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Unique Intracellular Activation of the Potent Anti-Human Immunodeficiency Virus Agent 1592U89

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The anabolism of 1592U89, (−)-(1*S*,4*R*)-4-[2-amino-6-(cyclopropylamino)-9H-purin-9-yl]-2-cyclopentene-1-methanol, a selective inhibitor of human immunodeficiency virus (HIV), was characterized in human T-lymphoblastoid CD4$^+$ CEM cells. 1592U89 was ultimately anabolized to the triphosphate (TP) of the guanine analog (−)-carbovir (CBV), a potent inhibitor of HIV reverse transcriptase. However, less than 2% of intracellular 1592U89 was converted to CBV, an amount insufficient to account for the CBV-TP levels observed. 1592U89 was anabolized to its 5′-monophosphate (MP) by the recently characterized enzyme adenosine phosphotransferase, but neither its diphosphate (DP) nor its TP was detected. The MP, DP, and TP of CBV were found in cells incubated with either 1592U89 or CBV, with CBV-TP being the major phosphorylated species. We confirmed that CBV is phosphorylated by 5′-nucleotidase and that mycophenolic acid increased the formation of CBV-TP from CBV 75-fold. However, mycophenolic acid did not stimulate 1592U89 anabolism to CBV-TP. The adenosine deaminase inhibitor erythro-9-(2-hydroxy-3-nonyl)adenine (EHNA) did not inhibit CBV-TP formation from CBV or 1592U89, whereas the adenylate deaminase inhibitor 2′-deoxycoformycin selectively inhibited 1592U89 anabolism to CBV-TP and reversed the antiviral activity of 1592U89. 1592U89-MP was not a substrate for adenylate deaminase but was a substrate for a distinct cytosolic deaminase that was inhibited by 2′-deoxycoformycin-5′-MP. Thus, 1592U89 is phosphorylated by adenosine phosphotransferase to 1592U89-MP, which is converted by a novel cytosolic enzyme to CBV-MP. CBV-MP is then further phosphorylated to CBV-TP by cellular kinases. This unique activation pathway enables 1592U89 to overcome the pharmacokinetic and toxicological deficiencies of CBV while maintaining potent and selective anti-HIV activity.

Human immunodeficiency virus (HIV), the causative agent of AIDS, is a retrovirus that requires the unique viral enzyme reverse transcriptase (RT) to catalyze the conversion of viral RNA to proviral DNA. The RT of HIV has therefore been an attractive target in the chemotherapy of AIDS, and a variety of inhibitors, including purine and pyrimidine nucleoside analogs and nonnucleosides, have been identified (3, 12, 34). The nucleosides 3′-azido-3′-deoxythymidine (AZT), 2′,3′-dideoxyinosine, 2′,3′-dideoxycytosine, 2′,3′-dideoxy-2′,3′-didehydrothymidine, and (−)-(2′*R*,5′*S*)-1-[2-(hydroxymethyl)-1,3-oxathiolan-5-yl]cytosine require metabolism to 5′-triphosphates (5′-TPs) for activity. Since HIV does not encode viral kinases, the compounds must be phosphorylated by host cell kinases and phosphotransferases (3, 12, 24, 25, 34). Following intracellular conversion to nucleoside analog 5′-TPs, the compounds act selectively against HIV both as competitive inhibitors of the natural deoxynucleoside triphosphates at the RT and as DNA chain terminators following incorporation of the inhibitor into viral DNA, due to the lack of a 3′-hydroxyl required for further DNA chain elongation (19, 20, 34, 36).

We recently described the potent and selective anti-HIV activity of the novel carbocyclic dideoxynucleoside (−)-(1*S*,4*R*)-4-[2-amino-6-(cyclopropylamino)-9H-purin-9-yl]-2-cyclopentene-1-methanol (1592U89) (Fig. 1) (9, 10, 43). 1592U89 possesses a number of attributes which are desirable for an anti-HIV agent (10). In vitro, 1592U89 is equipotent to AZT against clinical isolates of HIV type 1 (HIV-1), is synergistic when used in combination with AZT and other anti-HIV agents (10, 43), and has an attractive cross-resistance profile (42, 43). Preclinical pharmacokinetic, disposition, and toxicology studies (8, 23) demonstrated that 1592U89 has significant advantages over the structurally related guanine analog (1*R*,4*S*)-9-[4-(hydroxymethyl)-2-cyclopenten-1-yl]guanine (carbovir [CBV]) (7, 26, 47). In the studies reported here, the cellular anabolism of 1592U89 is characterized and compared to that of CBV.

(Preliminary reports of this work have been presented previously [14, 15].)

MATERIALS AND METHODS

Materials. 1592U89, [5′-^{3}H]1592U89, (1*S*,4*R*)-4-[2,6-diamino-9H-purin-9-yl]-2-cyclopentene-1-methanol (aminoCBV), and CBV; their monophosphates (MPs), diphosphates (DPs), and TPs; and erythro-9-(2-hydroxy-3-nonyl)adenine (EHNA) were synthesized at Glaxo Wellcome Inc. (10). [8-^{3}H]1592U89, [8-^{3}H]aminoCBV, and [8-^{3}H]CBV were radiolabeled by Moravek Biochemicals, Inc. (Brea, Calif.). The radiolabeled compounds used were >98% pure. The radiochemicals were typically used at a specific activity of 1 to 2 Ci/mmol. [5′-^{3}H]1592U89-MP and [8-^{3}H]aminoCBV-MP were prepared at Glaxo Wellcome Inc. from the radiolabeled parent nucleosides by a previously described method (33). Nucleoside 5′-TPs were purchased from Sigma Chemical Co. (St. Louis, Mo.). One-Phor-All buffer, phosphodiesterase I, and calf intestinal alkaline phosphatase were obtained from Pharmacia Biotech, Inc. (Piscataway, N.J.). 2′-Deoxycoformycin (dCF) was from Parke-Davis (Morris Plains, N.J.), coformycin (CF) was from Calbiochem (San Diego, Calif.), and mycophenolic acid (MA) was from Eli Lilly (Indianapolis, Ind.). The MPs of dCF and CF were synthesized at Glaxo Wellcome Inc. as described previously (17). All other chemicals were reagent grade or better.

Cell culture. Human T-lymphoblastoid CD4$^+$ CEM cells (CEM-T4) were obtained from the National Institutes of Health AIDS Research and Reference Reagent Program (Rockville, Md.). The cells were grown in Cellgro RPMI 1640 medium with L-glutamine (Mediatech, Washington, D.C.) supplemented with 10% heat-inactivated fetal bovine serum (Hyclone Laboratories, Logan, Utah) and were maintained in logarithmic growth by passaging twice weekly. Experiments were conducted in uninfected CD4$^+$ CEM cells after showing that CBV-TP levels in these cells exposed to 10 μM 1592U89 were unaffected by HIV-1 infection (10).

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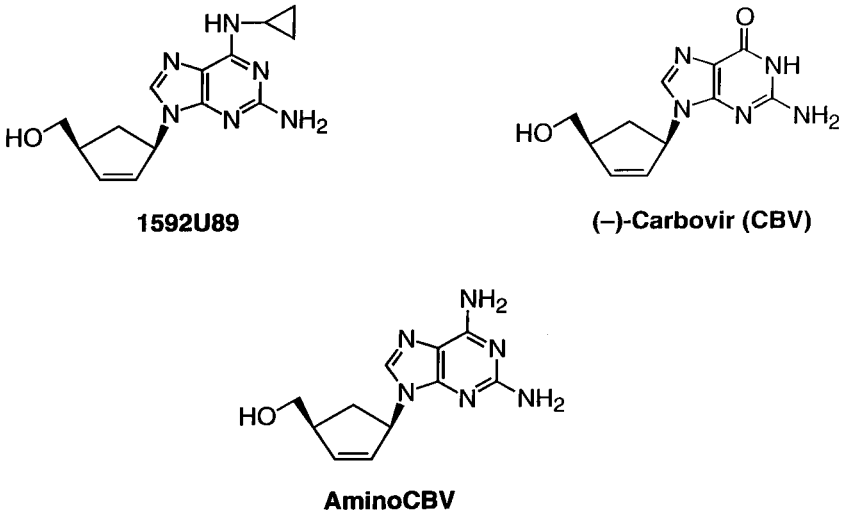

FIG. 1. Structures of 1592U89, aminoCBV, and CBV.

Anabolism in CD4$^+$ CEM cells. CD4$^+$ CEM cells were seeded at 2.5×10^5 cells/ml, and after 16 to 18 h of growth, the cultures were treated with [^{3}H]1592U89 or [^{3}H]CBV at the desired concentrations. At designated times following the addition of drugs, cell counts were determined with a hemocytometer, and the cells were pelleted by centrifugation, rinsed twice with ice-cold phosphate-buffered saline, and extracted with 3 ml of ice-cold 80% acetonitrile for 10 min. The extracts were centrifuged to remove cellular debris and evaporated to dryness in a Speed-Vac Concentrator (Savant, Farmingdale, N.Y.). The dried samples were stored at −80°C until analyzed by high-performance liquid chromatography (HPLC). The recovery of nucleoside 5′-TPs by this method was greater than 95%, as determined by the addition of recovery standards to replicate cell extracts.

HPLC. Dried nucleotide extracts were reconstituted in deionized water, and the MPs, DPs, and TPs were analyzed by strong anion-exchange (SAX) HPLC using a Partisil-10 SAX 4.6 by 250-mm analytical column (Alltech Associates Inc., Deerfield, Ill.) eluted at 1 ml/min by using the following multistep gradient: step 1, a 10-min isocratic elution in 100% mobile phase A; step 2, a 75-min linear gradient to 60% mobile phase B; step 3, a 10-min linear gradient to 100% mobile phase B; and step 4, a 5-min isocratic elution in 100% mobile phase B. Aqueous mobile phase A contained 2.5 mM KCl, 5 mM $MgCl_2$, 0.05% acetonitrile, 20% ethanol, and 10 mM ammonium phosphate, pH 3.8; aqueous mobile phase B contained 250 mM KCl, 100 mM $MgCl_2$, 5% acetonitrile, 20% ethanol, and 250 mM ammonium phosphate, pH 3.0. An abbreviated form of the above method, consisting of a 30-min linear gradient from 25% mobile phase B to 100% mobile phase B, was used to quantitate nucleoside TPs only. The mobile phases for the abbreviated gradient were identical to those above, except that the pH of mobile phase B was 3.4. Aliquots of the reconstituted samples were also analyzed by reversed-phase (RP) HPLC to identify nucleosides and nucleoside MPs, using a Rainin Microsorb MV 4.6 by 250-mm, 5-μm C_{18} column eluted at 1 ml/min by using the following multistep gradient: step 1, an isocratic elution for 5 min in 100% mobile phase C; step 2, a 15-min linear gradient to 35% mobile phase D; step 3, a 10-min linear gradient to 100% mobile phase D; and step 4, a 10-min isocratic elution in 100% mobile phase D. Aqueous mobile phase C was 25 mM ammonium acetate and 0.1% triethylamine, pH 4.25; mobile phase D was 50 mM ammonium phosphate, 0.1% triethylamine (pH 7.2), and 35% acetonitrile in deionized water. The UV absorbance of the column effluent was monitored at 254 and 294 nm, and fractions were collected at 30-s to 1-min intervals. The radioactivity in each collected fraction was determined by liquid scintillation counting.

Quantitation and identification of compounds. The amount of phosphorylated compound present per 10^6 cells was calculated from the amount of radioactivity in each peak by using the predetermined specific activity of each nucleoside analog. Because the specific activities and the absolute number of cells analyzed varied somewhat for each experiment, the limit of detection of radiolabeled compound also varied but was generally less than 0.004 pmol/10^6 cells. To convert concentration units from picomoles per 10^6 cells to micromolar concentrations, a factor of 0.8 μl of intracellular water per 10^6 CEM cells was used (11, 38).

Radioactive peaks were tentatively identified by comparing their retention times to those of authentic standards. The identities of CBV-DP and CBV-TP were also confirmed by peak shift analysis. Radioactive peaks eluting at the retention times for the DP and TP were collected, and the mobile-phase salts were precipitated by the addition of 0.1 volume of concentrated ammonium hydroxide and 3 volumes of methanol. The resulting supernatants were evaporated to dryness (Speed-Vac), redissolved in One-Phor-All buffer, and digested overnight at 37°C with 4 U of phosphodiesterase I and 20 U of calf intestinal alkaline phosphatase. The resulting samples were deproteinated by centrifugation in Ultrafree cellulose membrane filters (molecular weight cutoff, 30,000; Millipore Corp., Bedford, Mass.). Authentic, nonlabeled nucleoside standards were added, and the samples were analyzed by RP HPLC as described above.

Incubations with enzyme inhibitors. CD4$^+$ CEM cells were incubated for 30 min in the presence or absence of 5 μM MA prior to addition of [^{3}H]1592U89 or [^{3}H]CBV to a final concentration of 10 μM (5). A control group of CD4$^+$ CEM cells was also treated with 5 μM MA to assess the effect of the inhibitor on intracellular IMP pools. The MA remained in the medium for the duration of the experiment. Duplicate cultures were harvested at 6 and 24 h, and cell extracts were prepared and analyzed as described above.

CD4$^+$ CEM cells were treated with 10 μM [^{3}H]1592U89, [^{3}H]CBV, or [^{3}H]aminoCBV. Triplicates in each group were either untreated or treated with 1 μM EHNA or dCF 30 min prior to receiving the ^{3}H-labeled compounds. The EHNA or dCF remained in the medium for the duration of the experiment. All treatment groups were harvested 24 h following the addition of the ^{3}H-labeled compounds, and cell extracts were prepared and analyzed by SAX HPLC and RP HPLC as described above.

The antiviral activities of 1592U89, CBV, and aminoCBV were determined in CD4$^+$ CEM cells by quantitation of HIV-1 (strain IIIB) RT protein in a 5-day assay, as described elsewhere (13). Replicates in each group were either untreated or treated with 0.1 or 1 μM EHNA or dCF 30 min prior to addition of compounds. The EHNA or dCF remained in the medium for the duration of the experiment.

Subcellular fractionations. Subcellular fractionation of rat liver was performed by the differential sucrose gradient method of Fleischer and Kervina (16), using five livers from fasted male CD rats (Charles River Breeding Laboratories, Wilmington, Mass.). Cytosol was prepared from human peripheral blood lymphocytes (PBLs; approximately 10^9 cells) isolated by density gradient centrifugation on Ficoll-Paque (Pharmacia LKB, Uppsala, Sweden) from a single healthy donor buffy coat. Cytosol was also isolated from CD4$^+$ CEM cells (2×10^9 cells). The collected cells were lysed in 6 ml of 0.1 M potassium phosphate, pH 7.4, with a Dounce homogenizer followed by brief sonication. The cell homogenates were centrifuged (105,000 × *g*, 45 min), and the supernatant was recovered and stored at −80°C. Protein concentrations were determined by the Bradford method (6).

Enzyme assays. The assays for adenosine phosphotransferase (from human placenta), 5′-nucleotidase (inosine phosphotransferase; from human placenta), and GMP kinase (from hog brain) activities were performed as described elsewhere (22, 33). The assay mixtures for thymidine kinase (from CEM cells) and deoxyguanosine kinase (from human brain mitochondria) contained 25 μM nucleoside and 10 mM ATP (28). Thymidine kinase reactions were tested at pH 7.5, and deoxyguanosine kinase reactions were tested at pHs 5.5 and 8.0. The assay mixtures for deoxycytidine kinase from calf thymus contained 1 mM nucleoside and 5 mM ATP (21). The assay mixtures for adenosine kinase from rabbit liver contained 0.1 mM nucleoside and 1 mM ATP (32). AMP deaminase (AMPDA) bound to myosin was prepared and assayed as described elsewhere (39). The assay for adenosine deaminase (ADA) from calf intestine was performed as previously described (41). The assay for human erythrocyte purine nucleoside phosphorylase was performed as described elsewhere (45); the phos-

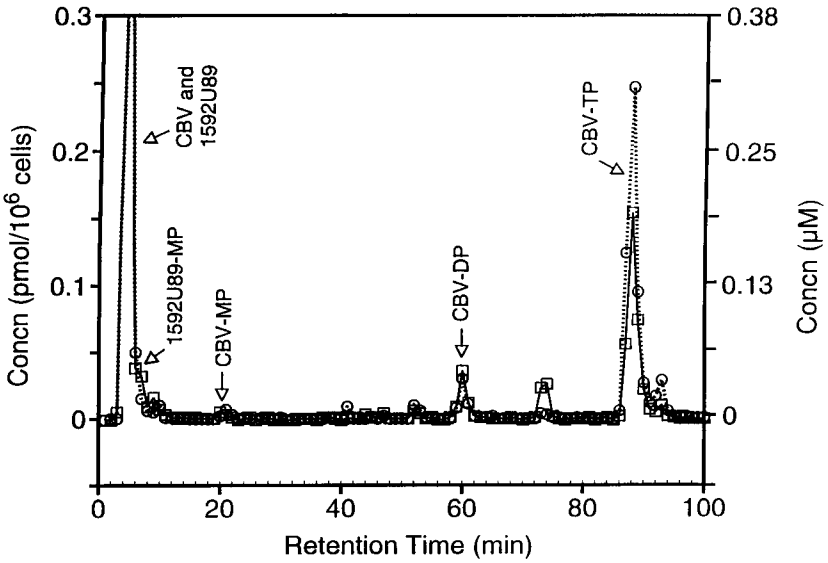

FIG. 2. SAX HPLC radioactivity elution profiles of intracellular extracts from CD4$^+$ CEM cells incubated with [^{3}H]1592U89 or [^{3}H]CBV. CD4$^+$ CEM cells incubated with 10 μM [8-^{3}H]1592U89 or [^{3}H]CBV for 48 h were extracted and analyzed by SAX HPLC as described in Materials and Methods. Circles, CBV; squares, 1592U89.

phorolysis of 1592U89 (at 0.1 and 0.33 mM) was monitored at 261.5 nm ($\Delta\varepsilon = -2.87$ mM^{-1} cm^{-1}). Deamination of [^{3}H]1592U89, [^{3}H]1592U89-MP, and [^{3}H]aminoCBV-MP by subcellular fractions was assessed at 37°C in mixtures containing a 0.1 to 20 μM concentration of each substrate and 2 mg of protein per ml in 50 mM potassium phosphate, pH 7.4. Aliquots of the deamination reaction mixtures were assayed at various times by RP HPLC coupled to a radiochemical detector (FloOne Beta; Packard Instruments, Meriden, Conn.) to determine the percent conversion of starting substrate to a deaminated species.

RESULTS

Intracellular metabolism in CD4$^+$ CEM cells. Typical SAX HPLC radioactivity elution profiles of acetonitrile extracts from CD4$^+$ CEM cells treated for 48 h with a 10 μM concentration of either [^{3}H]1592U89 or [^{3}H]CBV are shown in Fig. 2. Elution profiles of extracts from cells incubated with these nucleoside analogs for other times (3, 6, 12, 25, and 72 h) were qualitatively similar. The retention times for authentic CBV, CBV-MP, CBV-DP, and CBV-TP were approximately 5, 22, 60, and 87 min, respectively. The elution times for authentic 1592U89, 1592U89-MP, 1592U89-DP, and 1592U89-TP were approximately 5, 6, 48, and 71 min, respectively. Due to the lack of resolution with SAX HPLC, 1592U89-MP and the two nucleoside analogs were quantitated by RP HPLC. Neither 1592U89-DP nor 1592U89-TP was detected following incubations of cells with 10 μM 1592U89. In the SAX radiochromatograms, the peak eluting near 72 min in this gradient was also seen in extracts of cells incubated with CBV. The peak eluting immediately following CBV-TP in the profiles was GTP, most likely resulting from a minor guanosine-related contaminant, as observed by others (31, 37, 48).

The identities of CBV-DP and CBV-TP formed during incubations of CD4$^+$ CEM cells with 10 μM [^{3}H]1592U89 were confirmed by peak shift analysis on RP HPLC. A single peak of radioactivity coeluting with authentic CBV was obtained from both the DP and TP peak digests, thus verifying that the anabolites were phosphorylated CBV. Similar results were obtained from CEM cells treated with [^{3}H]CBV.

The time course of anabolism of 10 μM [^{3}H]1592U89 or [^{3}H]CBV to CBV-TP in CD4$^+$ CEM cells is shown in Fig. 3. The CBV-TP levels produced from 1592U89 (0.16 to 0.55 pmol/10^6 cells) were up to threefold lower than the CBV-TP levels formed from CBV during the first 48 h. However, similar CBV-TP levels were found at 72 h in CD4$^+$ CEM cells treated with either 1592U89 or CBV. The decline in CBV-TP levels at 72 h may be related to slower growth as the cells approach saturation density.

The profile of intracellular metabolites produced in CD4$^+$ CEM cells treated for 24 h with either 10 μM [^{3}H]1592U89 or [^{3}H]CBV is shown in Table 1. The levels of phosphorylated CBV species generated from CBV reported here agree well with those previously reported (4, 37). Both parent compounds appeared to be concentrated in the cells to 21 to 22 μM, relative to the 10 μM medium concentration. The concentration of CBV in the 1592U89-treated cells was less than 1.5% of that of 1592U89 and less than 1.5% of the CBV levels in CBV-treated cells. Both compounds produced CBV-MP, CBV-DP, and CBV-TP, with CBV-TP as the major phosphorylated form. Although the amount of CBV in 1592U89-treated cells was only 1.2% of that in CBV-treated cells, CBV-TP levels in 1592U89-treated cells were 50% of the CBV-TP levels in CBV-treated cells. In 1592U89-treated cells, 1592U89-MP was also detected at levels near 0.1 pmol/10^6 cells, which is eightfold higher than the levels of CBV-MP produced from 1592U89 in these same cells. However, 1592U89-DP and 1592U89-TP were not detected in these cell extracts. AminoCBV and its MP were also observed following incubation with 1592U89. As expected, the modified nucleobase of 1592U89, 2-amino-6-(cyclopropylamino)-9H-purine, was not detected in the cellular extracts. This is consistent with the finding that, like CBV (31), 1592U89 was not a substrate of human purine nucleoside phosphorylase (results not shown).

Specificity of phosphorylation of 1592U89. 1592U89 exhibited a unique enzymatic phosphorylation profile compared to other known antiviral nucleosides. Adenosine phosphotransferase from human placenta (22) enantioselectively phosphorylated 1592U89 at a rate 780-fold higher than that of the inactive (+) enantiomer (Table 2). The maximum velocity (V_{max}) for 1592U89 was 55% and the substrate efficiency (V_{max}/K_m) was 3.6% of the corresponding values for adenosine. The efficiency

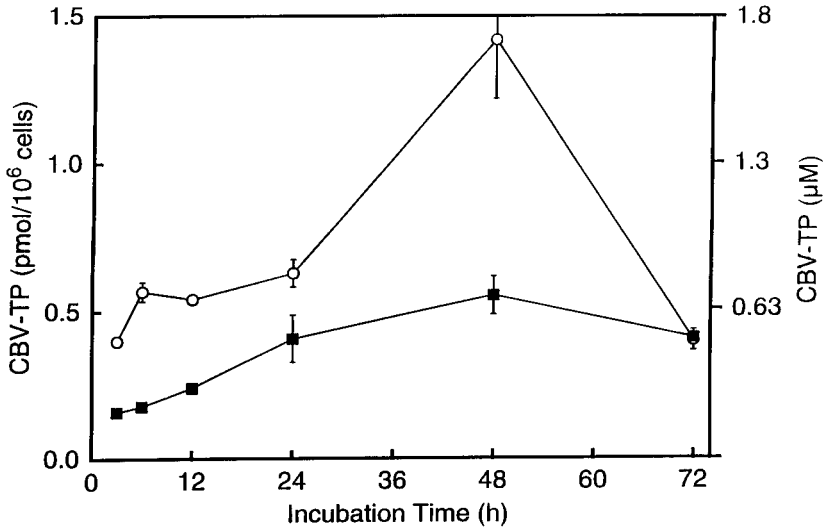

FIG. 3. Levels of CBV-TP formed in CD4$^+$ CEM cells incubated with 10 μM [^{3}H]1592U89 or [^{3}H]CBV. CD4$^+$ CEM cells were incubated with 10 μM [5′-^{3}H]1592U89 or [^{3}H]CBV, and at the times shown triplicate cultures were extracted and analyzed as described in Materials and Methods. Results shown are the means ± standard deviations from three samples at each point. Squares, 1592U89; circles, CBV.

of phosphorylation of aminoCBV was half that of 1592U89. In contrast to 1592U89 and aminoCBV, CBV was not a detectable substrate for adenosine phosphotransferase.

5′-Nucleotidase (inosine phosphotransferase) was shown to efficiently phosphorylate CBV (Table 2), as previously described (27, 33). 5′-Nucleotidase was enantioselective, phosphorylating 1592U89 but not its (+) enantiomer. However, the rate of phosphorylation was very low (1% of the rate with inosine) and close to the lower limit of detection for the assay. Because of low phosphorylation rates, the K_m could not be calculated for either 1592U89 or aminoCBV.

As previously reported (33), CBV-MP (but not its enantiomer) was a good substrate for GMP kinase from pig brain (Table 2). The K_m for CBV-MP was lower than that of the natural substrate GMP, but the V_{max} was only 1.9% of that of GMP, resulting in a substrate efficiency (V_{max}/K_m) 38% of that of GMP. In contrast, 1592U89-MP was an inefficient substrate for phosphorylation by GMP kinase, with a K_m >20-fold higher than and a V_{max} only 0.1% of that for GMP, resulting in a relative substrate efficiency of only 0.005%. The enantiomer of 1592U89-MP was an even poorer substrate for GMP kinase.

TABLE 1. Levels of intracellular anabolites in CD4$^+$ CEM cells incubated for 24 h with 10 μM [8-^{3}H]1592U89 or [^{3}H]CBV

Compound	Mean amt (pmol/10^6 cells) ± SEM (n)[a] from:	
	1592U89	CBV
1592U89	17 ± 2 (6)	ND
Nucleobase[b]	ND	ND
1592U89-MP	0.073 ± 0.008 (6)	ND
1592U89-DP	ND	ND
1592U89-TP	ND	ND
AminoCBV	0.18 ± 0.02 (6)	ND
AminoCBV-MP	0.10 ± 0.03 (3)	ND
CBV	0.22 ± 0.04 (6)	18 ± 2 (5)
CBV-MP	0.009 ± 0.003 (6)	0.053 ± 0.008 (8)
CBV-DP	0.083 ± 0.026 (9)	0.13 ± 0.03 (8)
CBV-TP	0.16 ± 0.02 (9)	0.33 ± 0.06 (8)

[a] n, number of samples analyzed. ND, not detected.
[b] 2-Amino-6-(cyclopropylamino)purine from 1592U89 or guanine from CBV.

1592U89 was not a substrate for enzymes known to phosphorylate other nucleoside analogs, namely, thymidine kinase, deoxycytidine kinase, adenosine kinase, or mitochondrial deoxyguanosine kinase (data not shown). The MPs of CBV and 1592U89 were not substrates for AMP kinase, dCMP kinase, or nucleoside MP kinase (data not shown). These data are consistent with the lack of observable 1592U89-DP or -TP in CD4$^+$ CEM cells incubated with 1592U89.

TABLE 2. Unique enzymatic phosphorylation profile for 1592U89

Activity	Substrate	K_m (mM)	V_{max}		V_{max}/K_m
			nmol/min/mg	% of natural substrate[a]	
Adenosine phosphotransferase[b]	Adenosine	0.22	87	100	390
	1592U89	3.2	47	55	14
	(+)-1592U89	NQ[c]	0.06[d]	0.07	NQ
	CBV	NQ	<0.02[d]	<0.02	NQ
	AminoCBV	6.4	47	55	7.4
5′-Nucleotidase[e]	Inosine	3.8	720	100	180
	1592U89	NQ	7.3[f]	1	NQ
	(+)-1592U89	NQ	<3.3[f]	<0.5	NQ
	CBV	6.3	400	56	63
	AminoCBV	NQ	3.5[f]	0.5	NQ
GMP kinase[g]	GMP	22	30,000	100	1,400
	1592U89-MP	500	37	0.1	0.07
	(+)-1592U89-MP[h]	NQ	<1	<0.003	NQ
	CBV-MP	1.1	580	1.9	530
	(+)-CBV-MP[h]	270	11	0.04	0.04

[a] For adenosine phosphotransferase, percentage of adenosine; for 5′-nucleotidase, percentage of inosine; for GMP kinase, percentage of GMP.
[b] From human placenta (22). AMP was the phosphate donor at 5 mM.
[c] NQ, not quantifiable.
[d] Rate at 0.5 mM nucleoside, compared with rate at 75 μM adenosine.
[e] Inosine phosphotransferase from human placenta (33). IMP was the phosphate donor at 5 mM.
[f] Rate at 0.5 mM nucleoside, compared with rate at 1 mM inosine.
[g] From pig brain (33). ATP was the phosphate donor at 4 mM.
[h] MP prepared from the (+) enantiomer of 1592U89 or CBV.

TABLE 3. Effects on CBV-TP levels produced from 10 μM [5′-^{3}H]1592U89 or [^{3}H]CBV

Treatment		pmol of CBV-TP/10^6 cells[a]	
Drug	Time (h)	Untreated	MA treated
CBV	6	0.32 ± 0.04	10 ± 0.4
	24	0.33 ± 0.06	25 ± 1.7
1592U89	6	0.09 ± 0.01	0.05 ± 0.03
	24	0.26 ± 0.04	0.22 ± 0.07

[a] Results are the means ± standard deviations (n = 2).

Effects of enzyme inhibitors on the formation of CBV-TP. CD4$^+$ CEM cells treated with 5 μM MA had an 80- to 100-fold increase in the intracellular pool of IMP at both 6 h (from 4.2 ± 0.5 to 410 ± 18 pmol/10^6 cells) and 24 h (from 2.3 ± 0.2 to 190 ± 28 pmol/10^6 cells). Concomitant with the increase in IMP levels, the intracellular levels of GTP fell 5- to 10-fold (from 470 ± 36 to 49 ± 4 pmol/10^6 cells at 6 h and from 400 ± 12 to 78 ± 8 pmol/10^6 cells at 24 h), as would be expected from the MA block of the de novo GTP synthetic pathway (2). The ATP levels in the cells remained essentially unchanged by MA. The other endogenous nucleotide pools were not examined in this experiment.

In CD4$^+$ CEM cells treated with 10 μM CBV, MA produced dramatic 32- and 75-fold increases in CBV-TP levels at 6 and 24 h, respectively (Table 3). In contrast to the results obtained with CBV, the addition of MA to incubations with 10 μM 1592U89 did not stimulate the anabolism of 1592U89 to CBV-TP.

The ability of ADA or AMPDA to produce CBV or CBV-MP from 1592U89 and aminoCBV and their MPs was investigated. The effect of the ADA-specific inhibitor EHNA (1) or the adenosine/adenylate deaminase inhibitor dCF (18) on the anabolism of 10 μM [^{3}H]CBV, [^{3}H]1592U89, and [^{3}H]amino CBV to CBV-TP was examined in CD4$^+$ CEM cells treated for 24 h (Table 4). The generation of CBV-TP from 1592U89 was inhibited 95% by dCF but was not inhibited by EHNA. Since dCF blocks metabolic deamination at the MP stage while EHNA cannot, these results suggest that 1592U89 is phosphorylated prior to deamination. As expected, neither ADA nor AMPDA had a direct role in the anabolism of CBV, as evidenced by the fact that neither EHNA nor dCF inhibited CBV-TP formation from CBV. (The observed slight stimulation of CBV anabolism to CBV-TP in the EHNA and dCF groups may be due to perturbations in the pools of donor phosphates.)

As expected with aminoCBV, a known ADA substrate, incubations with either EHNA or dCF abolished the production of CBV-TP. Greater than 90% of the aminoCBV in CD4$^+$ CEM cells was metabolized to CBV (data not shown). The addition of either EHNA or dCF inhibited production of CBV and its phosphates from aminoCBV by greater than 95%. Despite the resultant large increase in intracellular aminoCBV following EHNA or dCF treatments, aminoCBV-MP levels were not raised above the levels found in untreated cultures, and formation of CBV-TP was blocked. Thus, in aminoCBV-treated cells CBV-TP is produced via CBV rather than via aminoCBV-MP.

TABLE 4. Effects of 1.0 μM EHNA or dCF on CBV-TP levels formed from 10 μM [5′-^{3}H]1592U89, [^{3}H]CBV, or [^{3}H]aminoCBV in CD4$^+$ CEM cells

Compound	pmol of CBV-TP/10^6 cells[a]		
	Untreated	EHNA treated	dCF treated
CBV	0.81 ± 0.006[b]	1.5 ± 0.07	0.96 ± 0.03
1592U89	0.26 ± 0.04	0.31 ± 0.01	0.016 ± 0.002
AminoCBV	0.36 ± 0.003	0.013 ± 0.003	0.002 ± 0.003

[a] Results are the means ± standard deviations (n = 3, except where noted).
[b] n = 2.

TABLE 5. Specificity of conversion of 1592U89-MP to CBV-MP by cytosolic preparations from CD4$^+$ CEM cells, human PBLs, and rat liver

Cytosol	% Conversion[a]		
	1592U89-MP to CBV-MP	AminoCBV-MP to CBV-MP	1592U89 to CBV
CEM cell	29	<0.1	3.2
Human PBL	11	<0.1	<0.1
Rat liver	70	<0.1	0.9

[a] Incubations were for 2 h with 1 μM [^{3}H]1592U89-MP, [^{3}H]aminoCBV, or [5′-^{3}H]1592U89.

The effects of EHNA and dCF on the antiviral activities of 1592U89, CBV, and aminoCBV were examined in HIV-1 (strain IIIB)-infected CD4$^+$ CEM cells. EHNA, at 0.1 and 1.0 μM, did not significantly affect the antiviral activity of 1592U89 (data not shown). In contrast, the activity of 1592U89 was nearly abolished by 1 μM dCF. Both EHNA and dCF inhibited the activity of aminoCBV, but neither inhibited the activity of CBV. These results correlate the antiviral activities of 1592U89, aminoCBV, and CBV with the formation of CBV-TP (Table 4).

Deamination of 1592U89 and aminoCBV and their MPs. AminoCBV (at 100 μM) and aminoCBV-MP (at 2.0 mM) were poor substrates for ADA and AMPDA, with respective rates of deamination 0.08 and 0.07% of those of adenosine and AMP (both at 100 μM). 1592U89 (at 100 μM) and 1592U89-MP (at 2.0 mM) were exceedingly poor substrates for ADA and AMPDA, respectively. The relative rate of deamination of 1592U89 was 0.00003% of that of adenosine; the rate of deamination of 1592U89-MP was 0.000004% of that of AMP.

dCF reversal of the antiviral activity of 1592U89 would seem to imply involvement of AMPDA in the activation pathway. However, the extremely poor deamination of 1592U89-MP by this enzyme led us to look for an alternative enzymatic activity with sensitivity to dCF. Cytosolic preparations from CD4$^+$ CEM cells and also from human PBLs were found to contain a deaminating activity that converted 1592U89-MP to CBV-MP (Table 5). [^{3}H]1592U89-MP was used in these incubations at low concentrations (0.1 to 20 μM; only 1 μM results shown) to mimic the intracellular levels found in treated cells. The deamination activity was destroyed by heating at 100°C for 5 min or by treatment with proteinase K (not shown). The protein catalyzing the deamination was highly specific for 1592U89-MP; neither 1592U89 nor aminoCBV-MP was a substrate for deamination. The minor amounts of CBV formed from 1592U89 in both CEM and rat liver cytosol are likely due to the deamination by ADA of a small [^{3}H]aminoCBV contaminant present in these incubations (data not shown). Since CEM cell cytosol and PBL cytosol are not suitable as large-scale sources of this enzymatic activity, rat liver was examined for activity. As shown in Table 5, rat liver cytosol also contained deaminating activity specific for 1592U89-MP. Therefore, further characterization of this activity was performed with rat liver.

The localization of the enzymatic activity was confirmed to be cytosolic by examining subcellular fractions isolated from

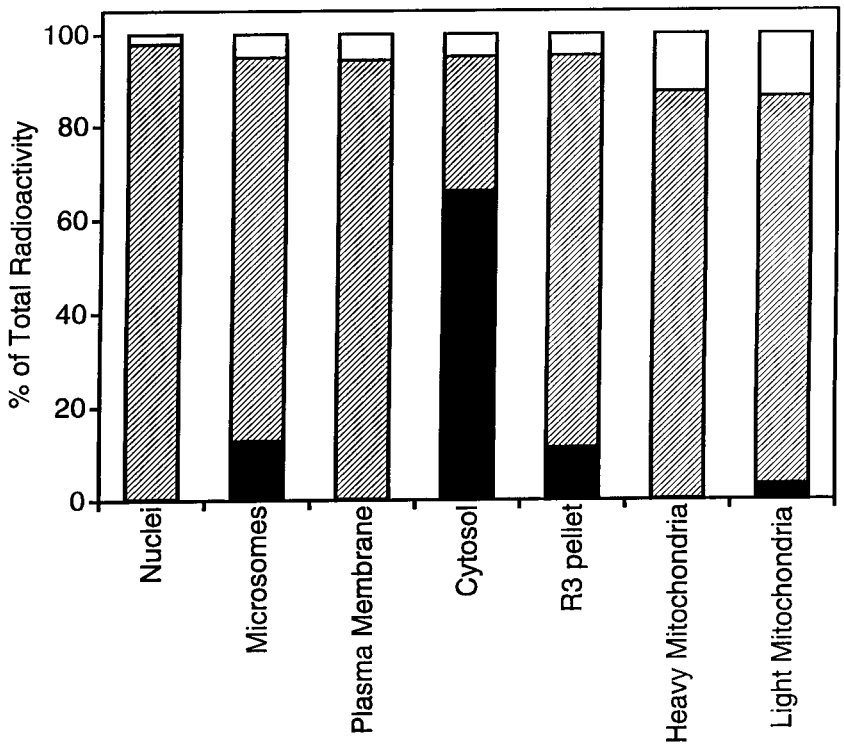

FIG. 4. Subcellular localization of 1592U89-MP deamination activity. The enzymatic activity in rat liver subcellular fractions was determined in incubations with 1 μM [^{3}H]1592U89-MP, and the fraction of radioactivity present as 1592U89, 1592U89-MP, and CBV-MP was determined as described in Materials and Methods. Solid bars, CBV-MP; open bars, 1592U89; hatched bars, 1592U89-MP (starting substrate).

rat liver (Fig. 4). [^{3}H]1592U89-MP was converted to [^{3}H]CBV-MP with protein from the cytosolic, microsomal, and R3 pellet fractions of rat liver. The microsomal and R3 pellet fractions are those last in contact with the cytosol during the fractionation procedure (16), and thus activity present in these fractions most likely represents residual cytosolic contamination. 1592U89 was also produced from [^{3}H]1592U89-MP by the action of phosphatases in the fractions. [^{3}H]1592U89-MP was nearly completely converted to CBV-MP after 4 h of incubation (data not shown). The deamination of 1 μM [^{3}H]1592U89-MP was linear over the first 1 h of incubation, with an apparent rate of 1.4 pmol of 1592U89-MP deaminated per min per mg of protein.

The effects of inhibitors on the deamination of [^{3}H]1592U89-MP were examined in cytosolic preparations of rat liver, CD4$^+$ CEM cells, and PBLs. At concentrations up to 1 μM, EHNA, dCF, and CF did not inhibit the conversion of 1592U89-MP to CBV-MP. In contrast to the nucleosides, the MPs of dCF and CF were potent inhibitors in all three cytosolic preparations. The conversion of 1 μM [^{3}H]1592U89-MP to [^{3}H]CBV-MP in rat liver cytosol was 95% complete in 4 h. In the presence of 1 nM dCF-MP or CF-MP, the conversion was inhibited 60 or 67%, respectively. Formation of [^{3}H]CBV-MP was completely prevented by 1 μM dCF-MP or CF-MP. Thus, the inhibitory effects of dCF on 1592U89 anabolism to CBV-TP (Table 4) and on antiviral activity in CD4$^+$ CEM cells are most likely mediated by intracellularly produced dCF-MP (40, 46).

DISCUSSION

1592U89 is a selective inhibitor of HIV-1 (IIIB) replication in vitro (9, 10, 43) with a 50% inhibitory concentration of 3.8 μM in CD4$^+$ CEM cells. However, 1592U89-TP, which competes with dATP for incorporation into viral DNA, is a poor inhibitor of HIV RT (10) and is not found in CD4$^+$ CEM cells incubated with 1592U89. Instead, 1592U89 is ultimately anabolized to CBV-TP, a potent inhibitor of HIV RT that acts both as a competitive inhibitor of dGTP for DNA incorporation and as a DNA chain terminator following incorporation (33, 35, 36, 49). The anabolism of 1592U89 to CBV-TP occurs via a unique pathway involving four steps (depicted in Fig. 5). 5′-Monophosphorylation was shown to precede the conversion of the modified N^6-substituted 2,6-diaminopurine moiety to a guanine. Intrinsic to this pathway is the nature of the N^6 modification of the 1592U89 purine ring.

Enzyme and inhibitor studies elucidated the anabolic pathway for 1592U89. The metabolism of 1592U89 was compared to that of CBV in human CD4$^+$ CEM cells. The relative intracellular levels of the phosphorylated species of CBV (TP > DP > MP) (Table 1) suggest that once CBV-MP is formed, either from CBV or 1592U89, the subsequent conversions to CBV-DP and CBV-TP are not rate limiting. The anabolism of CBV to CBV-TP is a known, direct pathway. CBV is anabolized to CBV-MP by cytosolic 5′-nucleotidase (Table 2), for which IMP is the preferred and limiting phosphate donor (4, 27, 33). The IMP dehydrogenase inhibitor MA greatly enhanced the anabolism of CBV to CBV-TP in CD4$^+$ CEM cells by raising IMP levels, a result consistent with previous reports (4, 27, 37). CBV-MP is efficiently anabolized further by cellular kinases to the DP and TP forms (33). In contrast to CBV, the anabolism of 1592U89 to CBV-TP in CD4$^+$ CEM cells was not stimulated by treatment with MA (Table 3). This result argues against 1592U89 anabolism proceeding through CBV and is in agreement with the fact that 1592U89 itself is a very poor substrate for 5′-nucleotidase (Table 2). Furthermore, the observation that EHNA inhibits CBV-TP formation from amino

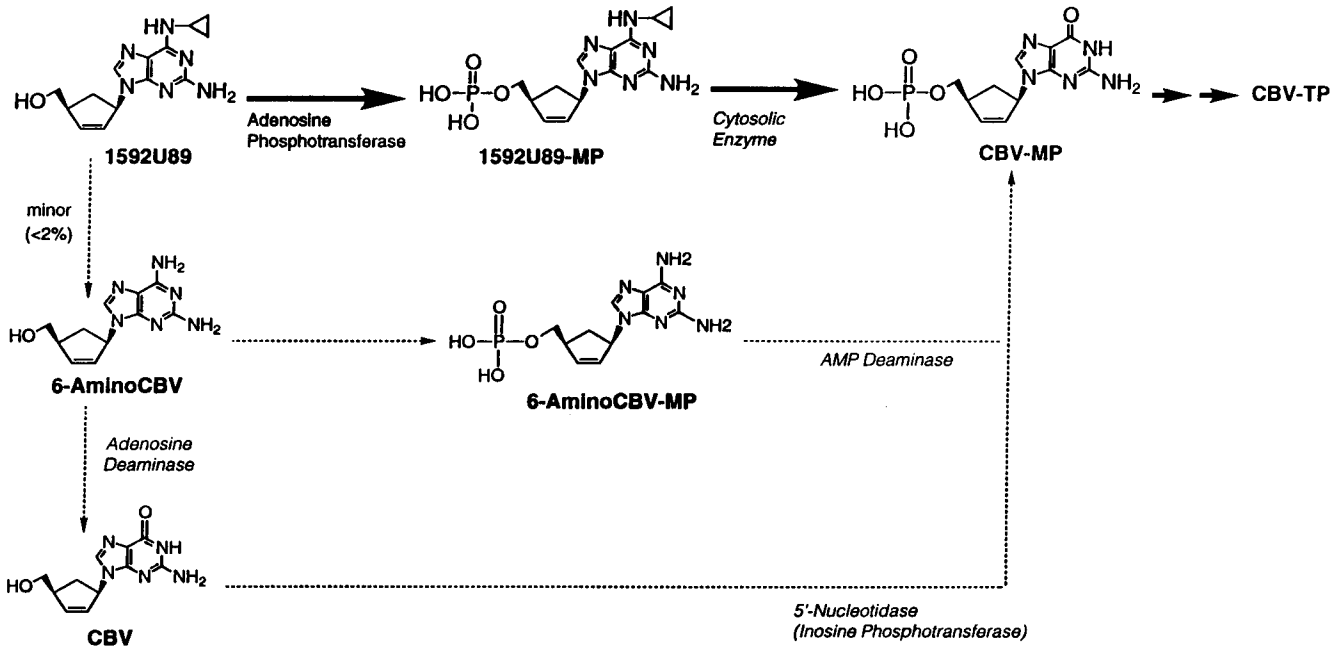

FIG. 5. Proposed pathway for the anabolism of 1592U89 to CBV-TP in human cells. 1592U89 is phosphorylated by adenosine phosphotransferase to 1592U89-MP, which is converted to CBV-MP by a cytosolic deaminase. CBV-MP is then efficiently anabolized to CBV-TP.

CBV but not from 1592U89 indicates that the anabolic pathway for 1592U89 does not involve aminoCBV.

The aminoCBV-MP found in $CD4^+$ CEM cells incubated with 1592U89 could provide an alternate path to CBV-MP (Fig. 5). The present data do not rule out a small contribution to the formation of CBV-TP through this pathway. However, aminoCBV-MP is not a substrate for the cytosolic enzyme responsible for converting 1592U89-MP to CBV-MP (Table 5). Since aminoCBV-MP is an inefficient substrate for AMPDA, formation of CBV-TP via aminoCBV-MP is predicted to be a minor contributor to the levels of CBV-TP generated from 1592U89 in the cell. Consistent with this prediction is the observation that CBV-TP levels were unaffected by EHNA in $CD4^+$ CEM cells incubated with 1592U89, whereas EHNA inhibited CBV-TP formation by 95% in aminoCBV incubations (Table 4) without affecting levels of aminoCBV-MP (data not shown).

While CBV-TP was the active species generated from 1592U89, 1592U89 did not act as a direct prodrug of CBV. 1592U89 was an exceedingly poor substrate for deamination, and less than 1.5% of intracellular 1592U89 was present as CBV in $CD4^+$ CEM cells treated with 10 μM 1592U89 (Table 1). It is readily apparent from CBV dose-response data (10) that this amount of CBV (approximately 0.25 μM) is insufficient to generate the levels of CBV-TP observed in cells treated with 10 μM 1592U89. It is relevant to note that the plasma and urinary CBV levels detected in animals dosed with 1592U89 were also less than 2% of the corresponding levels of 1592U89 (23).

1592U89 was a substrate for phosphorylation by the novel enzyme adenosine phosphotransferase (Table 2), an enzyme that has only recently been described (22). This is a unique first step of phosphorylation that other antiviral nucleosides do not share, since they are phosphorylated by nucleoside kinases or other phosphotransferases. Thus, it was of interest to assess whether this activity could account for the amount of phosphorylated anabolites of 1592U89 that were observed when 1592U89 was incubated with $CD4^+$ CEM cells. When $CD4^+$ CEM cell extract was assayed directly for adenosine phosphotransferase with 0.5 mM 1592U89, the rate of 1592U89 phosphorylation was about 0.6 pmol/min/10^6 cells. An extrapolation with the Michaelis-Menten equation would reduce the rate to about 18 pmol/24 h/10^6 cells when 1592U89 was at 10 μM. When $CD4^+$ CEM cells were incubated with 10 μM 1592U89 for 24 h, approximately 0.4 pmol of total phosphorylated anabolites per 10^6 cells was measured. Thus, adenosine phosphotransferase could account for the total amount of 1592U89 phosphorylation in $CD4^+$ CEM cells. Although 1592U89-MP was produced in $CD4^+$ CEM cells, further anabolism to 1592U89-DP and 1592U89-TP was not detectable. This observation is consistent with the finding that 1592U89-MP is a very poor substrate for nucleoside MP kinases.

Inhibitor studies with the compounds dCF and CF also suggested that 1592U89 is phosphorylated prior to deamination. The formation of CBV-TP from 1592U89 was almost completely prevented by dCF (an inhibitor of both ADA and AMPDA [18]), whereas EHNA (a selective inhibitor of ADA [1]) did not inhibit CBV-TP formation from 1592U89. In accord with these anabolism results, the anti-HIV activity of 1592U89 was also inhibited by coincubation with dCF but not EHNA. However, although dCF and CF are known potent inhibitors of isolated AMPDA (18), this enzyme was apparently not responsible for the deamination of 1592U89-MP. Instead, another cytosolic enzyme that catalyzed the conversion of 1592U89-MP to CBV-MP was identified. The specificity of this enzyme for 1592U89-MP (Table 5) and its selective inhibition by only the MPs of dCF and CF suggested that the activity was distinct from AMPDA. Ongoing studies have further distinguished this activity from that of AMPDA (32a), and work is in progress to characterize this novel enzyme. A cautionary note is therefore added here for interpretation of any studies in which assessment of AMPDA activity is based solely on inhibition by dCF and/or CF.

Both 1592U89 and CBV appeared to be concentrated ap-

proximately twofold in $CD4^+$ CEM cells during drug incubations. While CBV has been shown to permeate the membrane in erythrocytes by facilitated diffusion, primarily via a nucleobase carrier (29), 1592U89 enters both erythrocytes and $CD4^+$ CEM cells by nonfacilitated diffusion with a relatively high rate constant (30). The intracellular levels of CBV and 1592U89 found in $CD4^+$ CEM cells are in agreement with those observed in the cellular transport studies, where cytosolic protein binding has been suggested as a mechanism for concentrative accumulation of purine nucleoside analogs (29).

The contrasting in vivo pharmacokinetic and toxicological properties of 1592U89 and CBV provided early evidence that 1592U89 was not simply enhancing the oral delivery of CBV. Toxicology studies with 1592U89 succinate (8) did not produce the renal and cardiac toxicities observed with CBV (44), even at the highest doses studied (1,000 mg/kg of body weight per day in mice and 440 mg/kg/day in monkeys for 3 months). The properties of enhanced solubility and increased lipophilicity imparted to 1592U89 by the cyclopropylamino group are likely key factors in its improved oral bioavailability and central nervous system penetration compared to CBV (10). The elucidation of the unique mechanism by which 1592U89 delivers the guanine TP analog to cells while bypassing the guanine nucleoside CBV now provides a biochemical explanation for the marked in vivo differences between the two compounds. The guanine nature of 1592U89 is essentially masked until phosphorylation can occur. Crossover to the guanine at the level of the monophosphorylated species ultimately provides the level of CBV-TP required for inhibition of the RT without producing potentially toxic levels of CBV (CBV levels are $<2\%$ of 1592U89 in animals [23] and cells). The existence of this unique activation pathway enables 1592U89 to overcome deficiencies of CBV (which include low oral bioavailability, minimal brain penetration, and renal and cardiac toxicities) while maintaining potent and selective anti-HIV activity.

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