Nonnucleoside reverse transcriptase inhibitors that potently and specifically block human immunodeficiency virus type 1 replication

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ABSTRACT Certain bis(heteroaryl)piperazines (BHAPs) are potent inhibitors of the human immunodeficiency virus type 1 (HIV-1) reverse transcriptase (RT) at concentrations lower by 2–4 orders of magnitude than that which inhibits normal cellular DNA polymerase activity. Combination of a BHAP with nucleoside analog HIV-1 RT inhibitors suggested that together these compounds inhibited RT synergistically. In three human lymphocytic cell systems using several laboratory and clinical HIV-1 isolates, the BHAPs blocked HIV-1 replication with potencies nearly identical to those of 3'-azido-2',3'-dideoxythymidine or 2',3'-dideoxyadenosine; in primary cultures of human peripheral blood mononuclear cells, concentrations of these antiviral agents were lower by at least 3–4 orders of magnitude than cytotoxic levels. The BHAPs do not inhibit replication of HIV-2, the simian or feline immunodeficiency virus, or Rauscher murine leukemia virus in culture. Evaluation of a BHAP in HIV-1-infected SCID-hu mice (severe combined immunodeficient mice implanted with human fetal lymph node) showed that the compound could block HIV-1 replication *in vivo*. The BHAPs are readily obtained synthetically and have been extensively characterized in preclinical evaluations. These compounds hold promise for the treatment of HIV-1 infection.

The reverse transcriptase (RT) encoded by human immunodeficiency virus type 1 (HIV-1) catalyzes the conversion of the viral genomic RNA into proviral DNA (1, 2). Since RT is essential for virus replication and has no closely related identified cellular homolog, it has been the prime target for antiviral therapy against the acquired immunodeficiency syndrome (AIDS; refs. 3 and 4). This strategy is appropriate since 3'-azido-2',3'-dideoxythymidine (AZT), a nucleoside analog inhibitor of reverse transcription, was the first drug shown to benefit HIV-1-infected individuals (5). Other nucleoside analog RT inhibitors also show promise in clinical evaluations (6, 7). However, the administration of these drugs to patients is usually limited by serious toxicities (7, 8). In addition, HIV-1 with reduced AZT-sensitivity has been obtained from AZT-treated patients, suggesting the emergence of resistant virus will limit the drug's efficacy (9, 10). Thus, effective prolonged treatment of HIV-1 infection likely requires the discovery of other, perhaps multiple, RT inhibitors. To this end, we and others (11, 12) have sought to identify other nonnucleoside HIV-1 RT inhibitors.

MATERIALS AND METHODS

Cell Culture and Virus Infections. The cell cultures were maintained at 37°C in 5% CO₂/95% air. The HIV-1 infectivity studies were conducted in MT-2 cells, peripheral blood mononuclear cells (PBMC), and H9 cells as described (9,

13–15). In brief, MT-2 or H9 cells were infected with HIV-1 (IIIb isolate) at a multiplicity of infection of 0.001. In MT-2 cells syncytium formation was determined 4 days after infection at the peak of the viral cytopathic effect (13). In H9 cells, growth medium with fresh drug was replaced every 3–4 days, and at 14 days—the time of peak HIV-1 RT activity in the culture supernatants—the levels of RT activity and p24 in supernatants were determined (14). Cell viability was determined by trypan blue exclusion. In PBMC, 1×10^6 cells were infected with an inoculum containing 1 ng of p24 of the HIV-1 D34 isolate. The level of HIV-1 replication was determined 4 days after infection by measuring the levels of supernatant p24 and HIV-1 RNA as described (15, 16); cell viability was determined by measuring the levels of mitogen-stimulated cell proliferation (15). Titered stocks of AZT-sensitive virus [HIV-1 A018 pre-AZT; AZT 50% effective antiviral dose (ED₅₀) = 0.01 μ M] and AZT-resistant virus (HIV-1 A018 post-AZT; ED₅₀ = 4.4 μ M) were obtained from Douglas Richman, University of California, San Diego and used to infect MT-4 cells at 10–100 tissue culture 50% infective dose (TCID₅₀) per 10^5 cells (9). To obtain HIV-1 clinical isolates, PBMC were obtained by Ficoll/Hypaque gradient centrifugation of blood samples obtained from HIV-1-infected patients. HIV-1 isolates were amplified by cocultivating these infected PBMC with uninfected human PBMC obtained from normal donors; culture supernatants containing virus were harvested after less than five cell passages. For the infectivity studies, an inoculum of 10–50 ng of p24 of each isolate per 10^6 cells was used.

Inhibition of Enzymes *in Vitro*. A recombinant HIV-1 RT (17) was purified and assayed as described (18) with poly(rA)-oligo(dT) as template-primer. DNA polymerases α and δ and proliferating cell nuclear antigen were purified from fetal calf thymus and assayed as described (19) with poly(rA)-oligo(dT) as template-primer. To determine whether synergistic, additive, or antagonistic effects were achieved with combinations of drugs, the multiple drug effect analysis of Chou and Talalay (20) was used to calculate combined drug effects. This method involves plotting dose-response curves for each drug and for multiple diluted fixed-ratio combinations of the drugs by using the median-effect equation. The slopes of the plots (m), which signify the shapes of the dose-response curves, and the x intercepts of the plots (IC₅₀), which signify the potency of the drugs and combinations, were then used to calculate the combination indices (CI): CI < 1 indicates synergy, CI = 1 indicates additivity, and CI > 1 indicates antagonism.

Chemistry. The BHAPs were synthesized by coupling the appropriate carboxylic acids with the substituted (pyri-

Abbreviations: HIV, human immunodeficiency virus; RT, reverse transcriptase; AZT, 3'-azido-2',3'-dideoxythymidine; AZTTP, AZT triphosphate; ddTTP, 2',3'-dideoxythymidine triphosphate; PBMC, peripheral blood mononuclear cells; SCID-hu, severe combined immunodeficient mice implanted with human fetal lymph node; BHAP, bis(heteroaryl)piperazine; CI, combination indices.

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diyl)piperazines using 1,1'-carbonyldiimidazole or 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) in tetrahydrofuran. Aromatic nucleophilic substitution of 2-chloro-3-nitropyridine with excess piperazine in acetonitrile provided 1-(3-nitro-2-pyridyl)piperazine, which was subsequently N-protected with [2-(*tert*-butoxycarbonyloxyimino)-2-phenylacetone] (BOC-ON) or di-*tert*-butyl dicarbonate. Reduction of the 3-nitro group to the amine was accomplished with hydrogen (40 psi; 1 psi = 6.89 kPa) and 10% palladium on carbon. Reductive amination (acetaldehyde/sodium cyanoborohydride/methanol or acetone/sodium cyanoborohydride/acetic acid/methanol) and deprotection (trifluoroacetic acid/methylene chloride or hydrochloric acid/methanol) yielded the substituted (pyridyl)piperazines. The indole-2-carboxylic acids are available commercially (Aldrich). The 3,5-dimethyl-4-methoxy-benzoic acid was obtained in three steps beginning with the bromination of 2,6-dimethylanisole in chloroform to provide 4-bromo-2,6-dimethylanisole. Formation of the Grignard reagent (magnesium turnings/tetrahydrofuran) and quenching with ethyl chloroformate provided ethyl 3,5-dimethyl-4-methoxybenzoate. Basic hydrolysis (aqueous sodium hydroxide) yielded the desired benzoic acid. Methylene derivatives were prepared by reduction (lithium aluminum hydride in tetrahydrofuran or diisobutylaluminum hydride in toluene/tetrahydrofuran) of the corresponding amides or alkylation (potassium carbonate/acetonitrile; reflux) of the substituted piperazine with the appropriate alkyl halide. The structures of the intermediates and final products were confirmed by standard analytical means (^1H NMR, MS, IR, combustion analysis, etc.).

Evaluation of a BHAP in HIV-1-Infected SCID-hu Mice. This study was conducted at SyStemix, Palo Alto, CA, essentially as described (21) in collaboration with Mike McCune. In brief, groups of five to eight severe combined immunodeficient mice implanted with human fetal lymph node (SCID-hu) received no treatment (NIL group) or were administered HIV RT inhibitor U-87201 (see Fig. 1; 50 or 100 mg/kg of body weight, twice daily by oral gavage), or AZT (ad libitum at 1 mg/ml in drinking water; about 200 mg/kg per day). These treatments were initiated 1 day prior to i.v. infection with HIV-1 (JR-CSF isolate) and continued for 14 days. For the detection of HIV by RNA polymerase chain reaction (PCR), viral pellets were prepared by centrifugation of plasma obtained from individual mice. The pellets were resuspended in buffer (500 mM NaCl/10 mM Tris-HCl, pH 7.5/1 mM EDTA/1% sodium dodecyl sulfate) containing 100 μg of proteinase K, 20 μg of glycogen and 1 μl of RNasin (Promega) per ml. The samples were extracted with 1:1 (vol/vol) phenol/chloroform, and the RNA was precipitated with ethanol. Samples were processed for cDNA synthesis by using Moloney murine leukemia virus RT for 1 hr at 37°C and the priming oligonucleotide 661 from the conserved U5-gag region of the HIV genome (5'-CCTGCGTC-GAGAGCTCCTCTGG-3'). For detection of HIV-1 DNA, primer 661 was used in combination with primer 667 also from the HIV-1 U5-gag region (5'-GGCTAACTAGG-GAACCCACTG-3') during 60-cycle PCR (95°C for 1 min, 55°C for 1 min, 72°C for 1.5 min), and the amplified DNAs were resolved by gel electrophoresis and visualized by ethidium bromide staining as described (21). For the detection of HIV by DNA PCR, 0.25 μg of DNA isolated from implanted lymph nodes from individual mice was amplified as described (21).

RESULTS AND DISCUSSION

Discovery of U-80493E as a Leading Nonnucleoside Inhibitor of the HIV-1 RT. To discover nonnucleoside HIV-1 RT inhibitors, we screened roughly 1500 compounds selected

from the Upjohn chemical inventory by a computational dissimilarity-similarity analysis to ensure they represented multiple, distinct structural classes (22). These compounds were evaluated for inhibition of recombinant HIV-1 RT activity *in vitro* (17), and 100 inhibitors were identified. These inhibitors were next evaluated for inhibition of the cellular DNA polymerases α and δ (19, 23), cytotoxicity to human lymphocytic cell lines (MT-2 and CEM), and anti-HIV activity in an assay based on the formation of HIV-1 (IIIb isolate)-induced syncytia in MT-2 cells (13). Selected leading compounds that exhibited at least a 50% reduction in syncytia formation (ED_{50}) at noncytotoxic concentrations were further evaluated for anti-HIV activity with other human cells (H9 and PBMC) and viral isolates [i.e., D34 (16), JR-CSF (24), and clinical isolates].

From this work we identified the arylpiperazine U-80493E, *N*-ethyl-2-[4-[(4-methoxy-3,5-dimethylphenyl)methyl]-1-piperazinyl]-3-pyridinamine, as a lead compound (Fig. 1 *Upper*). Evaluation of U-80493E in 3- to 4-day HIV-1 replication assays (13, 15) or 14-day assays involving more rounds of viral infection (14) showed that U-80493E exhibited low anti-HIV activity compared with AZT (ED_{50} of 1–10 μM versus 0.001–0.07 μM , respectively; Table 1) and a cytototoxic ratio of about 10-fold. However, the concentration required to inhibit 50% of recombinant HIV-1 RT activity *in vitro* (IC_{50}) was lower by a factor of 30–60 than those for DNA polymerase α or δ (Table 2), and U-80493E was devoid of other significant pharmacological activities when evaluated in a broad panel of bioassays routinely used at Upjohn (data not shown).

Chemical Modification of U-80493E Yields Potent and Selective HIV-1 RT Inhibitors That Block Viral Replication in Cell Culture. Because of the novelty of U-80493E's structure compared with those of the nucleoside analog HIV-1 RT inhibitors and because of its selectivity towards RT, we synthesized many arylpiperazine congeners and systematically explored the relationship between structure and anti-HIV activity. Initially compounds with only minor variations from U-80493E were prepared and evaluated; for example, replacing the methylene group linking the piperazine and phenyl rings with a carbonyl group (U-85199E, Fig. 1 *Upper*) maintained anti-HIV activity, whereas varying ring substitution or extending the carbon linker significantly reduced activity (data not shown). Pyridylpiperazines were more

Fig. 1. Structures of the arylpiperazine HIV-1 RT inhibitors.

Table 1. BHAPs potentially block HIV-1 replication in human lymphocytes

Compound	MT-2/IIIb		PBMC/D34		H9/IIIb	
	ED ₅₀ , μM	CC ₅₀ , μM	ED ₅₀ , μM	CC ₅₀ , μM	ED ₅₀ , μM	CC ₅₀ , μM
U-80493E	2	15	1–10	>10	1	10
U-85961	0.3	>30	0.01	>10	0.06	>10
U-87201	<0.2	>20	0.001	>10	0.04	>10
U-88204	0.3	>27	0.001	>10	0.04	>10
U-88352	0.3	>26	0.003	>10	0.05	>10
U-88353	0.3	>25	0.001	>10	0.06	63
AZT	0.07	123	0.001	10	0.03	63
ddA	0.2	409	0.01	>10	0.01	>400

HIV-1 infectivity and drug cytotoxicity studies were conducted in MT-2 and H9 cells with the HIV-1 IIIb isolate and in PBMC with the HIV-1 D34 isolates as described. CC₅₀ is defined as the concentration of drug required to decrease cell viability 50% compared to uninfected controls; in most cases CC₅₀ is estimated since at the highest drug concentration tested, cell viability was >50%. All data represent the median values from at least two or three independent determinations with standard deviations ≈ 10% or less. ddA, dideoxyadenosine.

inhibitory than phenylpiperazines (data not shown). Replacing the phenyl ring of U-85199E with indole yielded U-85961 with greatly enhanced inhibitory activity (Fig. 1 *Lower*; Tables 1 and 2). Indole substituted for the aryl group was more inhibitory than pyrrole, benzimidazole, and benzofuran (data not shown). Several analogs designed to block potential sites of metabolism on U-85961, such as ring hydroxylation or N-dealkylation, were also synthesized (25, 26). These included U-87201, U-88204, U-88352, and U-88353, all of which exhibited potent anti-HIV activities (Fig. 1 *Lower*; Tables 1 and 2; below). These analogs are easily prepared in sequences as short as four steps with unoptimized overall yields of 30–45% (data not shown).

The above bis(heteroaryl)piperazines (BHAPs) inhibited HIV-1 RT activity at concentrations lower by factors of 10 to 100 compared with U-80493E (IC₅₀ values of about 0.2–2 μM versus 20 μM; Table 2); moreover, the inhibition of RT by U-88204, U-88352, and U-88353 was similar to that determined for 3'-azido-2',3'-dideoxythymidine triphosphate (AZTTP). Compared with AZTTP or 2',3'-dideoxythymidine triphosphate (ddTTP), the BHAPs were consistently less inhibitory to DNA polymerases α (IC₅₀ values of 60–100 μM versus 170–1300 μM) or δ (IC₅₀ values of 0.1–0.14 mM versus 1.9–2.5 mM). The levels of BHAPs that inhibited these normal polymerases were also about 10²- to 10⁴-fold higher than those that inhibited RT.

Table 2. *In vitro* inhibition of HIV-1 RT and normal cellular DNA polymerases (pol) by BHAPs

Compound	HIV-1 RT, μM	BHAP IC ₅₀ , μM		Selectivity index	
		pol α	pol δ	pol α/RT	pol δ/RT
U-80439E	20	600	>1,250	30	>60
U-85961	0.85	1,300	2,400	1,500	2,800
U-87201	1.3	260	1,976	200	1,500
U-88204	0.25	270	2,500	1,100	10,000
U-88352	0.21	170	2,100	810	10,000
U-88353	0.25	380	2,000	1,500	8,000
AZTTP	0.15	60	140	400	930
ddTTP	0.025	100	100	4,000	4,000

The BHAP IC₅₀ values were determined by using recombinant HIV-1 RT and synthetic poly(rA)-oligo(dT) template-primer as described. DNA polymerases α and δ were assayed as described (18, 19); DNA polymerase δ was assayed in the presence of 1.6 μg of proliferating cell nuclear antigen per ml. The IC₅₀ values were derived from the slopes of median-effect plots (20) from at least two independent determinations. In these studies the correlation coefficients for the plots were ≥0.98. Selectivity index = IC₅₀ (cellular DNA polymerase)/IC₅₀ (HIV-1 RT).

Since the structures of the BHAPs and nucleoside analogs are very different, we expected they would inhibit RT differently. Detailed analysis of RT inhibition by U-85961 indicated that it had a K_i of about 1 μM and that inhibition was noncompetitive with respect to dTTP and template-primer, suggesting that U-85961 does not interact with the nucleoside triphosphate binding site on RT (data not shown). Consistent with this interpretation, combinations of AZTTP or ddTTP with U-85961 were consistently more inhibitory to RT than either drug alone. The results of a synergy analysis of these data summarizing the slopes (*m*), IC₅₀ values, and correlation coefficients (*r*) obtained from median-effect plots as well as the results of multiple drug effect analysis developed by Chou and Talalay (20) are shown in Table 3. The interaction between two inhibitors was determined by calculating the combination index (CI) under both mutually exclusive and mutually nonexclusive assumptions. The CI values for both AZTTP or ddTTP and U-85961 (1:3) at 50%, 70%, 90% inhibition levels were ≤1, suggesting moderately synergistic effects. In contrast, the CI values for AZTTP and ddTTP (2:1) at these inhibition levels were ≥1, suggesting moderately antagonistic effects; these effects were concentration dependent and increased with increasing concentration. Finally, in contrast to the potent inhibition of RT by the BHAPs, no inhibition of HIV-1 ribonuclease H activity was obtained with these compounds at concentrations up to 100 μM (data not shown; ref. 18).

Table 3. Synergy analysis of AZTTP, ddTTP, and U-85961 combinations against HIV-1 RT activity

Inhibitor	Parameter			CI at % inhibition		
	<i>m</i>	IC ₅₀	<i>r</i>	50	70	90
AZTTP	0.68	0.51	0.96			
ddTTP	0.75	0.30	0.98			
U-85961	0.75	0.85	0.99			
U-85961/AZTTP (3:1)	0.98	0.64	1.00	0.80 (0.92)	0.59 (0.66)	0.36 (0.38)
U-85961/ddTTP (3:1)	0.93	0.38	0.99	0.65 (0.75)	0.52 (0.59)	0.37 (0.41)
AZTTP/ddTTP (2:1)	0.64	0.42	0.99	1.06 (1.33)	1.12 (1.43)	1.22 (1.59)

HIV-1 RT activity was assayed as described except that activated calf thymus DNA was used as template-primer and dATP, dGTP, dCTP, and dTTP were present at 1 μM. Drug combinations were varied at fixed molar ratios of U-85961:AZTTP (3:1); U-85961:ddTTP (3:1), and AZTTP:ddTTP (2:1). CI at 50%, 70% and 90% inhibition of enzyme activity were calculated under both mutually exclusive and mutually nonexclusive (numbers in parentheses) according to Chou and Talalay (20) as described; *m* is the slope, IC₅₀ is the median inhibitory concentration, and *r* is the correlation coefficient as determined from the median effect plot. CI < 1, synergistic; CI = 1, additive; CI > 1, antagonistic.

As expected, the BHAPs are potent inhibitors of HIV-1 replication in human lymphocytes (Table 1). In MT-2 cells the ED_{50} values for inhibiting the formation of HIV-1-induced syncytia were about 0.3 μ M; the cytotoxic concentrations were at least 100-fold higher. In PBMC the BHAP ED_{50} values were 1–10 nM as determined by measuring the levels of HIV-1 (D34 isolate) p24 and RNA 4 days after infection in culture supernatants (Table 1; ref. 15). The cytotoxic concentrations were at least 10^3 - to 10^4 -fold higher. In this cell system, the BHAPs exhibit similar anti-HIV potencies compared to AZT (ED_{50} of 1 nM) or 2',3'-dideoxyadenosine (ED_{50} of 10 nM). We also obtained similar antiviral data in a 14-day assay in H9 cells (14). In this cell system, the BHAP ED_{50} values were 40–60 nM and nearly identical to those determined for AZT and dideoxyadenosine (ED_{50} values of 10–30 nM). We also determined that U-87201 inhibited to a similar extent in MT-2 cells (ED_{50} values about 1–20 nM) replication of both AZT-sensitive and highly AZT-resistant isolates (about 450-fold AZT-resistant) obtained from the same patient during the course of prolonged drug therapy (9). U-87201 also exhibited similar antiviral potency against the HIV-1 IIIb isolate and four different clinical isolates recovered from patient PBMC and used prior to extensive culture adaptation (data not shown). Finally, in this series of compounds we noted a good correlation between anti-RT and antiviral activity providing additional evidence that HIV-1 RT was a primary target in infected cells.

In contrast to the potent inhibition of HIV-1 replication in human lymphocytes, no inhibition of HIV-2 (ROD isolate)-induced syncytia formation was observed in MT-2 cells at BHAP concentrations up to 10 μ M (13); for comparison, the ED_{50} for AZT in this assay was 0.01 μ M. Consistent with this lack of antiviral activity, the BHAPs did not inhibit recombinant HIV-2 RT activity *in vitro* (data not shown; ref. 27). The BHAPs were similarly inactive in blocking replication of the simian immunodeficiency virus (SIV_{mac251}, B670), Rauscher murine leukemia virus, and the feline immunodeficiency virus in cell culture (data not shown).

A BHAP Can Block HIV-1 Replication *in Vivo*. To investigate whether a BHAP could block HIV-1 replication *in vivo*, we evaluated the antiviral activity of U-87201 in HIV-1-infected SCID-hu mice. A standard protocol previously developed to study the antiviral efficacy of AZT in this animal model was used (21). In this system 100% of SCID-hu mice examined (40/40) were positive for HIV-1 2 weeks after infection when analyzed by the PCR. In our experiment U-87201 was administered twice daily orally beginning 1 day before the i.v. infection of mice with HIV-1 (JR-CSF isolate) and continuing for 14 days. At this time, venous blood and human tissues were obtained from individual mice and used for detection of HIV-1 by RNA and DNA PCR. Under these conditions, HIV-1 is undetectable in 50% of mice receiving about 60 mg of AZT per kg of body weight per day administered *ad libitum* in their drinking water. As expected in our experiment, all of the mice (5/5) that did not receive any drug treatment were positive for HIV-1 by RNA PCR (Fig. 2A, NIL lanes 1–5). In contrast, the five mice administered AZT (about 200 mg/kg per day *ad libitum* in their drinking water) were all negative for HIV-1 (Fig. 2A, AZT lanes 6–10). We also determined that three of eight mice that received U-87201 at 200 mg/kg per day were negative for HIV by RNA PCR (Fig. 2B, lanes 11, 14, and 18). Human DNA was successfully isolated from implanted lymph nodes from six of the eight mice treated with U-87201 at 200 mg/kg, which included the animals found to be HIV-negative by RNA PCR. We confirmed that the same three mice that were HIV-negative by RNA PCR were also HIV-negative by DNA PCR (Fig. 3); that is, the samples analyzed by RNA PCR from individual mice in lanes 11, 14, and 18 in Fig. 2 correspond to samples from the same mice analyzed by DNA PCR in lanes

FIG. 2. Evaluation of U-87201 in HIV-1-infected SCID-hu mice by RNA PCR. Groups of five to eight SCID-hu mice received no treatment (lanes NIL in A) or were administered U-87201 (100 mg/kg, twice daily by oral gavage) (B) or AZT (1 mg/ml in drinking water, about 200 mg/kg/day) (lanes AZT in A) 1 day prior to and 14 day after i.v. infection with HIV-1 JR-CSF isolate. Viral pellets were prepared from plasma samples from individual mice, and the presence of HIV was assessed by RNA PCR as described. The amplified DNAs were analyzed on 3% agarose gels for the presence of the expected 202-base-pair (bp) HIV-1 DNA fragment (21). Lane M shows DNA size markers in bp. Data were provided by SyStemix under contract DT-9 from Upjohn.

1, 3, and 6 in Fig. 3. In addition, the levels of HIV-1 DNA amplified by DNA PCR with samples from the three mice treated with U-87201 that were HIV-positive (Fig. 3, lanes 2, 4, and 5) were lower than those from the nontreated animals (Fig. 3, lanes 7 and 8); thus, under these PCR conditions and with detection of DNA by ethidium bromide staining after gel electrophoresis as an end point, the drug-treated animals consistently had lower levels of HIV DNA by factors of 5–10 (data not shown). No inhibition of HIV-1 replication was observed in mice treated with U-87201 at 100 mg/kg per day (data not shown), and no drug-related toxicity was observed

FIG. 3. Evaluation of U-87201 in HIV-1-infected SCID-hu mice by DNA PCR. Groups of SCID-hu mice were treated with AZT or U-87201 or received no drug treatment (NIL) as described in Materials and Methods and the legend to Fig. 2. DNA was isolated from implanted human lymph nodes, and the presence of HIV-1 DNA was assessed by PCR exactly as described (21) except that 0.25 μ g of DNA from individual mice was used in each amplification. The amplified DNAs were analyzed on 8% polyacrylamide gels for the presence of the expected 202-bp HIV-1 DNA fragment (21). Lanes M, DNA size markers in bp; 1–6, DNA from individual drug-treated mice (one-fifth of the amplified DNA was analyzed); (-), no DNA was included in the amplification mixture; 7 and 8, DNA from two representative samples that received no drug (1/10th of the amplified DNA was analyzed); 9 and 10, samples from two representative mice receiving AZT. To ensure that the 202-bp fragments were HIV-1 DNA, we determined that they specifically hybridized to a 32 P-labeled oligonucleotide probe designed from an internal 23-bp sequence of the predicted amplified DNA.

in any of the drug-treated animals at either dose. These data indicate that U-87201 at 200 mg/kg per day can block the replication of HIV-1 *in vivo*. Compared with AZT, U-87201 was not as effective in blocking HIV-1 replication in this experiment, which may relate to the *ad libitum* administration of AZT vs. only twice-daily administration of U-87201.

Recent work by Pauwels *et al.* (11) and Merluzzi *et al.* (12) identified two other classes of nonnucleoside HIV-1 RT inhibitors related to benzodiazepines and dipyridodiazepinones, respectively. These compounds are similar to the BHAPs regarding their anti-HIV potencies and cytotoxic ratios. Interestingly, none of these compounds inhibit HIV-2 RT despite the fact that the HIV-1 and HIV-2 RTs share about 60% similarity in their primary sequence (28). It is unknown presently whether these inhibitors use similar or distinct mechanisms to inhibit HIV-1 RT.

In summary, we have identified the BHAPs as a class of nonnucleoside HIV-1 RT inhibitors. We believe these compounds hold promise for the treatment of HIV-1 infection. In laboratory animals the BHAPs exhibit good oral bioavailability, drug serum levels in great excess of those required for *in vitro* antiviral activity are maintained for prolonged periods, and toxicity has been characterized by multiple dose studies (data not shown). Plans for the clinical evaluation of the BHAPs are in progress.

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