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Alkylglycerol Prodrugs of Phosphonoformate Are Potent In Vitro Inhibitors of Nucleoside-Resistant Human Immunodeficiency Virus Type 1 and Select for Resistance Mutations That Suppress Zidovudine Resistance

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Phosphonoformate (foscarnet; PFA) is a potent inhibitor of human immunodeficiency virus type 1 (HIV-1) reverse transcriptase (RT), but its use for the treatment of HIV-1 infection is limited by toxicity and the lack of an orally bioavailable formulation. Alkylglycerol-conjugated prodrugs of PFA (1-*O*-octadecyl-*sn*-glycero-3-PFA [B-PFA]) having *sn*-2 substituents of hydrogen (deoxybatyl-PFA [DB-PFA]), methyl (MB-PFA), or ethyl (EB-PFA) are more-potent inhibitors of wild-type HIV-1 in vitro than unmodified PFA and are orally bioavailable in mice. We have evaluated the activities of these compounds against a panel of nucleoside-resistant HIV-1 variants and have characterized the resistant variants that emerge following in vitro selection with the prodrugs. Except for an HIV-1 variant encoding the K65R mutation in RT that exhibited 3.3- to 8.2-fold resistance, the nucleoside-resistant viruses included in the panel were sensitive to the PFA prodrugs (<3-fold increase in 50% inhibitory concentration), including multinucleoside-resistant variants encoding the Q151M complex of mutations or the T69S[SA] insert. Viruses resistant to the PFA prodrugs (>10-fold) were selected in vitro after 15 or more serial passages of HIV-1 in MT-2 cells in escalating prodrug concentrations. Mutations detected in the resistant viruses were S117T, F160Y, and L214F (DB-PFA); M164I and L214F (MB-PFA); and W88G and L214F (EB-PFA). The S117T, F160Y, and M164I mutations have not been previously identified. Generation of recombinant viruses encoding the single and double mutations confirmed their roles in prodrug resistance, including 214F, which generally increased the level of resistance. When introduced into a zidovudine (AZT)-resistant background (67N 70R 215F 219Q), the W88G, S117T, F160Y, and M164I mutations reversed AZT resistance. This suppression of AZT resistance is consistent with the effects of other foscarnet resistance mutations that reduce ATP-dependent removal of AZT monophosphate from terminated template primers. The favorable activity and resistance profiles of these PFA prodrugs warrant their further evaluation as clinical candidates.

Phosphonoformic acid (PFA; foscarnet) is a potent inhibitor of herpesvirus, influenza, and retroviral polymerases (7) and has antiviral activity in human infections caused by herpes simplex virus, cytomegalovirus (CMV), and human immunodeficiency virus type 1 (HIV-1) (3, 32, 33). PFA is an analog of pyrophosphate, which is a byproduct of nucleotide polymerization and is composed of the β and γ phosphates cleaved from the incoming nucleotide triphosphate during incorporation into the nascent DNA strand. PFA is thought to inhibit HIV-1 replication by binding to reverse transcriptase (RT), preventing pyrophosphate release and thereby blocking further catalysis (7).

Mutations in HIV-1 RT that reduce sensitivity to PFA have been identified in resistant viruses selected in vitro and in isolates from HIV-infected patients treated with PFA. These mutations include W88G/S, E89K, L92I, S156A, Q161L, and H208Y (20, 29). In the three-dimensional structure of HIV-1 RT, these mutations cluster near the deoxynucleotide triphosphate (dNTP) and template-primer binding sites of RT (20). Most of the mutations that reduce susceptibility to PFA increase susceptibility to 3′-azido-3′-deoxythymidine (zidovudine; AZT) and suppress AZT resistance when introduced into an AZT-resistant genetic background (30).

Several potential mechanisms have been proposed whereby PFA or PFA resistance mutations could suppress or reverse AZT resistance. First, PFA may directly inhibit the removal of terminating nucleotide analogs via pyrophosphorylsis by competing with pyrophosphate for binding to RT (1, 2). Second, PFA resistance mutations may decrease the binding of ATP to RT, thereby reducing ATP-dependent removal of AZT from terminated template primers (21, 22). Alternatively, PFA resistance mutations may affect the binding of the terminated template primer to RT, thus reducing the efficiency of the removal reaction. These interactions between PFA and AZT resistance may explain why HIV-1 coresistant to PFA and AZT

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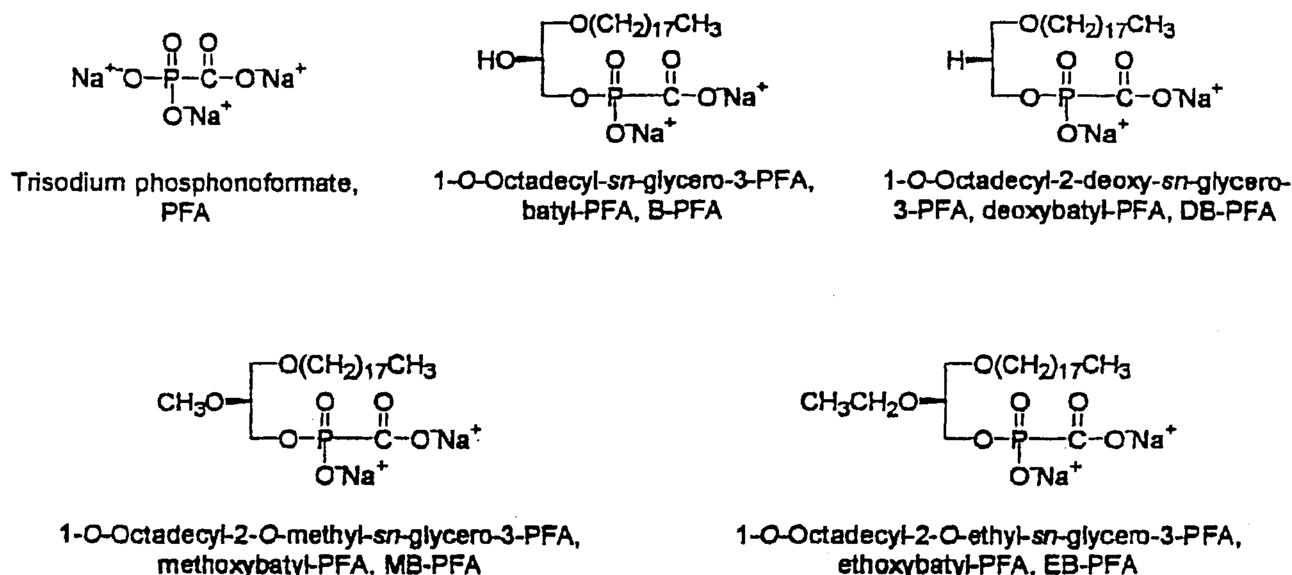

FIG. 1. Alkylglycerol PFA prodrug structures.

has not been isolated in vitro and has been reported only for one patient who received more than 20 months of combination therapy with PFA and AZT (31).

PFA has been used successfully to treat CMV retinitis and acyclovir-resistant herpes simplex virus, and it reduces HIV-1 RNA levels in HIV-infected patients (9, 10, 26), but several limitations prevent its practical use as an antiretroviral agent. These include the lack of an orally bioavailable formulation, necessitating intravenous administration, and toxicity manifested as electrolyte abnormalities (11) and renal failure (6). These limitations of PFA are likely due to its polyanionic nature at physiological pH. Its highly negative charge limits its ability to permeate cellular membranes and its gastrointestinal absorption. Its negative charge also results in the chelation and elimination of physiologically important cations such as calcium and magnesium, resulting in hypocalcemia, hypomagnesemia, and other electrolyte abnormalities (11).

To address these limitations of PFA, alkylglycerol prodrugs of PFA (Fig. 1) have been synthesized and evaluated. The prodrugs have the PFA moiety linked to 1-0-alkylglycerol at the *sn*-3 position of glycerol. This modification improves cellular uptake of PFA by decreasing its charge at physiologic pH from -3 to -2. Once the prodrug is inside the cell, intracellular enzymes metabolize it to yield free PFA and alkylglycerol (12). This release of PFA does not occur in fetal bovine serum (FBS)-supplemented tissue culture medium or conditioned MRC-5 medium (12). Modifications at the *sn*-1 and *sn*-2 sites of the glycerol backbone can improve antiviral activity and reduce cytotoxicity (13, 16). Previous structure-activity studies involving these compounds have shown that prodrugs containing alkyl chains of 14 to 22 carbons at the *sn*-1 position and alkyl chains of 1 to 3 carbons at the *sn*-2 position have optimal activity, with submicromolar 50% inhibitory concentrations (IC_{50}s) against wild-type HIV-1 and better selectivity indexes than unmodified PFA (13, 16). These prodrugs also have greater oral bioavailability in mice than unmodified PFA (K. Y. Hostetler, K. N. Wright, M. F. Gardner, and J. R. Beadle, Antivir. Ther. **46**, abstr. 111, p. A70, 2000). The objective of the present work was to further evaluate the in vitro activities of these compounds against a panel of PFA- and nucleoside analog reverse transcriptase inhibitor (NRTI)-resistant HIV-1 variants and to characterize resistant HIV-1 variants that are selected by the prodrugs in vitro.

MATERIALS AND METHODS

Chemicals. The following compounds were prepared as 5 mM liposomal preparations as previously reported (13, 16): 1-*O*-octadecyl-*sn*-glycero-3-PFA (B-PFA), 1-*O*-octadecyl-propanediol-3-PFA (DB-PFA), 1-*O*-octadecyl-2-*O*-methyl-*sn*-glycero-3-PFA (MB-PFA), and 1-*O*-octadecyl-2-*O*-ethyl-*sn*-glycero-3-PFA (EB-PFA). The compounds were stored at 4°C and warmed to 37°C immediately before use. AZT and PFA were purchased from Sigma Chemical Company, St. Louis, Mo. AZT and PFA were prepared as 10 or 30 mM stock solutions in dimethyl sulfoxide (DMSO) and sterile water, respectively, and stored at -20°C. Immediately before use, the compounds were warmed to 37°C and diluted to the desired concentrations in RPMI 1640 medium.

Cells. MT-2 cells (AIDS Research and Reference Reagent Program, National Institute of Allergy and Infectious Diseases, National Institutes of Health) were maintained in RPMI 1640 supplemented with 10% FBS (JRH Biosciences, Lenexa, Kans.), 10 mM HEPES buffer, 50 IU of penicillin/ml, and 50 μg of streptomycin/ml.

Viruses. Stock viruses were prepared by electroporating (with a Bio-Rad [Hercules, Calif.] Gene Pulser) MT-2 cells (1.3×10^7 cells) with 5 to 10 μg of plasmid DNA encoding an infectious proviral clone of HIV-1_{LAI} (23, 24). At peak cytopathic effect (CPE) (generally 5 to 7 days posttransfection), culture supernatants were harvested and stored at -80°C. Viral infectivity titers were determined in threefold endpoint dilution assays conducted in MT-2 cells (six wells per dilution). The 50% tissue culture infective dose ($TCID_{50}$) was calculated using the Reed and Muench equation (25).

Antiviral susceptibility assays. The antiviral activity of each compound was determined by inoculating MT-2 cells with HIV-1_{LAI} at a multiplicity of infection (MOI) of 0.01 $TCID_{50}$/cell, followed by incubation in the presence of threefold serial drug dilutions (three wells per dilution) (20). Five or 7 days after infection, culture supernatants were harvested, lysed with 0.5% Triton X-100, and assayed for p24 antigen concentration using a commercial enzyme-linked immunosorbent assay (ELISA) (DuPont, NEN Products, Wilmington, Del.). The antiviral activity of each compound is expressed as the IC_{50}, which is the concentration required to inhibit 50% of p24 antigen production. The fold resistance of a test

TABLE 1. Susceptibility of NRTI-resistant HIV-1 to PFA prodrugs

HIV-1 variant[a]	IC_{50}[b] (fold resistance)[c]			
	B-PFA	MB-PFA	EB-PFA	Free PFA
Wild type	1.79 ± 0.81	0.50 ± 0.48	0.65 ± 0.36	17.66 ± 10.12
K65R	14.68 ± 6.61 (8.2)	1.66 ± 0.72 (3.3)	2.91 ± 1.30 (4.5)	67.0 ± 22.79 (3.8)
L74V	4.45 ± 2.26 (2.5)	0.88 ± 0.63 (1.8)	1.21 ± 0.85 (1.9)	32.7 ± 9.00 (1.9)
M184V	1.93 ± 0.54 (0.7)	1.51 ± 0.45 (1.7)	1.36 ± 0.93 (0.7)	33.4 ± 18.7 (1.7)

[a] $HIV\text{-}1_{LAI}$ encoding the indicated resistance mutations.
[b] In micromolar concentrations; determined by measuring inhibition of p24 antigen production in MT-2 cells. See Materials and Methods for details. Values are means ± standard deviations from at least three independent experiments.
[c] Relative to wild-type virus.

virus is calculated by dividing the IC_{50} of the test virus by the IC_{50} of the $HIV\text{-}1_{LAI}$ control virus. Fold resistance values of 3 or greater were statistically significant. This is based on calculating the 95% confidence intervals (95% CIs) for the mean IC_{50}s of control and test viruses from at least three independent experiments. The 95% CIs for control and test viruses did not overlap if there was a threefold or greater difference in the mean IC_{50}s.

Selection of resistant viruses. Selections were initiated by inoculating 1.0×10^6 MT-2 cells at an MOI of 0.1 with plasmid-derived $HIV\text{-}1_{LAI}$ that had been passaged as cell-free virus 10 times in MT-2 cells in the absence of compound (4). Cells were pretreated with drug for 2 h prior to inoculation with virus. For each selection, the starting concentration was the IC_{50} of the compound, and the selective pressure (i.e., drug concentration) was doubled every three passages. Viral CPEs were monitored daily. At 2+ CPE (≥2 syncitia per 100× field), cell-free virus supernatant was harvested and used to initiate a new cycle of infection in fresh MT-2 cells. The passaged virus was monitored regularly for a reduction in susceptibility to the compounds by determining the IC_{50} relative to unpassaged $HIV\text{-}1_{LAI}$ (20).

Genetic analyses. Virions were pelleted from culture supernatants by centrifugation at 25,000 × *g* for 1 h. Total RNA was extracted from the virus pellet using TRIZOL Reagent (Gibco BRL, Grand Island, N.Y.) and resuspended in diethyl pyrocarbonate-treated sterile water. After cDNA synthesis, the full-length coding region of RT (amino acids [aa] 1 to 550) was amplified using PCR (4). The bulk PCR products were then purified using a commercially available kit (Wizard PCR Purification System; Promega, Madison, Wis.) and sequenced (aa 1 to 350) using an automated sequencer (Perkin-Elmer Biosciences, San Francisco, Calif.).

Generation of mutant recombinant HIV-1. HIV-1 containing the desired mutations were generated by oligonucleotide-directed mutagenesis (Altered Sites II; Promega) as previously described (19). After mutagenesis, mutant RT was subcloned into the $pxxHIV\text{-}1_{LAI}$ cloning vector using the silent *Xma*I and *Xba*I restriction sites located at the 5′ and 3′ ends of RT. Clones were DNA sequenced to verify the presence of the desired mutation(s) and were electroporated into MT-2 cells as described above to generate infectious mutant recombinant HIV-1.

RESULTS

Activities of PFA prodrugs against NRTI-resistant HIV-1. Octadecyl alkylglycerol analogs of PFA with -OH (B-PFA), -OCH_3 (MB-PFA), and $\text{-OCH}_2\text{CH}_3$ (EB-PFA) moieties at the *sn*-2 position of the glycerol backbone (Fig. 1) are potent inhibitors of wild-type $HIV\text{-}1_{LAI}$. The IC_{50}s for B-PFA, MB-PFA, and EB-PFA against $HIV\text{-}1_{LAI}$ are 1.40, 0.28, and 0.39 μM, respectively (13). In the present study, we evaluated the activities of these three analogs against a panel of NRTI-resistant HIV-1 variants.

The NRTI-resistant panel consisted of viruses containing mutations conferring resistance to single or multiple NRTIs (Tables 1 to 3). Of the viruses tested, only those encoding K65R demonstrated significant resistance to the prodrugs and unmodified PFA, with fold resistance values ranging from 3.3 to 8.2 (IC_{50}s, 1.66 to 14.68 μM). Viruses resistant to 3TC or ddI/ddC (containing M184V and L74V resistance mutations, respectively) were sensitive to both the PFA prodrugs and unmodified PFA (fold resistance, <3.0) (Table 1). The compounds were also evaluated against a panel of three multinucleoside-resistant (MNR) viruses (Table 2). The MNR panel consisted of a virus containing the mutations V75I, F77L, F116Y, and Q151M, a virus containing the T69S[SA] insert, and a clinical isolate carrying the classic MNR genotype (62V 75I 77L 116Y 151M). Each of the PFA prodrugs retained potency against these viruses with fold resistance values of <2.0. The sole exception was EB-PFA, which showed 4.5-fold loss of activity against the virus containing 75I 77L 116Y 151M.

AZT-resistant viruses tested against the prodrugs consisted

TABLE 2. Susceptibility of MNR HIV-1 to PFA prodrugs

HIV-1 variant	IC_{50}[a] (fold resistance)[b]			
	B-PFA	MB-PFA	EB-PFA	Free PFA
$HIV\text{-}1_{LAI}$[c]	1.8 ± 0.81	0.5 ± 0.48	0.7 ± 0.36	17.7 ± 10.12
NL4-3[c,g]	2.6 ± 1.5	2.3 ± 1.9	0.2 ± 0.2	15.1 ± 6.1
11163p3[d]	1.1 ± 0.53 (0.6)	0.4 ± 0.28 (0.9)	0.5 ± 0.06 (0.8)	15.5 ± 5.04 (0.9)
K[e,g]	4.2 (1.6)	2.0 ± 0.6 (0.87)	0.9 ± 0.7 (4.5)	21.4 ± 15.6 (0.7)
9GC[f,g]	4.1 ± 1.2 (1.6)	2.3 ± 0.5 (4.6)	0.2 ± 0.3 (0.3)	13.2 ± 12.8 (1.1)

[a] In micromolar concentrations; determined by measuring inhibition of p24 antigen production in MT-2 cells. See Materials and Methods for details. Values are means ± standard deviations from two or three independent experiments.
[b] Relative to wild-type virus.
[c] Wild-type virus.
[d] An MNR clinical isolate with the mutations A62V, V75I, F77L, K103N, F116Y, Q151M, Y181C, and M184V.
[e] A recombinant MNR virus with the mutations V75I, F77L, F116Y, and Q151M.
[f] A recombinant MNR virus with the mutations D67E, S68T, T69S[SA insert], and T215Y.
[g] Kindly provided by Mark Winters, Palo Alto, Calif.

TABLE 3. Susceptibility of AZT-resistant HIV-1 to PFA prodrugs

HIV-1 variant[a]	IC_{50}[b] (fold resistance)[c]			
	B-PFA	MB-PFA	EB-PFA	Free PFA
Wild type	1.79 ± 0.81	0.50 ± 0.48	0.65 ± 0.36	17.66 ± 10.12
M41L T215Y	0.94 ± 1.08 (0.5)	0.18 ± 0.11 (0.4)	0.30 ± 0.35 (0.5)	13.86 ± 4.14 (0.8)
4×AZT[d]	1.47 ± 0.63 (0.8)	0.58 ± 0.22 (1.2)	0.34 ± 0.24 (0.5)	12.7 ± 8.07 (0.7)
4×AZT M184V	2.29 ± 1.59 (1.3)	0.59 ± 0.36 (1.2)	0.56 ± 0.08 (0.9)	22.74 ± 15.81 (1.3)
4×AZT K103N	3.87 ± 2.36 (2.2)	0.60 ± 0.38 (1.2)	0.88 ± 0.14 (1.4)	19.22 ± 8.85 (1.1)
G2-3g[e]	1.67 ± 0.95 (0.9)	0.40 ± 0.26 (0.8)	0.30 ± 0.04 (0.5)	8.85 ± 3.49 (0.5)

[a] $HIV\text{-}1_{LAI}$ encoding the indicated resistance mutations.
[b] In micromolar concentrations; determined by measuring inhibition of p24 antigen production in MT-2 cells. See Materials and Methods for details. Values are means ± standard deviations from at least three independent experiments.
[c] Relative to wild-type virus.
[d] 4×AZT = D67N K70R T215Y K219Q.
[e] A molecularly cloned isolate coresistant to AZT and 3TC; M41L D67N M184V H208Y L210W R211K L214F T215Y 1293V E297A.

of $HIV\text{-}1_{LAI}$-derived recombinants with double (M41L T215Y) and quadruple (D67N K70R T215Y K219Q) mutations (Table 3). These AZT-resistant viruses were susceptible to each of the prodrugs and unmodified PFA. Virus containing both the quadruple AZT resistance mutations and the 3TC resistance mutation M184V ($HIV_{4XAZT\ M184V}$) or the nonnucleoside reverse transcriptase inhibitor (NNRTI) mutation K103N ($HIV_{4XAZT\ K103N}$) also showed sensitivity to the prodrugs (fold resistance, <3.0). Additionally, a molecularly cloned clinical isolate coresistant to AZT and 3TC (G2-3g) (15) was sensitive to each of the compounds, with fold resistance values of <1.0 (IC_{50}s, 0.30 to 1.67 μM).

Activities of PFA prodrugs against PFA-resistant and PFA- and AZT-resistant HIV-1. The PFA prodrugs were also tested against a panel of $HIV\text{-}1_{LAI}$-derived recombinants resistant to PFA (20, 29) (Table 4). As anticipated, PFA-resistant viruses showed similar levels of resistance to the PFA prodrugs. Virus containing the E89G mutation was least sensitive to the compounds, with fold resistance values ranging from >17.2 to >39.0. The compounds were also tested against a panel of $HIV\text{-}1_{LAI}$-derived recombinant viruses containing both PFA and AZT resistance mutations. As before, the viruses demonstrated similar levels of cross-resistance to both unmodified PFA and the PFA prodrugs. In general, the presence of AZT resistance mutations decreased the levels of cross-resistance to the PFA prodrugs. In the AZT-resistant genetic background, mutations at codon 89 (G or K) in RT conferred the greatest degree of resistance to both unmodified PFA and PFA prodrugs, with fold resistance values ranging from 5.6 to 11.1.

Selection of prodrug-resistant virus. Viruses resistant to the PFA prodrugs as well as unmodified PFA were selected in vitro by serial passage of $HIV\text{-}1_{LAI}$ in MT-2 cells in the presence of escalating concentrations of compound (Table 5). Virus exhibiting 27-fold resistance to MB-PFA was isolated after 18 rounds of cell-free virus passage. DNA sequencing of the RT gene (aa 1 to 350) from MB-PFA-resistant virus identified three mutations: V75L, M164I, and L214F. Recombinant viruses encoding the V75L, M164I, and L214F mutations were constructed and tested for susceptibility to MB-PFA (Table 6). Virus containing both M164I and L214F exhibited 9.3-fold resistance (IC_{50} = 8.1 μM). The V75L mutation alone or in combination with L214F or M164I did not cause significant (>3-fold) resistance to MB-PFA (data not shown). The selection of MB-PFA-resistant virus was repeated; after 15 passages

TABLE 4. PFA prodrug susceptibility of PFA and PFA/AZT Resistant HIV-1

HIV-1 variant[a]	IC_{50}[b] (fold resistance)[c]			
	B-PFA	MB-PFA	EB-PFA	Free PFA
Wild type	1.74 ± 0.79	0.77 ± 0.33	0.90 ± 0.73	13.06 ± 5.59
W88G	>30 (>17.2)	5.88 ± 4.42 (7.6)	6.8 ± 0.64 (7.6)	152.24 ± 14.28 (11.7)
W88S	20.7 ± 1.20 (11.9)	2.49 ± 0.12 (3.2)	2.39 ± 0.15 (2.7)	67.09 ± 6.76 (5.1)
E89G	>30 (>17.2)	>30 (>39.0)	>26.11 (>29.0)	241.25 ± 54.1 (18.5)
E89K	>30 (>17.2)	7.21 ± 0.12 (9.4)	5.21 ± 0.16 (5.8)	90.2 ± 46.24 (6.9)
Q161L	>30 (>17.2)	8.78 ± 1.58 (11.4)	6.73 ± 0.04 (7.5)	114.6 ± 14.14 (8.8)
Q161L H208Y	>30 (>17.2)	12.84 ± 9.00 (17.7)	7.02 ± 1.82 (7.8)	126.4 ± 33.09 (9.7)
4×AZT[d]	1.47 ± 0.63 (0.8)	0.58 ± 0.22 (1.2)	0.34 ± 0.24 (0.5)	12.7 ± 8.07 (0.7)
4×AZT W88G	16.37 ± 6.1 (6.2)	2.46 ± 0.11 (2.9)	3.31 ± 1.30 (4.2)	86.2 ± 12.16 (4.9)
4×AZT W88S	4.09 ± 1.84 (1.6)	1.54 ± 0.81 (1.8)	1.51 ± 0.21 (1.9)	18.44 ± 10.40 (1.0)
4×AZT E89K	17.39 ± 14.9 (6.6)	9.28 ± 9.20 (11.1)	4.39 ± 4.59 (5.6)	133.75 ± 122.72 (7.6)
4×AZT Q161L	6.64 ± 2.10 (2.5)	1.87 ± 0.33 (2.2)	1.18 ± 0.61 (1.5)	39.93 ± 9.40 (2.3)
4×AZT Q161L H208Y	6.2 ± 0.85 (2.4)	2.3 ± 0.14 (2.7)	2.02 ± 0.54 (2.6)	43.13 ± 17.50 (2.4)

[a] $HIV\text{-}1_{LAI}$ encoding the indicated resistance mutations.
[b] In micromolar concentrations; determined by measuring inhibition of p24 antigen production in MT-2 cells. See Materials and Methods for details. Values are means ± standard deviations from at least three independent experiments.
[c] Relative to wild-type virus.
[d] 4×AZT = D67N K70R T215Y K219Q.

TABLE 5. Mutations and altered susceptibility of PFA prodrug-resistant HIV-1 selected in vitro

Compound	Selection no.	Virus passage	Fold resistance[a]	Codon Δ[b]	aa Δ[b]
MB-PFA	1	15	27.0	GTA→TTA	V75L
				ATG→ATA	M164I
				CTT→TTT	L214F
	2	15	31.0	ATG→ATA	M164I
				CTT→TTT	L214F
DB-PFA	1	18	10.8	TCA→ACA	S117T
				CTT→TTT	L214F
	2	15	39.0	TCC→TAC	F160Y
				CTT→TTT	L214F
EB-PFA	1	15	41.0	TGG→GGG	W88G
				CTT→TTT	L214F
	2	15	13.0	TGG→GGG	W88G
				CTT→TTT	L214F
PFA	1	17	23.2	TGG→GGG	W88G
	2	15	23.2	TCA→ACA	S117T

[a] Relative to resistance of baseline virus (HIV-1_{LAI} at passage 0).
[b] Δ, change relative to baseline virus sequence (HIV-1_{LAI} at passage 0).

the selected virus exhibited 31-fold MB-PFA resistance and encoded the M164I and L214F mutations but not the V75L mutation (Table 5).

Virus exhibiting 10.8-fold resistance to DB-PFA was isolated after 18 rounds of cell-free passage. DNA sequencing identified two mutations in RT: S117T and L214F. Recombinant virus encoding S117T demonstrated 10.0-fold resistance to DB-PFA (IC_{50} = 9.0 μM) (Table 6). Addition of the L214F mutation to the virus encoding S117T did not increase the level of DB-PFA resistance (9.7-fold). Selection of DB-PFA-resistant virus was repeated; after 15 passages the selected virus exhibited 39-fold DB-PFA resistance and encoded the F160Y and L214F mutations. Recombinant virus encoding F160Y did not show resistance to DB-PFA (IC_{50} = 0.8 μM). Addition of the L214F mutation to the F160Y virus caused 17-fold resistance to DB-PFA (IC_{50} = 15.3 μM) (Table 6).

Virus exhibiting 41-fold resistance to EB-PFA and having W88G and L214F mutations was isolated after 15 rounds of cell-free passage. Recombinant virus encoding the W88G mutation showed 9.4-fold resistance, which was increased to 15.5-fold resistance (IC_{50} = 9.1 μM) when the L214F mutation was added (Table 6). EB-PFA-resistant virus was selected a second time after 15 passages. This virus also contained the W88G and L214F mutations.

As controls for the prodrug selections, HIV-1_{LAI} was passaged in the presence and absence of unmodified PFA. Virus demonstrating 23-fold resistance to PFA was selected after 15 and 17 cycles of cell-free passage in two independent selections. DNA sequence analysis of RT from these PFA-resistant viruses identified single mutations in RT: W88G (first selection) and S117T (second selection). The L214F mutation was not selected. Recombinant viruses having the W88G and S117T mutations showed 6.2- and 4.7-fold resistance to PFA, respectively. None of the mutations selected by the prodrugs or unmodified PFA were detected in control viruses passaged in the absence of drug.

Cross-resistance between PFA analogs and unmodified PFA. Recombinant viruses containing the mutations selected

TABLE 6. Susceptibility of mutant recombinant HIV-1_{LAI} to PFA analogs

HIV-1 variant[a]	IC_{50}[b] (fold resistance)[c]			
	DB-PFA	MB-PFA	EB-PFA	Free PFA
Wild type	0.89 ± 0.61	0.77 ± 0.32	0.59 ± 0.17	9.52 ± 6.68
L214F	5.04 ± 1.1 (4.7)	1.71 ± 1.15 (2.0)	1.95 ± 1.24 (3.3)	14.55 ± 2.68 (1.5)
S117T	13.11 ± 1.1 (12.3)			44.41 ± 4.85 (4.7)
S117T L214F	8.72 ± 3.4 (8.2)			46.60 ± 24.97 (4.9)
F160Y	0.8 ± 0.3 (0.9)			34.6 ± 19.6 (3.6)
F160Y L214F	15.0 ± 2.7 (17)			91.06 ± 34.9 (9.6)
M164I		2.14 ± 0.37 (2.5)		22.35 ± 14.18 (2.3)
M164I L214F		8.10 ± 1.47 (9.3)		62.74 ± 21.00 (6.6)
W88G			7.30 ± 5.1 (10.6)	59.02 ± 39.04 (6.2)
W88G L214F			14.32 (20.8)	121.37 ± 7.88 (12.7)

[a] HIV-1_{LAI} encoding the indicated resistance mutations.
[b] In micromolar concentrations; determined by measuring inhibition of p24 antigen production in MT-2 cells. See Materials and Methods for details. Values are means ± standard deviations from at least three independent experiments.
[c] Relative to wild-type virus.

TABLE 7. Susceptibility of mutant recombinant HIV-1_{LAI} to AZT

HIV-1 variant[a]	AZT IC_{50}[b] (μM)	Fold resistance[c]
Wild type	0.021 ± 0.008	
L214F	0.035 ± 0.033	1.7
S117T	0.017 ± 0.007	0.8
S117T L214F	0.022 ± 0.010	1.1
F160Y	0.005 ± 0.002	0.3
F160Y L214F	0.003 ± 0.001	0.2
M164I	0.012 ± 0.004	0.6
M164I L214F	0.008 ± 0.003	0.4
W88G	0.019 ± 0.013	0.9
W88G L214F	0.015 ± 0.015	0.7
Wild type	0.040 ± 0.006	
4×AZT[e]	0.290 ± 0.114	7.3
S117T 4×AZT	0.057 ± 0.057	1.4
F160Y 4×AZT	0.023 ± 0.012	0.6
M164I 4×AZT	0.043 ± 0.022	1.1
W88G 4×AZT	0.060 ± 0.018	1.5

[a] HIV-1_{LAI} encoding the indicated resistance mutations.
[b] Determined by measuring inhibition of p24 antigen production in MT-2 cells. See Materials and Methods for details. Values are means ± standard deviations from at least three independent experiments.
[c] Relative to wild-type virus.
[e] 4×AZT = D67N K70R T215Y K219Q.

by the PFA prodrugs were evaluated for their cross-resistance to unmodified PFA (Table 6). The S117T, F160Y, and W88G mutations were sufficient to confer significant resistance to unmodified PFA (4.7-, 3.6-, and 6.2-fold, respectively); however, the M164I mutation was not (2.3-fold resistance). The level of cross-resistance to PFA was increased by the addition of the L214F mutation (Table 6). In addition, each of the combinations of mutations selected by specific prodrugs conferred similar levels of cross-resistance to the other prodrugs, indicating general cross-resistance between members of the PFA prodrug class (data not shown).

Cross-resistance of PFA prodrug-selected mutations to AZT. Recombinant viruses containing the mutations selected by the PFA prodrugs were also evaluated for susceptibility to AZT (Table 7). None of the mutations selected by the prodrugs, including L214F, reduced sensitivity to AZT. The resistance mutations selected by the PFA prodrugs were also introduced into an AZT-resistant background (D67N K70R T215Y K219Q) to evaluate their effects on AZT resistance (Table 7). The S117T, F160Y, M164I, and W88G mutations all suppressed AZT resistance from 7.3-fold to 1.4-, 0.6-, 1.1-, and 1.5-fold, respectively.

DISCUSSION

We evaluated the in vitro activity of a series of alkylglycerol PFA analogs against a panel of NRTI- and PFA-resistant HIV-1 variants. The compounds were potent inhibitors of most NRTI-resistant viruses, including those encoding MNR mutations. Although unmodified PFA was also active against these NRTI-resistant variants, the potency of unmodified PFA was an order of magnitude lower than of the alkylglycerol analogs. All AZT-resistant variants tested, including those containing the 41L 215Y and 67N 70R 215Y 219Q mutations, were susceptible to the prodrugs.

The only NRTI-resistant virus showing cross-resistance to the prodrugs and PFA (3.3- to 8.2 fold) encodes the K65R mutation. The recently published "closed" RT crystal structure indicates that the K65 residue interacts with the γ phosphate of the incoming nucleotide triphosphate, and this interaction is important for the correct positioning of the incoming nucleotide (14). Because PFA is a structural analog of the β and γ phosphates of nucleotide triphosphates, it follows that mutations at residue 65 will reduce PFA binding. The K65R mutation was also recently shown by Sluis-Cremer and colleagues to decrease both the efficiency of the pyrophosphorylysis reaction by purified RT and the susceptibility of RT to PFA inhibition (27). Thus, the K65R mutation, although not selected by PFA or PFA prodrugs, causes resistance to both pyrophosphate and nucleoside/nucleotide analogs. The K65R mutation is rarely detected, however, in clinical isolates from patients treated with antiretroviral agents that are known to select for the mutation in vitro. The rarity of the K65R mutation may be related to its effects on dNTP binding, which could reduce the catalytic efficiency of RT and overall viral replicative fitness.

The resistance profiles of alkylglycerol PFA prodrugs with -H, $-OCH_3$, and $-OCH_2CH_3$ substitutions at the *sn*-2 position of the prodrugs were also evaluated in this study. RT mutations W88G, S117T, F160Y, M164I, and L214F were selected by the PFA prodrugs during in vitro passage in MT-2 cells in the presence of escalating concentrations of the compounds. These mutations were found to be necessary for resistance to the prodrugs when introduced via site-directed mutagenesis into a wild-type HIV-1_{LAI} background. Codons W88, S117, F160, and M164 are highly conserved in HIV-1_{LAI} RT, whereas L214F is a polymorphism previously associated with AZT-3TC coresistance (15, 28). W88G has been previously identified as a PFA resistance mutation (29), but S117T, F160Y, and M164I have not been previously associated with resistance to PFA or any RT inhibitor. These findings expand the number of mutations that are associated with HIV-1 resistance to PFA to 12: W88G/S, E89G/K, L921, S117T, S156A, F160Y, Q161L, M164I, H208Y, and L214F.

The L214F mutation was consistently selected by the PFA prodrugs ($n = 5$) but not by unmodified PFA ($n = 2$), both in this study and in prior studies on PFA resistance (20, 29). The L214F mutation appears to be necessary for high-level resistance to the prodrugs (Table 6). Although it was not selected by unmodified PFA, L214F also increases the level of resistance conferred by S117T, F160Y, M164I, and W88G to unmodified PFA. L214F may not have been selected by unmodified PFA because of the relatively low potency and selective pressure exerted by PFA compared with the prodrugs. The virus G2-3g, an AZT-3TC-coresistant virus included in the AZT-resistant virus panel, contains the mutation L214F in the context of AZT resistance mutations (Table 3). This virus was sensitive to the PFA prodrugs; therefore, the presence of the L214F mutation in an AZT-resistant background does not appear to result in coresistance to AZT and PFA.

None of the resistance mutations selected by the prodrugs conferred cross-resistance to AZT. When introduced into an AZT-resistant genetic background, W88G, S117T, F160Y, and M164I suppressed the AZT resistant phenotype. This result is consistent with the effects of other PFA resistance mutations that have also been found to reverse AZT resistance when present in an AZT-resistant genetic background (18, 30). This

phenotypic reversion is suspected to be due to a reduction in the ATP-dependent removal of AZT monophosphate from terminated template primers (21, 22). The PFA and PFA prodrug resistance mutations may reduce the binding of ATP, which catalyzes chain terminator removal.

Prior studies with PFA analogs indicate that the compounds are cleaved intracellularly to yield unmodified PFA (12). In vitro resistance selection studies, such as those conducted here, are a useful means of examining the interaction of an inhibitor with its target enzyme. In the present study, we have obtained several lines of additional evidence that support our prior findings that the prodrugs are cleaved intracellularly to yield free and active PFA. First, we have shown that the PFA prodrugs inhibit HIV-1 in vitro (13). Second, the prodrugs have activity profiles against a panel of drug-resistant mutants similar to that of unmodified PFA. Third, the prodrugs select for similar, although not identical, resistance mutations as unmodified PFA. Fourth, the resistance mutations selected for by the prodrugs also confer resistance to unmodified PFA. An explanation for why the prodrugs and PFA selected overlapping but not identical resistance mutations may be differences in the potency and thus selective pressure exerted by the prodrugs relative to unmodified PFA (13).

The data from this study allow several important conclusions. First, alkylglycerol PFA prodrugs are potent inhibitors of both wild type HIV-1_{LAI} and most NRTI-resistant variants. Second, the resistance mutations selected by the PFA prodrugs do not confer cross-resistance to AZT and have similar antagonistic interactions with AZT resistance mutations as those selected by unmodified PFA. Third, the PFA analogs are being cleaved intracellularly to yield free PFA, as evidenced by their activity profiles against drug-resistant HIV-1 being similar to that of unmodified PFA and by the similar resistance mutations that are selected in vitro.

Preliminary results from ongoing pharmacokinetic studies in mice indicate that the PFA analogs have greater oral bioavailability than unmodified PFA (Hostetler et al., Antivir. Ther. **46**, abstr. 111, 2000). Additional studies planned include animal toxicity profiling. The favorable activity and resistance profiles of these PFA analogs warrant their further evaluation as clinical candidates, particularly for use in salvage therapy and AZT combination regimens.

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