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Anti-Human Immunodeficiency Virus Type 1 Activity and In Vitro Toxicity of 2′-Deoxy-3′-Thiacytidine (BCH-189), a Novel Heterocyclic Nucleoside Analog

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We describe a novel nucleoside analog, 2′-deoxy-3′-thiacytidine (BCH-189), in which the 3′ carbon of the ribose ring of 2′-deoxycytidine has been replaced by a sulfur atom. In MT-4 T cells, this compound had significant time- and dose-dependent antiviral activity against five different strains of human immunodeficiency virus type 1 (HIV-1) (mean 50% inhibitory dose, 0.73 μM); known 3′-azido-3′-deoxythymidine (AZT)-resistant HIV-1 variants did not exhibit cross-resistance to it. BCH-189 also suppressed HIV-1 replication in the U937 monocytoid cell line as well as in primary cultures of human peripheral blood mononuclear cells; in these latter systems, suppression was fuller and longer lasting than that induced by AZT. Moreover, BCH-189 was less toxic than AZT in cell culture. BCH-189 may be a promising drug for the treatment of HIV-1-associated disease.

Nucleoside analogs represent the most closely studied family of antiviral agents. Of these, zidovudine (3′-azido-3′-deoxythymidine [AZT]) has emerged as the drug of choice for combatting human immunodeficiency virus type 1 (HIV-1) infection. There is good evidence that AZT therapy can delay although not necessarily stop the onset of AIDS, the late and fatal outcome of infection by HIV-1 (2, 3, 12, 14). AZT is phosphorylated upon entering the cell and can then act as a strong inhibitor of reverse transcription, a key step in the retroviral life cycle (4, 22). In addition, there is a demonstrated potential for emergence of drug-resistant virus strains in patients who have received AZT over long periods (9, 16, 19). Several other nucleoside analogs, such as 2′,3′-dideoxyinosine and 2′,3′-dideoxycytidine, have also been shown to possess significant anti-HIV activity in vitro (6, 8, 13); both 2′,3′-dideoxyinosine and 2′,3′-dideoxycytidine have been studied in clinical trials (21). Toxic side effects have been dose-limiting features with each of these agents.

The present study describes a novel nucleoside analog, 2′-deoxy-3′-thiacytidine (BCH-189), in which the 3′ carbon in the ribose ring of 2′-deoxycytidine has been replaced by a sulfur atom (Fig. 1). We now demonstrate that this new compound has potent in vitro anti-HIV-1 activity in both T-lymphoid and monocytoid cell lines, as well as in primary cultures of human peripheral lymphocytes. Moreover, BCH-189 is less toxic than AZT in tissue culture, which may make it a promising candidate drug for the treatment of HIV-1-associated disease.

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MATERIALS AND METHODS

Cell cultures. U937 cells (18) were obtained from the American Type Culture Collection. MT-4 cells (7), chosen for their rapid susceptibility to most strains of HIV-1, were kindly provided by J. A. Levy, University of California at San Francisco. All cells were cultivated at 37°C in RPMI 1640 medium (Flow Laboratories, Mississauga, Ontario, Canada), supplemented with 10% heat-inactivated fetal calf serum (GIBCO, Toronto, Canada), 25 mM L-glutamine, 50 IU of penicillin per ml, and 50 μg of streptomycin (Flow Laboratories) per ml.

Human peripheral blood mononuclear cells (PBMCs) were obtained by Ficoll-Hypaque (Pharmacia Labs, Montreal, Canada) density gradient centrifugation of blood collected from male HIV-1-seronegative volunteers. The PBMCs were stimulated with 0.1% phytohemagglutinin in supplemented RPMI 1640 medium and subsequently maintained in the presence of 5% recombinant interleukin-2 (Boehringer-Mannheim, Montreal, Canada) for 72 h prior to infection, as previously described (5, 16, 17).

HIV-1 strains and infection. The HIV-III_B isolate of HIV-1 was kindly supplied by R. C. Gallo, National Institutes of Health, Bethesda, Md. UHC-1 virus was derived from a cell clone of U937 cells that had been chronically infected with HIV-III_B (1) and was employed because of its high infectiousness for U937 cells. We also studied two pairs of AZT-susceptible and -resistant clinical isolates of HIV-1, obtained from infected asymptomatic subjects both prior to and following initiation of AZT therapy, as described previously (16). Both of the AZT-resistant viruses studied were mutated at amino acid positions 70 and 215 of viral reverse transcriptase (RT), as determined by polymerase chain reaction using published primer pairs and techniques (10). All viruses were pelleted by ultracentrifugation from clarified culture fluids. Infectivity of each isolate was quantified by end-point titration in MT-4 cells, and virus stocks were kept frozen at −70°C until used. All cells were infected at a

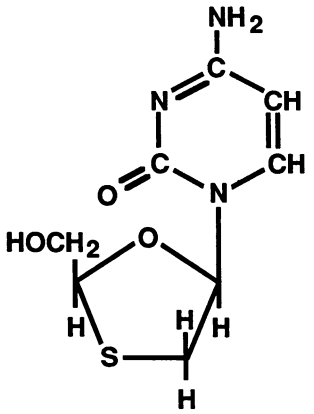

FIG. 1. Molecular structure of the cytosine analog BCH-189.

multiplicity of infection of 0.1 50% tissue culture infective dose per cell as previously described (1, 16).

Antiviral compounds. AZT was obtained from Burroughs-Wellcome Inc., Research Triangle Park, N.C. BCH-189 was a gift of IAF Biochem International, Laval, Quebec, Canada.

Susceptibility testing. To determine the levels of inhibition of HIV-1 replication by BCH-189, we infected MT-4 cell cultures (pretreated for 3 h with various concentrations of the drug) with a panel of five different isolates of HIV-1. RT activity in the culture fluids was measured, as previously described (11), at 5 days postinfection. AZT was tested in an identical fashion in parallel cultures. The results were expressed as the 50% inhibitory dose (ID_{50}), as measured by determinations of viral RT levels in culture fluids. All studies were carried out with three replicate samples, and the results were analyzed by Student's *t* test when applicable. Cell proliferation in untreated controls was not impaired in these studies.

The viral p24 antigen (Ag) present in culture fluids was quantitated by using a commercially available kit (Abbott Laboratories, North Chicago, Ill.) according to the manufacturer's instructions. All assays were performed with three replicate samples.

Toxicity testing. For toxicity testing, three replicate cultures of each of uninfected MT-4 cells, U937 cells, or phytohemogglutinin-stimulated PBMCs (2×10^5 cells) were incubated with various concentrations of AZT and BCH-189; cell viability was determined 6 days from drug addition by trypan blue exclusion, and the results were expressed as the 50% cell culture inhibitory dose ($CCID_{50}$) for each compound, i.e., the drug concentration at which a 50% inhibition of cell replication was observed.

TABLE 1. Inhibition of replication of various HIV-1 isolates in MT-4 cells by AZT and BCH-189

HIV-1 isolate[a]	ID_{50} (10^2 μM)[b] of:	
	BCH-189	AZT
III_B	37.0 ± 5.9	2.30 ± 0.55
AZT susceptible (A)	86.8 ± 13	2.17 ± 0.28
AZT resistant (A)	131 ± 20	19.3 ± 3.1
AZT susceptible (B)	54.5 ± 9.5	0.48 ± 0.084
AZT resistant (B)	57.6 ± 7.0	10.2 ± 0.85

[a] Paired isolates of AZT-susceptible and -resistant strains were obtained from the same asymptomatic patients (A and B), both prior to and at 52 weeks after initiation of AZT therapy.

[b] Results are presented as means (n = 3) ± standard deviations.

RESULTS

Antiviral activity and cytotoxicity in MT-4 cells. The ID_{50} of BCH-189 ranged from 0.370 to 1.31 μM (mean = 0.73 μM) for the isolates tested (Table 1). The ID_{50} of AZT ranged between 0.00480 and 0.0217 μM (mean = 0.017 μM) for the AZT-susceptible isolates, values which are generally in accordance with results obtained by other groups (9, 15). In contrast, the AZT-resistant variants were 10 to 20 times less susceptible to AZT as determined by ID_{50} than were their corresponding AZT-susceptible counterparts. No such differences were found between AZT-susceptible and AZT-resistant paired isolates exposed to BCH-189. Thus, the AZT-resistant strains in question did not display cross-resistance to BCH-189. Comparable results were obtained when p24 indirect immunofluorescence assays were performed to monitor the cell cultures (results not shown).

For MT-4 cells, the $CCID_{50}$ of BCH-189 was 405 μM, about 10-fold greater than that of AZT (37.6 μM) (Fig. 2). In

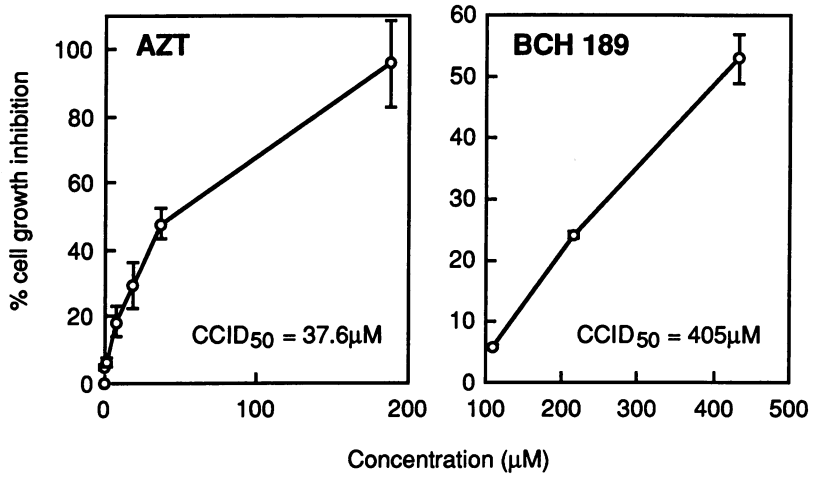

FIG. 2. Toxicity of BCH-189 and AZT for MT-4 cells. Results are expressed as means (n = 3) ± standard deviations.

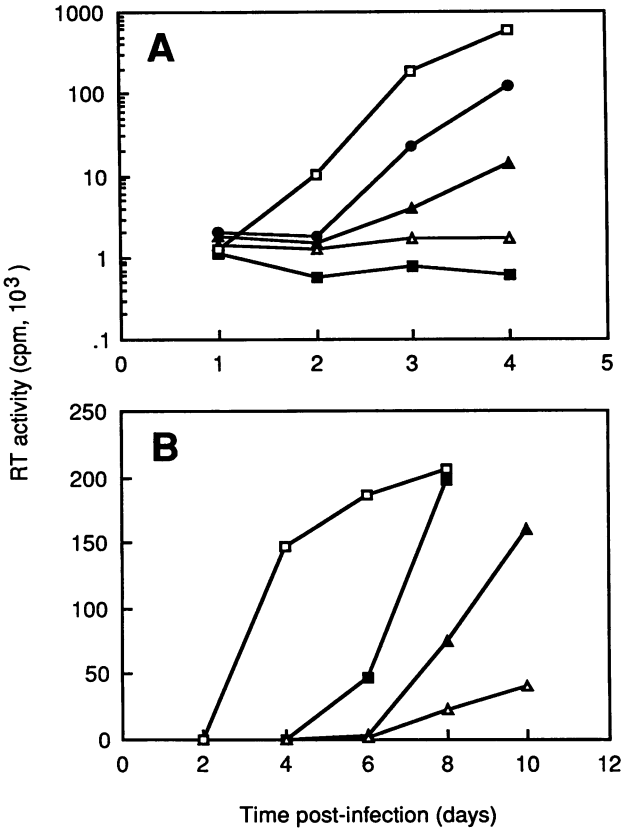

FIG. 3. Effects of drug pretreatment and maintenance on inhibition of HIV-1 replication by 10 μM BCH-189 in MT-4 cells. (A) Drug treatment was started 4 h prior to infection (■), 2 h postinfection (△), 6 h postinfection (▲), or 18 h postinfection (●) and maintained continuously thereafter with twice-weekly changes of medium; the control (□) had no drug. (B) Drug treatment was initiated at the end of a 2-h viral adsorption period and subsequently maintained for 4 h per day (■), 12 h per day (▲), or continuously (△); the control (□) had no drug. All cells were washed and fed with fresh medium, whether containing drug or not, at the same times.

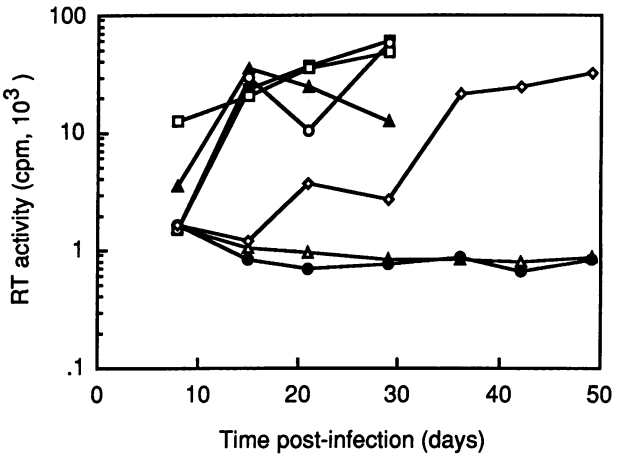

FIG. 4. Inhibition of HIV-1 replication in the U937 monocytoid cell line. Exposure of cells to either BCH-189 or AZT was initiated 2 h prior to infection and continued during a 2-h viral adsorption period; the cells were then washed free of drug and maintained thereafter in the absence of drug (■, 20 μM BCH-189; ▲, 5 μM BCH-189; ○, 4 μM AZT), or fresh drug was added at the end of the viral adsorption period and again at each biweekly cell passage (△, 20 μM BCH-189; ●, 5 μM BCH-189; ◇, 5 μM AZT; □, no drug).

contrast, the values of their respective therapeutic indices ($CCID_{50}/ID_{50}$, a standard measure of drug efficacy), as determined with the means obtained from Table 1, indicated that AZT was a more effective compound against HIV-1 replication in MT-4 cells than was BCH-189 (2,212 for AZT and 555 for BCH-189). Toxicity testing of BCH-189 was also carried out against both uninfected U937 cells and phytohemagglutinin-stimulated, interleukin-2-supported PBMCs and yielded $CCID_{50}$ values of 170 and 385 μM, respectively.

Timing of addition studies. To determine whether a correlation might exist between the degree of inhibition by BCH-189 and the time of exposure of cells to this drug, MT-4 cells were treated with 10 μM BCH-189 from 4 h preinfection to 18 h postinfection. A direct relationship between the time of drug administration and the magnitude of inhibition of RT activity in the cell culture supernatants was shown: the earlier the onset of treatment, the more complete the inhibition of HIV-1 production (Fig. 3A).

To determine whether the duration of BCH-189 maintenance in the tissue culture medium influenced the level of inhibition of HIV-1 replication, 10 μM BCH-189 was maintained in the culture medium for periods ranging from 4 to 24 h per day. The cells were washed free of compound at the end of each maintenance period. Again a direct correlation was demonstrated between the time of daily exposure to the drug and the extent of inhibition of RT activity in culture supernatants (Fig. 3B). Progeny HIV-1 which was produced by the continuously treated cultures after 8 days was isolated and itself tested for susceptibility to BCH-189. No evidence of drug resistance could be demonstrated (results not shown).

Activity in other cell types. The ability of both BCH-189 and AZT to inhibit HIV-1 production was tested in the U937 monocytoid cell line. Continuous treatment with 5 μM BCH-189 suppressed HIV-1 production for more than 45 days, as determined by production of viral RT activity. Similar results were obtained by p24 Ag capture assay. In contrast, the use of a similar concentration of AZT was somewhat less effective in these cells. Single-pulse pretreatment with either drug was largely ineffective (Fig. 4).

To determine whether the drug administration schedule could influence this inhibitory effect, U937 cells were infected and then treated with 10 μM BCH-189 once per 2 weeks, once per week, or twice per week. Our results indicate, on the basis of monitoring for RT activity, that the greater the frequency of exposure to fresh drug, the longer and more complete the inhibition of HIV-1 production (Fig. 5). Similar data were obtained by measurements of p24 Ag in culture fluids and through the use of multiplicities of infection of 1.0 and higher (data not shown).

Finally, we assessed the ability of BCH-189 to inhibit HIV-1 replication in freshly isolated primary cultures of PBMCs. Replicate cultures were infected with either the AZT-susceptible (from patient B) or AZT-resistant (from patient B) HIV-1 strain and then treated with either AZT (1 μM) or BCH-189 (10 μM), starting at various times postinfection. Both viral RT activity and p24 Ag levels were measured in culture fluids after 7 days. In cultures of PBMCs, BCH-189 completely prevented the growth of both AZT-susceptible and AZT-resistant HIV-1 variants, even

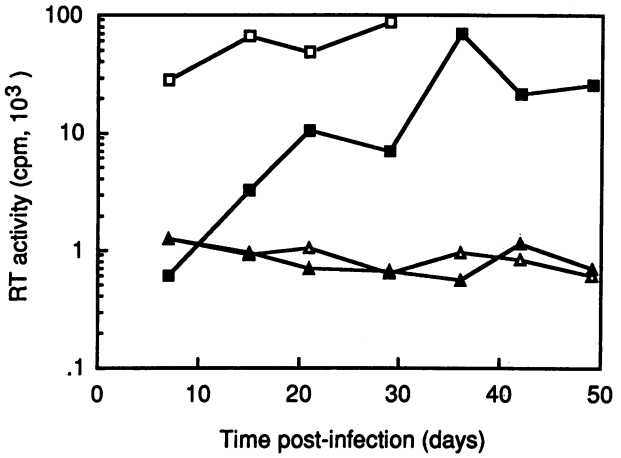

FIG. 5. Influence of frequency of drug treatment on inhibition of HIV-1 replication in U937 cells. Infection was carried out in the absence of BCH-189, which was added (10 μM) at the end of the adsorption period. Medium changes were carried out twice weekly and either included or excluded fresh drug according to the following schedule: BCH-189 (10 μM) was included at each medium change (▲); fresh drug added after 7 days and at subsequent weekly intervals (△); or fresh drug added after 14 days and at 14-day intervals (■). □, no-drug control.

when drug treatment started as late as 10 h postinfection. In contrast, AZT was unable to block the ultimate replication of either virus (Table 2).

DISCUSSION

We have shown that BCH-189 possesses lower anti-HIV activity in MT-4 cells than AZT; in contrast, BCH-189 appeared to be a more effective antiviral agent than AZT in both cultures of U937 cells and PBMCs. The growth of each of five different HIV-1 isolates was inhibited by BCH-189, and we confirmed that two known AZT-resistant HIV-1 strains did not exhibit cross-resistance to it. We showed that BCH-189 could effect a total suppression of HIV-1 production in U937 cells for a period of almost 50 days, something that could not be achieved with similar doses of AZT. We have also demonstrated that the time of onset of exposure to BCH-189 and the duration of such exposure can each play an important role in limiting HIV-1 replication.

In terms of toxicity, the 50% lethal dose of BCH-189 in MT-4 cells is more than 10 times higher than that of AZT.

TABLE 2. Inhibition of replication of AZT-susceptible and -resistant HIV-1 strains in PBMCs by AZT and BCH-189

Drug	Time of addition (h postinfection)	p24 Ag (pg/ml)[a] in:	
		AZT-susceptible strain	AZT-resistant strain
10 μM BCH-189	2	0	0
	5	0	0
	10	0	0
1 μM AZT	2	0	149
	5	9.10 ± 8.6	173 ± 56
	10	37.3 ± 0.47	287 ± 47
None		6,140 ± 644	3,443 ± 1,144

[a] Results are presented as means (n = 3) ± standard deviations.

This relatively low toxicity compensates in large part for the lower specific anti-HIV-1 activity of BCH-189, in comparison with that of AZT, in that system. Early results of toxicity testing in rats have shown that oral doses of BCH-189 as high as 100 mg/kg of body weight neither caused apparent organ dysfunction nor altered hematologic parameters (20). The half-life of BCH-189 in the circulation of rats is about 60 min, similar to that of AZT and other nucleoside analogs (20).

Our data suggesting that BCH-189 may be a more effective antagonist of HIV-1 replication in U937 cells than AZT must be understood in context. Little is known, for example, about the stability of extracellular BCH-189 or intracellular conversion rates to phosphorylated forms. Furthermore, the experiments on timing of drug addition were carried out using a multiplicity of infection of 0.1, which means that our measurements of virus production may represent the consequence of secondary rounds of infection.

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