM phosphate buffer, pH 6, and 5 mM tetrabutylammonium bromide) for 15 min and a flow rate of 1.2 ml/min and 40°C was used to separate the metabolites. The metabolites were identified by the comparison of their retention time with that of standards for TFV (t_R = 3.5 min), TFV-Ala (t_R = 6.3 min), TFVp (t_R = 8.8 min), TFVpp (t_R = 11.4 min) and GS-7340 (t_R = 13.6 min).

Preparation of PBMC Extract. Freshly isolated human PBMCs (4×10^8 cells) were washed twice with 15 ml of PBS and centrifuged (500*g*, 5 min). The pellet was extracted with 4 ml of 10 mM Tris-HCl buffer, pH 7.3, 150 mM NaCl, 2 mM $CaCl_2$, 1 mM DTT, and 1% Nonidet P-40 for 20 min on ice. The extract was cleared by centrifugation at 13,000*g* for 30 min and the supernatant was frozen at −70°C.

Subcellular Fractionation. MT-2 T cells and PBMCs were grown in RPMI 1640 medium supplemented with 100 mM HEPES and 10% fetal bovine serum at 37°C. To perform subcellular fractionation, cells were disrupted and fractionated using a modified version of a method described previously (Wex et al., 2001). In brief, 5×10^7 cells were washed twice with ice-cold PBS and once with buffer A (0.25 mM sucrose, 3 mM imidazole, pH 7.4, and 1 mM EDTA). The pellet was resuspended in 500 μl of buffer A and passed 10 times (MT-2 cells) or 35 times (PBMC cells) through a 26 gauge needle. This resulted in a disruption of >90% cells as monitored by microscopic examination. Disrupted cells were brought to a volume of 4 ml and spun at 1000*g* for 10 min at 4°C. The pellet containing nuclei and unbroken cells was discarded. The supernatant was removed, diluted with buffer A to a volume of 4.33 ml, and combined with 1.67 ml of 90% Percoll. The sample was centrifuged at 75,000*g* for 30 min at 4°C using a 50TI rotor on an ultracentrifuge (L7; Beckman Coulter, Fullerton, CA). A gradient of Percoll with a density of 1.040 to 1.15 g/ml was formed during the centrifugation. Fractions (0.25 ml) were collected from the top of the tube. To remove Percoll, each fraction was spun in a TLA100 rotor on a TL-100 ultracentrifuge at 100,000*g* for 1 h at 4°C. This step was necessary to prevent the interference of Percoll with enzyme marker assays.

Organelle Marker Assays. Lysosomal enzyme marker β-hexosaminidase was assayed using 5 μM fluorescent substrate 4-methylumbelliferyl-2-acetamide-2-deoxy-β-*O*-glucopyranoside in 100 mM sodium acetate and 0.1% TX-100, pH 4.5 (Merion and Sly, 1983). In addition, chloroquine was used as a small molecule marker for lysosomes (Matsuzawa and Hostetler, 1980). Cells were incubated with 20 nM [^{3}H]chloroquine for 1 h at 37°C followed by disintegration and gradient fractionation. The amount of chloroquine in each fraction was determined by scintillation counting. Mitochondrial succinate dehydrogenase was assayed using iodonitrotetrazolium chloride according to a previously published protocol (Pennington, 1961). NADPH cytochrome-c reductase was used as a marker for endoplasmic reticulum according to previously published methods (Ouar et al., 2003). The activity of GS-7340 hydrolase was evaluated using the same reaction condition as described for the hydrolase from PBMC extracts (see below).

In the experiment evaluating TFV metabolites distribution among the organelles, the cells were incubated with 10 μM [^{14}C]GS-7340 for 4 h at 37°C followed by disintegration and gradient fractionation. The total amount of TFV metabolites in each fraction was determined by scintillation counting.

Enzyme Assays. TFV prodrugs (100 μM) were incubated with each enzyme for 10, 30, and 120 min at 37°C. The reactions with leukocyte elastase (4 μg/ml), proteinase 3 (4.4 μg/ml), porcine liver carboxyl esterase (40.5 μg/ml), granzyme A (13.88 μg/ml), and granzyme B (13.88 μg/ml) was performed in a reaction buffer containing 25 mM Mes-Na^+, pH 6.5, 100 mM NaCl, and 1 mM DTT. The extract from PBMCs (134 μg/ml) was assayed under the same conditions except that Nonidet P-40 was present at a final concentration of 0.1%. To perform the inhibition experiments, PBMC extract was preincubated with 0, 1, 10, and 100 μM tested inhibitors for 10 min. GS-7340 was added to a final concentration of 100 μM and incubated with the treated extract for 5, 10, and 20 min. After sample analysis, the concentration of inhibitor reducing the hydrolysis of GS-7340 by 50% (IC_{50}) was determined. Mast cell chymase (1 μg/ml), cathepsin G (0.333 μg/ml), chymotrypsin (1.47 μg/ml), trypsin (16 μg/ml), and pancreatic elastase (7.8 μg/ml) were incubated with TFV prodrugs in a reaction buffer containing 25 mM Tris-HCl, pH 8.0, 100 mM NaCl, and 1 mM DTT. The conversion of TFV prodrugs by cysteine proteases cathepsin H (7.5 μg/ml), cathepsin L (7.35 μg/ml), cathepsin B (8.33 μg/ml), and cathepsin C (40 μg/ml) was performed in 25 mM Mes-Na^+, pH 6.5, 100 mM NaCl, and 5 mM DTT. Aspartic protease cathepsin D (5.2 μg/ml) was incubated with prodrugs in 25 mM acetate-Na^+ buffer, pH 4.0, containing 100 mM NaCl and 1 mM DTT.

After the incubation, reactions with all enzymes were quenched by adding ice-cold methanol (final concentration, 70%) and samples were processed as described above. Reaction products were quantified by HPLC analysis on C18 column (Vydac, Hesperia, CA) using a gradient of acetonitrile (5%–45%, 6 min; flow rate, 0.25 ml/min; λ 260 nm) in a buffer containing 25 mM KPO_4, pH 6.0, and 5 mM tetrabutylammonium bromide. The identity of corresponding TFV-amino acid conjugates was confirmed by LC-mass spectrometry analysis. Hydrolase specific activity was expressed as picomoles of metabolites produced per minute per microgram of total protein. For all tested enzymes, the prodrug hydrolysis rates were determined under the steady-state reaction conditions.

Covalent Labeling of Serine Hydrolases with [^{14}C]GS-7340. Bovine serum albumin (BSA), proteinase 3 (Pr3), leukocyte elastase (LE), pancreatic elastase (PE), and porcine liver carboxylesterase (PLCE) were diluted in 200 μl of 25 mM Mes-Na^+ buffer, pH 6.5, containing 100 mM NaCl and 1 mM DTT to a final concentration of

10 μg/ml and kept on ice. Half of each enzyme solution was denatured for 10 min at 80°C before the labeling was performed. Native and denatured PLCE was also preincubated with nonradioactive diisopropyl fluorophosphate (DFP) (final concentration, 100 μM) before labeling with GS-7340. The denatured and native samples were simultaneously preincubated for 2 min at 37°C and [^{14}C]GS-7340 (final concentration, 1.5 mM; 0.08 mCi/ml) was added. After an additional 2-min incubation at 37°C, the reaction was quenched with 0.3% SDS solution and proteins were precipitated with ice-cold trichloroacetic acid (10% final concentration). The samples were kept on ice for 30 min, spun at 13,000*g* for 30 min at 4°C, and protein pellets were washed twice with 700 μl of ice-cold acetone. The protein pellets were air-dried, dissolved in Nu-PAGE loading buffer (Invitrogen, Carlsbad, CA), and denatured for 10 min at 70°C. After electrophoresis in 4–12% Nu-PAGE BisTris gel with Mes buffer (Invitrogen), the gels were fixed in isopropanol/water/acetic acid (25:65:10) solution for 30 min and then soaked in Amplify Fluorographic Reagent (GE Healthcare) for 30 min. The gels were dried under vacuum at 80°C and exposed for 2 weeks to preflashed X-ray films. Native and denatured PLCE was also labeled with 50 μM [^{3}H]DFP (0.1 mCi/ml) for 30 min at 37°C. The labeled protein was then processed as described above.

Results

Metabolism of GS-7340 in Human PBMCs. During the incubation of quiescent or activated PBMCs with 10 μM [^{14}C]GS-7340, the concentration of intact prodrug reaches intracellular steady-state levels (≥ 8 μM) in less than 15 min (Fig. 2), indicating that GS-7340 readily enters the cells and achieves equilibrium with the extracellular prodrug. In addition to the intact prodrug, the formation of TFV-Ala, TFV, and TFVpp was detected in PBMCs. TFVp was present at levels below the limit of quantification.

The initial rate of GS-7340 hydrolysis to TFV-Ala observed in quiescent and activated PBMCs is approximately 0.21 and 0.16 pmol/10^6 cells/min, respectively (Table 1). The slightly higher rate of TFV-Ala production observed in quiescent cells results in approximately 1.3-fold higher total level of TFV metabolites compared with activated cells (Fig. 2). Although the initial rate of TFV formation (Table 1) is similar in both quiescent and activated PBMCs (0.11–0.13 pmols/10^6 cells/min), the equilibrium level of TFV-Ala in quiescent cells (55μM) was 2.5-fold higher than the equilibrium concentration of TFV-Ala reached in activated cells (22μM). This observation suggests that the conversion of TFV-Ala to TFV is more efficient in activated PBMCs, possibly because of a higher expression of the putative phosphoamidase in these cells. Despite the presence of higher concentrations of TFV in quiescent cells, the rate of intracellular accumulation of TFVpp is independent of the cell activation state (~0.05 pmols/10^6 cells/min), suggesting that TFV is slightly more efficiently phosphorylated in activated cells compared with quiescent cells.

Characterization of GS-7340 Hydrolase Activity in PBMC Extract. The rapid hydrolysis of GS-7340 observed in PBMCs and cellular extracts (Table 1 and 2) indicates the presence of intracellular hydrolytic enzyme(s) capable of catalyzing the hydrolysis of the carboxyl ester bond of GS-7340. The structural similarity between the phosphonophenyl ester isopropyl alanyl amidate and the tripeptide Phe-Ala-Ala (Fig. 3) suggests that the phenol ring, alanine moiety, and the isopropyl ester of GS-7340 may mimic P2, P1, and P1′ residues of a peptidic substrate. [Nomenclature for the substrate amino acid preference is P4, P3, P2, P1, P1′, P2′, P3′, and P4′. Amide bond hydrolysis occurs between P1 and P1′. S4, S3, S2, S1, S1′, S2′, S3′, and S4′ denote the corresponding enzyme binding sites (Schechter and Berger, 1967).] Together with the known fact that proteases and peptidases can efficiently hydrolyze ester in addition to amide bonds, these structural characteristics of GS-7340 suggest the potential involvement of specific cellular proteases and/or peptidases in the activation of GS-7340. To further characterize this hydrolytic activity, GS-7340 was incubated with PBMC extract in the presence and absence of various protease inhibitors.

Various inhibitors of cysteine proteases [E64; L-*trans*-epoxysuccinyl-leucylamido(4-guanidino)butane] (Barrett et al., 1982), aspartic proteases (pepstatin A) (Rich et al., 1982), and metalloproteases (EDTA, bestatin) (Powers and Harper, 1986) at concentrations up to 100 μM failed to inhibit the hydrolysis of GS-7340 in PBMC extract. In contrast, GS-7340 hydrolysis was inhibited by both DFP and 3,4-dichloroisocoumarin, two potent inhibitors of serine proteases (Powers et al., 2002), with IC_{50} values of 10.5 and 6.5 μM, respectively.

In addition, the hydrolytic activity present in PBMC extracts exhibited a substrate preference for TFV prodrugs containing linear aliphatic or aromatic residues (Ala, Gly, α-ABA, or Phe) compared with those containing aliphatic branched amino acid moieties (Leu, Ile, or Val) (Table 2, Fig.

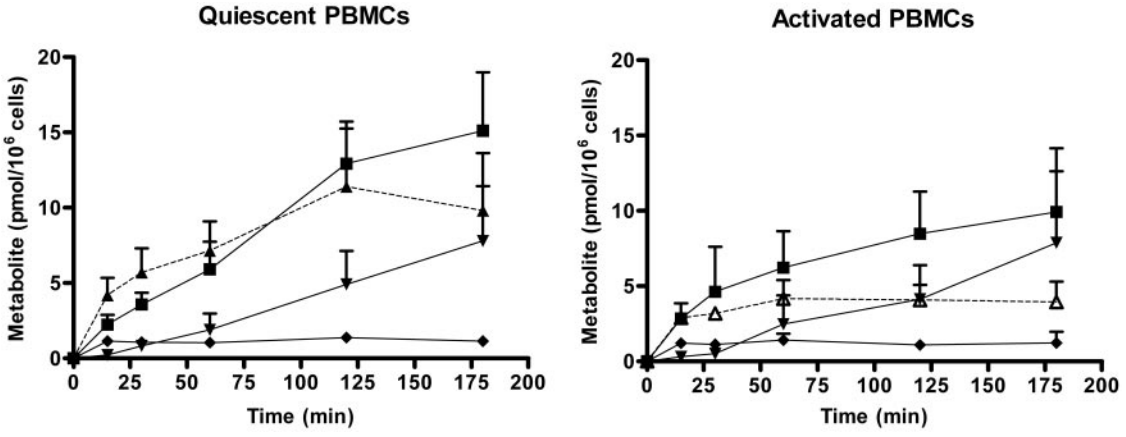

Fig. 2. Metabolism of GS-7340 in quiescent and activated PBMCs. 2 × 10^6 cells per ml were incubated with 10 μM [^{3}H]GS-7340 (0.52 μCi/ml) for 15, 30, 60, 120, and 180 min. Cells were extracted with 70% methanol and GS-7340 (♦), TFV-Ala (▲), TFV (■), and TFVpp (▼) were analyzed using high-performance liquid chromatography-liquid scintillation detection as described under *Materials and Methods.*

4). Together, these data suggest that the hydrolysis of the alanine isopropyl ester in GS-7340 as well as ester bonds in other amidate prodrugs of TFV may be mediated by specific proteases present in PBMC extract.

Subcellular Localization of GS7340 Hydrolase. Immune cells contain a variety of proteases localized in subcellular organelles such as lysosomes and secretory granules (Borregaard and Cowland, 1997; Blott and Griffiths, 2002). To test whether the GS-7340 hydrolase activity is localized in a distinct subcellular compartment, MT-2 T-cells and PBMCs were disintegrated, and the presence of GS-7340 hydrolase was determined in organelles fractionated using Percoll density gradient ultracentrifugation (Wex et al., 2001). In parallel, cells were incubated with [^{14}C]GS-7340 and fractionated to determine the intracellular distribution of TFV metabolites. Individual subcellular fractions were identified by measuring the levels of specific organelle markers (Merion and Sly, 1983). Lysosomal hexosaminidase activity was found predominantly in high-density fractions (fractions 18 and 20) (Fig. 5), well separated from the peak of the mitochondrial marker activity. Both of these markers were distinct from those for less dense organelles, including endoplasmic reticulum. GS-7340 hydrolase activity was present in a distinct peak overlapping with the lysosomal hexosaminidase marker. In addition, the fractionation of organelles of cells treated with a lysosomotropic agent [^{3}H]chloroquine (Matsuzawa and Hostetler, 1980) indicated the colocalization of chloroquine-containing fractions with both the hexosaminidase marker and the GS-7340 hydrolase activity, a further evidence for the lysosomal localization of GS-7340 hydrolase (Fig. 5). It is noteworthy that TFV metabolites were equally distributed across all Percoll gradient fractions from cells treated with [^{14}C]GS-7340, indicating their presence primarily in cytosol, which does not sediment in the Percoll gradient.

Hydrolysis of TFV Amidate Prodrugs by Proteases. The hydrolase capable of activating GS-7340 was isolated from human PBMCs and identified as a serine protease cathepsin A (Birkus et al., 2007). Consistent with the above results from the cell fractionation experiments, cathepsin A is localized primarily in lysosomes. However, there is a broad spectrum of cellular proteases known to be present in lysosomes and lysosome-related secretory organelles (azurophilic granules of neutrophils and cytotoxic granules of T-cells); therefore, selected lysosomal proteases in addition to cathepsin A were tested for their ability to hydrolyze GS-7340 and

TABLE 1

Rates of metabolite formation in quiescent and phytohemagglutinin/interleukin-2-activated human PBMCs

Quiescent and phytohemagglutinin/interleukin-2-activated PBMC were treated with 10 μM [^{14}C]GS-7340 and extracted with ice-cold 70% methanol. TFV metabolites were analyzed using ion-pair HPLC with on-line liquid scintillation detection. The rate of TFV-Ala formation = Σ of intracellular [TFV-Ala], [TFV], and [TFVpp] accumulated per minute of incubation time; the rate of TFV formation = Σ of intracellular [TFV] and [TFVpp] accumulated per minute of incubation time; and the rate of TFVpp formation is expressed as the intracellular [TFVpp] accumulated per minute of incubation time.

Metabolite	Activated PBMC		Quiescent PBMC	
	Rate of Formation	Steady-State Concentration	Rate of Formation	Steady-State Concentration
	pmol/min/10^6 cells	*μM*	*pmol/min/10^6 cells*	*μM*
GS-7340	N.D.	79	N.D.	77
TFV-Ala	0.16	22	0.21	55
TFV	0.11	55	0.13	N.R.
TFVpp	0.044	N.R.	0.045	N.R.

N.D., not determined; N.R., not reached.

TABLE 2

Conversion of tenofovir prodrugs by serine, cysteine, and aspartic proteases

Substrates (100 μM) were incubated with each tested enzyme under the reaction conditions described under *Materials and Methods*. The reaction products were analyzed by ion-pairing HPLC. Data are means from at least two independent determinations; S.D. values were <20% of the mean.

Amidate Moiety	GS-7003 Phe-Me	GS-7098 Leu-Me	GS-7340 Ala-iPr	GS-7095 Ile-Me	GS-7096 Val-Me	GS-7119 Gly-Et	GS-7120 ABA-Et
				pmol/min/μg			
Serine proteases							
Chymotrypsin	4138	604	0	0	0	123	22
Cathepsin G	8144	995	0	0	0	0	0
Chymase	4961	369	0	0	0	168	10
Leukocyte elastase	0	0	891	327	194	0	181
Proteinase 3	0	0	57	165	0	0	70
Pacreatic Elastase I	0	85	113	53	24	23	756
Granzyme A	0	0	0	0	0	0	0
Trypsin	0	0	0	0	0	0	0
Granzyme B	0	0	0	0	0	0	0
Cathepsin A[a]	4400	0	31,000	0	0	2449	2900
Cysteine proteases							
Cathepsin H	34	0	27	0	0	12	10
Cathepsin B	0	0	0	0	0	0	0
Cathepsin C	0	0	0	0	0	0	0
Cathepsin L	0	26	0	0	0	0	44
Aspartic proteases							
Cathepsin D	0	0	0	0	0	0	0
Serine hydrolases							
Porcine liver carboxylesterase	73	10	116	0	1	67	44
Cellular extract	6.8	0	21.5	0.2	0.2	3.7	5.3

[a] Data from Birkus et al. (2007).

other amino acid ester prodrugs of TFV (Table 2). To assess a potential effect of proteases on the intestinal absorption of TFV prodrugs, the ability of several major digestive proteolytic enzymes to hydrolyze GS-7340 and other TFV amidates was evaluated.

TFV prodrug containing Phe in the amidate moiety (i.e., in the putative P1 position) is cleaved most efficiently by chymotrypsin-like serine proteases (chymotrypsin, cathepsin G, and mast cell chymase) (Table 2). In contrast, a prodrug containing Leu was cleaved 7- to 13-fold less efficiently by these proteases. Chymotrypsin and mast cell chymase exhibited low activity against a prodrug with Gly and α-aminobutyric acid (α-ABA) in the P1 position. It is noteworthy that chymotrypsin-like proteases failed to hydrolyze GS-7340 with Ala as the P1-like residue. In contrast, elastase-like serine proteases (LE, Pr3, and PE I) exhibited a preference for TFV prodrugs containing Ala, Ile, Val, and α-ABA moieties at this position (Table 2). PE displayed the broadest substrate specificity toward TFV phosphonoamidate prodrugs, the α-ABA-containing prodrug being cleaved most efficiently. In contrast, Ala-containing GS-7340 was the best substrate for leukocyte elastase among the prodrugs tested.

It is noteworthy that none of the tested prodrugs were hydrolyzed by trypsin-like protease (trypsin, granzyme A) or

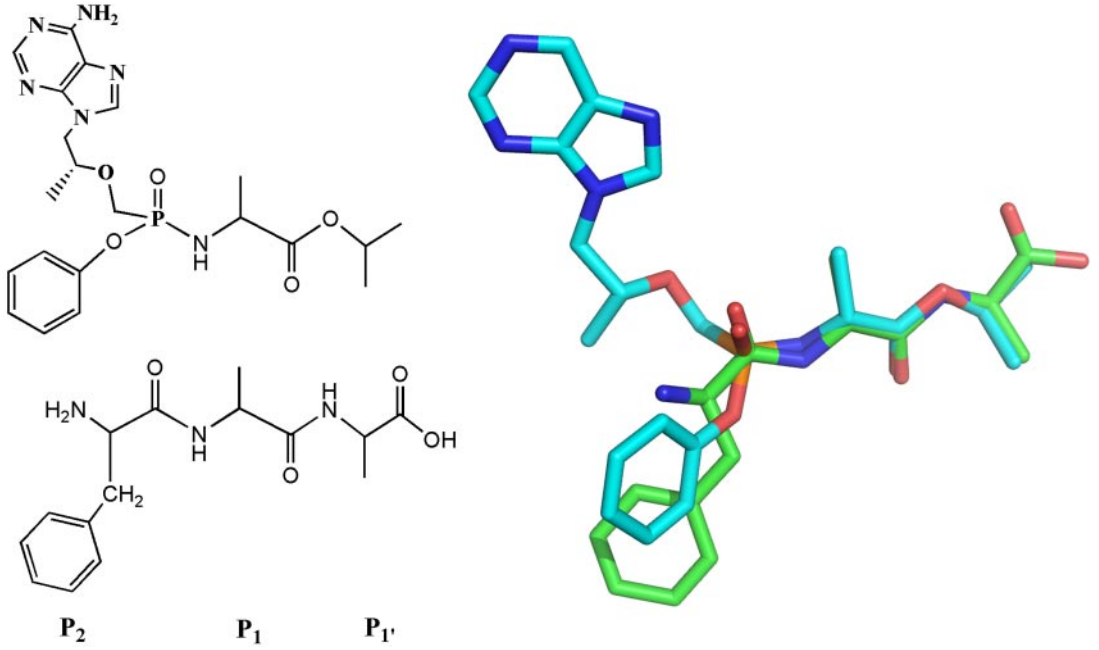

Fig. 3. Alignment of GS-7340 (in blue) and Phe-Ala-Ala tripeptide (green).

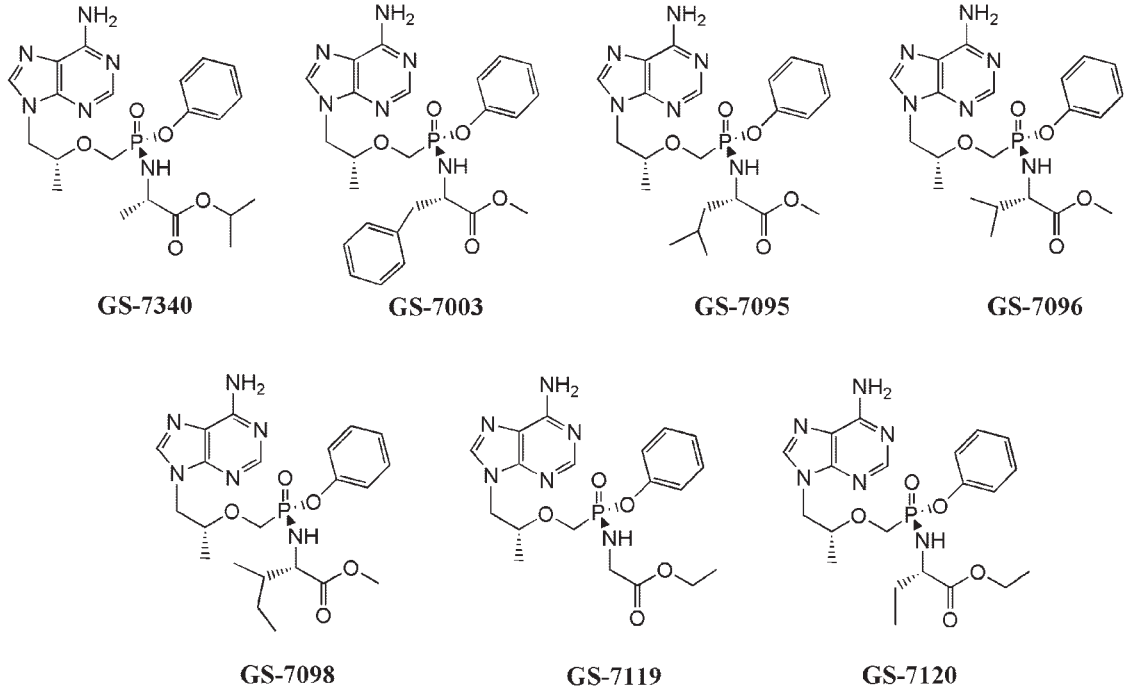

Fig. 4. Structures of tenofovir phoshonoamidate prodrugs.

aspase-like protease (granzyme B). Among cysteine proteases, cathepsins H and L were the only enzymes capable of hydrolyzing TFV prodrugs, albeit with a very low efficiency (Table 2). Cathepsin D, the only aspartic protease tested, hydrolyzed no TFV phosphonoamidate prodrugs. As expected, the nonselective liver carboxyl esterase exhibited a broad substrate specificity toward a wide range of tested TFV prodrugs (Table 2). However, the efficiency of prodrug hydrolysis was substantially lower compared with more specific proteases.

Cathepsin A exhibited the highest specific activity for the GS-7340 hydrolysis and was also able to effectively cleave TFV prodrugs containing Phe, α-ABA, or Gly. It is noteworthy that chymotrypsin, cathepsin G, and chymase hydrolyzed the Phe-containing prodrug with efficiency comparable with that of cathepsin A. On the other hand, TFV prodrugs with Val, Leu, and Ile were not substrates for cathepsin A, but they were hydrolyzed by LE, Pr3, and PE I.

Covalent Labeling of Hydrolases with GS-7340 Substrate. The reaction mechanism of serine hydrolases involves the formation of a covalent bond between serine in the active site and the substrate acyl group (Satoh and Hosokawa, 1998). Denaturing the enzyme during the course of reaction should allow for capturing this intermediate. Consistent with this assumption, we have recently demonstrated that cathepsin A can be covalently labeled with [^{14}C]GS-7340 (Birkus et al., 2007). Likewise, the other enzymes tested in this study and found capable of GS-7340 hydrolysis (PLCE, LE, Pr3, and PE) were successfully labeled with GS-7340 (Fig. 6). The specificity of this method was confirmed by the lack of labeling of heat-denatured enzymes or inert bovine serum albumin. In addition, the preincubation of enzymes with the inhibitor DFP prevents their labeling by [^{14}C]GS-7340. These observations further confirm the interactions of GS-7340 with multiple proteases observed in enzyme kinetic experiments.

Discussion

The cellular permeability and antiviral activity of TFV is markedly enhanced by masking its two negative charges by hydrolyzable hydrophobic moieties. After the clinical development of TDF, a diester prodrug of TFV, a new class of TFV prodrugs was successfully designed by using phenol and an esterified amino acid moiety as masking groups. GS-7340, the lead molecule of this new prodrug series, exhibits enhanced in vivo pharmacokinetic properties, particularly with respect to the delivery of parent nucleotide into cell types and tissues supporting HIV replication (Lee et al., 2005). In this study, we characterized the intracellular metabolism of GS-7340 in human PBMCs and conclusively demonstrated efficient intracellular conversion of the prodrug to the active metabolite TFVpp. In these experiments, GS-7340 rapidly achieves equilibrium between the media and cells indicating its rapid uptake across plasma membrane.

The initial rate of GS-7340 hydrolysis was marginally faster in quiescent cells than in activated PBMCs; however, the rate of TFV formation in cells was independent of their activation status. TFV-Ala reached a steady-state concentration approximately 2.5-fold higher in quiescent cells than in activated cells, suggesting higher activity of a phosphoamidase in activated cells (Chou et al., 2007). Alternatively, enhanced acidification of activated cells may cause more efficient conversion of TFV-Ala to TFV as a result of a limited stability of TFV-Ala under acidic conditions present in lysosomes. TFV-Ala exhibits $t_{1/2}$ of 72 and 28 min at pH 5.4 and 4.9, respectively (data not shown). Despite higher levels of

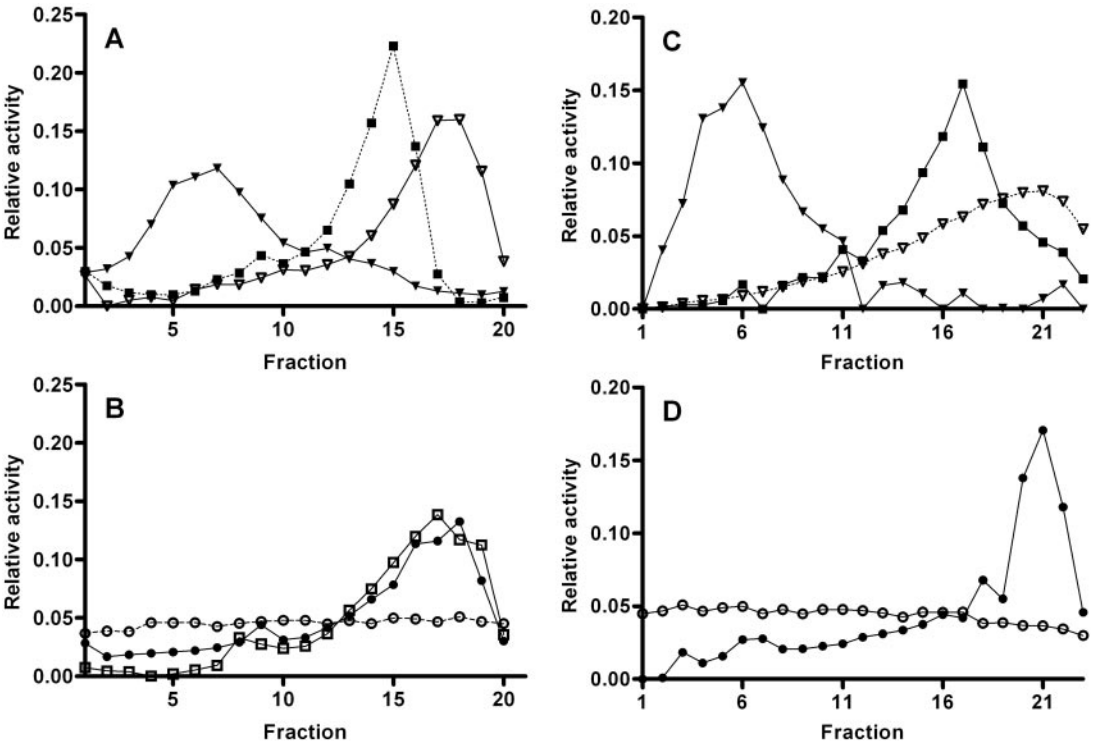

Fig. 5. Fractionation of MT-2 cells (A and B) and PBMCs (C and D) using Percoll gradient. Lysosomal markers hexosaminidase (▽) and chloroquine (□), mitochondrial marker succinate dehydrogenase (■), endoplasmic reticulum marker NADPH cytochrome *c* reductase (▼), GS-7340 hydrolase activity (●), and TFV metabolites (○)

TFV-Ala and TFV in quiescent cells, the overall rate of the intracellular accumulation of TFVpp was similar regardless of the PBMC activation state, suggesting more efficient phosphorylation of TFV in activated cells.

Hydrolysis of GS-7340 in PBMC extract was sensitive to serine protease inhibitors but not to inhibitors of cysteine, aspartic, or metalloproteases. In addition, subcellular fractionation of the MT-2 T-cells and PBMCs conclusively demonstrated that GS-7340 hydrolase is localized in the high-density lysosomal fraction (Fig. 4). Lysosomes and lysosome-related organelles are known to contain a wide variety of hydrolases, including serine proteases (Borregaard and Cowland, 1997; Blott and Griffiths, 2002). Because the prodrug moiety of GS-7340 can be viewed as a structural analog of Phe-Ala-Ala tripeptide (Fig. 3), these results suggested that lysosomal serine protease(s) are probably responsible for the hydrolysis of GS-7340. Consistent with this hypothesis, we have recently identified cathepsin A as an enzyme capable of effectively activating GS-7340 (Birkus et al., 2007). In this follow-up study, we identified multiple other lysosomal proteases that are able to cleave a set of structurally diverse phosphonoamidate prodrugs of TFV, including GS-7340.

Leukocyte elastase and proteinase 3 are elastase-like serine proteases and prefer peptide substrates containing small hydrophobic amino acids in P1 position (Blow, 1977; Harper et al., 1984; Kam et al., 1992). These proteases hydrolyzed TFV prodrugs containing Val, Ala, or α-ABA at P1-like position but not those with bulkier hydrophobic moieties such as Phe or Leu. Likewise, the chymotrypsin-related serine proteases cathepsin G and chymase, which prefer large hydrophobic residues in P1 position (Powers et al., 1985; Polanowska et al., 1998), efficiently hydrolyzed TFV prodrugs with Phe or Leu but not those with smaller amino acids, including GS-7340. In contrast, none of the tested TFV prodrugs were hydrolyzed by trypsin or granzymes A/B (Kam et al., 2000), which are known to display a strong preference for substrates with charged residues Lys/Arg or Asp at P1, respectively. Likewise, the relative specific activity of digestive serine proteases chymotrypsin and pancreatic elastase toward various TFV amidate prodrugs matched their preference for their peptide substrates (Bergman and Fruton, 1943). The susceptibility of TFV prodrugs to cleavage by digestive serine proteases may affect their stability in the gastrointestinal tract and consequently influence their oral bioavailability. In contrast to serine proteases, the primary structural determinant for the substrate specificity of papain-like cysteine endopeptidases is the P2 amino acid residue. Cathepsins B, H, and L all prefer hydrophobic amino acids, including Phe in the P2 position (Kärgel et al., 1980; Koga et al., 1992; Rothe and Dodt, 1992). Although all tested prodrugs contain phenol in the P2-like position that may mimic Phe residue, only cathepsins H and L were able to hydrolyze TFV amidates. This suggests that the phenol residue, which is shorter than natural Phe by one atom, may shift the scissile bond further from the enzyme catalytic dyad, thereby reducing the catalytic efficiency of substrate hydrolysis.

Cysteine protease cathepsin C and aspartyl protease cathepsin D both exhibit unique substrate specificity. The former cleaves two residues from the N terminus of polypeptide substrates (Turk et al., 1997). Cathepsin D has an elongated binding site cleft requiring interaction with up to seven amino acid residues in a polypeptide chain; therefore, it is inactive against shorter synthetic peptides (Press et al., 1960; Conner, 2004). Consistent with their substrate specificity, neither of these two enzymes hydrolyzed any of the tested TFV phosphonoamidate prodrugs. Taken together, these data demonstrate that the specificity of serine proteases toward TFV phosphonoamidate prodrugs closely matches their natural substrate specificity, indicating that nucleotide amidate prodrugs should be considered mimics of oligopeptide substrates.

PLCE, a close homolog of human liver carboxylesterase 1 (Lange et al., 2001), was shown to hydrolyze carboxyl ester bonds in a wide variety of substrates, including phosphoramidate prodrugs of nucleoside analogs such as 2′,3′-didehydro 3′-deoxythymidine (d4T) and 3′-azido 3′-deoxythymidine (AZT) (Balzarini et al., 1996; Valette et al., 1996). The current data indicate that PLCE displays relatively low specific activity against both GS-7340 and the other TFV prodrugs. However, because of its abundance in liver, the carboxylesterases may contribute to the hepatic clearance of this class of TFV prodrugs.

The present study demonstrates that multiple intracellular proteolytic enzymes exist that are capable of hydrolyzing GS-7340 and other TFV amidate prodrugs. More than 553 different human proteases have been identified thus far (Puente et al., 2003), many of them being specifically expressed only in a narrow range of tissues or body compartments. Even though cathepsin A is present in a variety of tissues, its expression is elevated in cells of the immune system (data not shown). This makes it an attractive target for the activation of prodrugs for HIV therapy. In contrast, other proteases have much more restricted expression patterns. For example, leukocyte elastase, proteinase 3, and cathepsin G are present in neutrophils (Owen and Campbell, 1999), granzymes in natural killer cells and cytotoxic T lymphocytes (Kam et al., 2000), and tryptases and chymases in mast cells (Sayama et al., 1987). Tissue-specific expression of prodrug hydrolases may allow for the rational design of nucleotide phosphonoamidate prodrugs that can be preferentially activated in relevant target cells. Furthermore, many viruses and other infectious agents produce their own specific proteases in host cells. These enzymes can be targeted for the activation of specific prodrugs, enhancing the therapeutic selectivity toward infected cells or tissues. Detailed knowl-

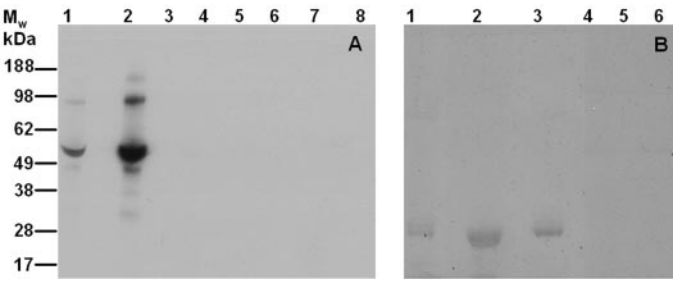

Fig. 6. Labeling of hydrolases with [^{14}C]GS-7340. Labeling of PLCE, BSA, Pr3, LE, and PE with [^{14}C]GS-7340 was performed as described under *Materials and Methods*. A, native PLCE labeled with: [^{14}C]GS-7340 (lane 1), [^{3}H]DFP (lane 2), preincubated with unlabeled DFP and labeled with [^{14}C]GS-7340 (lane 3); denatured PLCE labeled with: [^{14}C]GS-7340 (lane 4); [^{3}H]DFP (lane 5); preincubated with unlabeled DFP and labeled with [^{14}C]GS-7340 (lane 6); native (lane 7) and denatured (lane 8) BSA labeled with [^{14}C]GS-7340. B, native Pr3 (lane 1), PE (lane 2), and LE (lane 3) labeled with [^{14}C]GS-7340; denatured Pr3 (lane 4), PE (lane 5), and LE (lane 6) labeled with [^{14}C]GS-7340

edge about the localization and substrate preference of the targeted prodrug-activating proteases as well as hydrolytic enzymes potentially affecting absorption and/or metabolic elimination (e.g., digestive proteases and liver esterases) should facilitate the rational design of novel prodrugs with improved pharmacokinetic and therapeutic properties.

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