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BI-RG-587 Is Active against Zidovudine-Resistant Human Immunodeficiency Virus Type 1 and Synergistic with Zidovudine

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A series of dipyridodiazepinones have been shown to be potent inhibitors of human immunodeficiency virus type 1 (HIV-1) reverse transcriptase. The lead compound, BI-RG-587, had a 50% inhibitory concentration of 84 nM against HIV-1 reverse transcriptase activity. This compound reduced plaque formation of HIV-1 in HeLa cells expressing the CD4 receptor by 50% at 15 nM. BI-RG-587 at comparable concentrations inhibited the production of p24 antigen following the acute infection of CEM T-lymphoblastoid cells or primary human monocyte-derived macrophages with HIV-1. No inhibitory effects against HIV-2 or against three picornaviruses were detected. Zidovudine (3′-azido-3′-deoxythymidine [AZT])-susceptible and AZT-resistant isolates of HIV-1 were equally susceptible to BI-RG-587. AZT and BI-RG-587 exhibited synergistic inhibition of HIV-1_{BRU} at all concentrations examined.

Reverse transcriptase (RT) activity is essential for retroviral replication (6). Most effective inhibitors of RT have been nucleoside analogs that are converted by cellular enzymes to the 5′ triphosphate which inhibits the essential RT activity (14). The nucleoside analog, zidovudine (3′-azido-3′-deoxythymidine [AZT]), can prolong survival, reduce morbidity, and delay disease progression in patients infected with human immunodeficiency virus (HIV) (4, 5, 23). Despite the benefits documented in these studies, AZT use is associated with toxicity (18), incomplete suppression of viremia (7), and the emergence of drug-resistant strains of HIV-1 (11, 19).

A series of dipyridodiazepinones, of which BI-RG-587 is the lead compound, have been identified as nonnucleoside inhibitors of HIV-1 RT (13). BI-RG-587 specifically inhibits HIV-1 RT with exquisite specificity, not inhibiting human DNA polymerases alpha, beta, gamma, and delta and RT enzymes from other viruses (13). BI-RG-587 has also been shown to inhibit the production of syncytia and p24 antigen in T-cell lines infected with HIV-1 by using both laboratory strains and isolates from infected individuals (13). The present study extended this evaluation of BI-RG-587 to additional host cell types, to additional isolates of HIV-1, including AZT-resistant viruses, to HIV-2, and to the examination of synergism with AZT.

MATERIALS AND METHODS

HIV-1 RT. Recombinant HIV-1 RT was obtained from T. Steitz of Yale University. The enzyme was assayed with 5 nM primer sites on poly(rC)-oligo(dG) and 450 nM [^{3}H]dGTP as substrates in a reaction mixture consisting of 50 mM Tris, 1 mM dithiothreitol, 2 mM $MgCl_2$, 50 mM glutamic acid, and 0.02% (vol/vol) 3-[3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate at pH 7.8. After a 60-min reaction, products were precipitated by the addition of trichloroacetic acid to 5%, harvested onto glass fiber filters, and quantified by liquid scintillation counting. Inhibition was determined by comparison of the products formed in the presence or absence of BI-RG-587. The 50% inhibitory concentration (IC_{50}) was calculated by nonlinear regression by using the Sigmoid E_{max} model (8, 21).

Drug susceptibility assays by plaque reduction. HeLa cells expressing CD4 (HT4-6C cells) (1) were used in a plaque (syncytial focus) reduction assay with virus strains HIV-1_{BRU} and HIV-2_{ROD} and patient isolates that were propagated and whose titers were determined as previously described (10, 11). Briefly, 24-well culture plates containing HT4-6C cell monolayers were inoculated with virus in various concentrations of AZT or BI-RG-587 in medium. Duplicate wells were prepared for each dilution, and the percent plaque reduction was based upon the control value without drugs. The IC_{50} was calculated with the median effect plot (2).

Drug susceptibility assay as measured by p24 production. The reduction of HIV-1_{BRU} p24 antigen production in CEM T-lymphoblastoid cells (20) or of HIV-1_{Bal-85} in primary human monocyte-derived macrophages (9) was performed as previously described, infecting the cells with a multiplicity of infection of one 50% tissue culture infective dose per cell. Culture supernatants were harvested at 72 h after infection of CEM cells and 5 days after the infection of macrophages. P24 antigen production was assayed by enzyme-linked immunosorbent assay (Abbott, North Chicago, Ill.). Duplicate wells were prepared for each dilution of AZT and BI-RG-587, and the percent reduction of supernatant p24 antigen production was based upon the value from the control without drugs.

Compounds. AZT was the gift of S. Lehrman, Burroughs Wellcome. BI-RG-587 was synthesized at Boehringer Pharmaceuticals Inc., Ridgefield, Conn. The synthesis, structure and enzymatic activities have been reported elsewhere (13). BI-RG-587 was dissolved in dimethyl sulfoxide, diluted further in RPMI 1640, and used within 1 h. AZT was dissolved in RPMI 1640 and frozen in aliquots at 1 mM.

Cytopathogenic effect assay with picornaviruses. HeLa cells (CCL2 F 6333), rhinovirus 54 (VR-521), coxsackievirus A13 (VR-1019), and poliovirus type 1 (VR-192) were obtained

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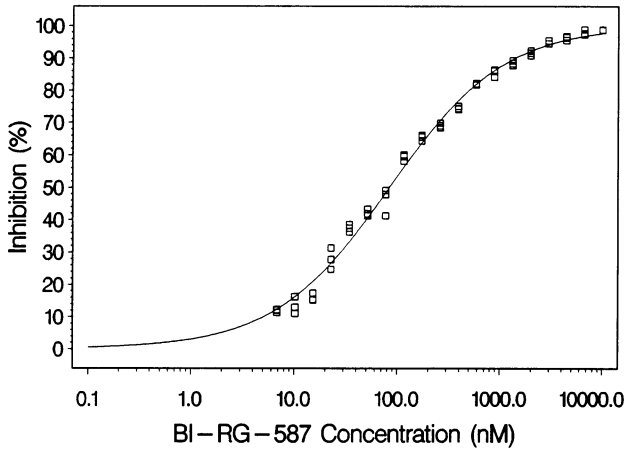

FIG. 1. Effect of BI-RG-587 on HIV-1 recombinant RT. Single data points of percent inhibition with triplicate values for each drug concentration are displayed (maximum inhibition, 100%), with an IC_{50} of 84 nM based on nonlinear regression analysis with the Sigmoid E_{max} model (8, 21).

from the American Type Culture and Collection, Rockville, Md. One hundred 50% tissue culture infectious doses were added to 70% confluent HeLa cell monolayers with various concentrations of BI-RG-587 in medium. After 4 days in culture, the cytopathogenic effect was determined as previously described (12, 22). Human fibroblast beta interferon was purchased from Lee Biomolecular Research, Inc., San Diego, Calif.

Analysis of drug interactions. The degrees of synergism and antagonism were quantitatively determined by the median-effect principle and the isobologram technique by using the combination index calculation as described by Chou and Chou (2) and Chou and Talalay (3). The method involves the conversion of dose-effect curves for each drug and for serially diluted fixed-ratio combinations of the agents into the median-effect plot. The slope of the plot, which signifies the shape of dose-effect curve, and the x intercept of the plot (log of the 50% effective dose), which signifies the potency of each compound and their combination, were then used to calculate the combination index. The combination index values of <1, $=1$, and >1 indicate synergism, additive effect, and antagonism, respectively (2, 3). Also, the data were analyzed by the isobologram technique, which evaluates drug interactions by a dose-oriented geometric method (2, 3).

RESULTS

With recombinant HIV-1 RT, BI-RG-587 inhibited enzyme activity by 50% at 84 nM (Fig. 1). The antiviral activity of BI-RG-587 and AZT against HIV-1 and HIV-2 were then quantified with a plaque reduction assay. Both compounds inhibited plaque formation by HIV-1_{BRU} with IC_{50}s of 16 nM (BI-RG-587) and 30 nM (AZT) (Table 1). Ninety-five percent inhibition was observed with 710 nM BI-RG-587 and 2,000 nM AZT. In contrast to the results with HIV-1, BI-RG-587 was inactive against HIV-2_{ROD} (Table 1). Although HIV-2 was slightly less susceptible to AZT in this assay (IC_{50} = 143 nM), BI-RG-587 had no activity against HIV-2 at 3,200 nM.

BI-RG-587 was also tested on three picornaviruses (rhinovirus type 54, poliovirus type 1, and coxsackievirus type A13) for the inhibition of cytopathic effects in culture.

TABLE 1. Reduction by BI-RG-587 and AZT of plaque formation by HIV-1 and HIV-2[a]

Virus	BI-RG-587 (nM)			AZT (nM)		
	IC_{50}	IC_{90}	IC_{95}	IC_{50}	IC_{90}	IC_{95}
HIV-1	16	450	710	30	710	2,000
HIV-2	>3,200			112	>3,200	

[a] The data are representative of six experiments with similar results. The linear correlation coefficients (r values) of the median effect plots were uniformly >0.95, most frequently >0.99.

BI-RG-587 should not affect replication of these viruses in HeLa cells, because these viruses are not dependent upon RT to replicate. As with HIV-2, none was inhibited by BI-RG-587, but all three viruses were inhibited by more than 50% with 100 U of beta interferon per ml (data not shown).

HIV p24 antigen production by both CEM T-lymphoblastoid cells and primary human monocytes or macrophages was quantified in the presence of BI-RG-587. This compound inhibited antigen production by the HIV-1_{BRU} strain in CEM cells and by the HIV-1_{Bal-85} strain in primary human monocytes or macrophages at comparable concentrations of drug (IC_{50} = 40 nM) (Table 2). The selectivity of the drug was marked. In CEM cells, proliferation and viability were unaffected at 32,000 nM and reduced 14% at 100,000 nM.

Isolates of HIV-1 from patients receiving prolonged therapy with AZT may exhibit drug resistance (11, 19). To examine whether AZT-resistant isolates would display cross resistance to BI-RG-587, paired isolates obtained from a patient before and after prolonged therapy were examined for susceptibility to BI-RG-587. The isolate that displayed an at least 100-fold reduction in susceptibility to AZT displayed no cross resistance to BI-RG-587 (Fig. 2). Two other isolates highly resistant to AZT also retained their susceptibility to BI-RG-587 (data not shown).

Because BI-RG-587 and AZT probably inhibit RT by different mechanisms, it is possible that the two drugs act synergistically and it is important to document that they do not antagonize each other. BI-RG-587 and AZT were assayed alone and in combination at a wide range of concentrations in the plaque reduction assay utilizing HIV-1_{BRU}. BI-RG-587 and AZT inhibited HIV-1 synergistically, as depicted by the isobologram method (Fig. 3). For example, the IC_{50} of AZT was 10 nM, and that of BI-RG-587 was 32 nM. In combination, 50% inhibition was attained with 0.3

TABLE 2. Effect of BI-RG-587 on HIV-1 p24 antigen production in CEM T-lymphoblastoid cells and primary human macrophages

Cell type[a] and BI-RG-587 concn (nM)	p24 (pg/ml)	% Reduction	IC_{50} (nM)
CEM			
1,000	3,000	98	
100	260,000	85	39
10	1,578,000	12	
0	1,785,000	0	
Macrophages			
1,000	280	99	
100	14,430	63	44
10	32,850	15	
0	38,663	0	

[a] CEM, CEM T-lymphoblastoid cells infected with HIV-1_{BRU}; Macrophages, primary human monocyte-derived macrophages infected with HIV-1_{Bal-85}.

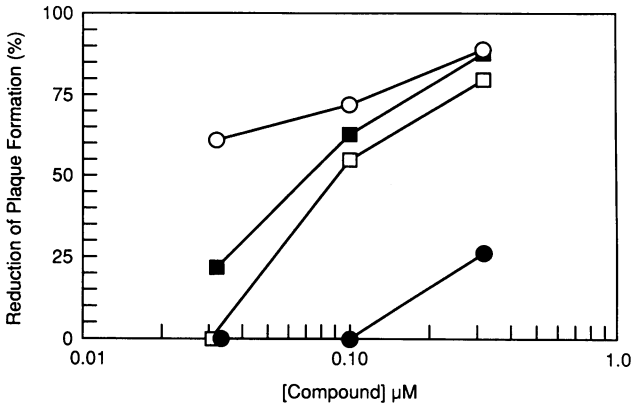

FIG. 2. Reduction by BI-RG-587 or AZT of plaque formation by an AZT-susceptible (A102B) isolate and an AZT-resistant (A012D) isolate. The susceptible isolate with AZT (○) or BI-RG-587 (□) and the resistant isolate with AZT (●) or BI-RG-587 (■) were obtained from the same patient after an interval of 24 months of AZT therapy (11).

nM AZT and 10 nM BI-RG-587 for a combination index of 0.24, which indicates significant synergism (2, 3). Synergism was observed at all combinations of drug tested with concentrations ranging from 10 to 1,000 nM for BI-RG-587 and from 0.3 to 100 nM for AZT (combination indices, 0.15 to 0.71).

DISCUSSION

BI-RG-587 represents a new class of compounds that inhibit HIV-1 RT and are distinct from dideoxynucleoside triphosphates and PP_i analogs like foscarnet. BI-RG-587 inhibits production of p24 antigen and syncytia in c8166 cells with a selectivity index of >8,000 (13). Enzyme reactions have shown that this compound is a noncompetitive inhibitor of HIV-1 RT while having no effect on the RT of simian immunodeficiency virus or feline leukemia virus and human DNA polymerases alpha, beta, gamma, and delta (13). The data presented here extend these previous observations to other cell types and other isolates of HIV-1, including those that are resistant to AZT. BI-RG-587 was also inactive against HIV-2 and picornaviruses. These data were not unexpected, because this compound did not inhibit the RT of simian immunodeficiency virus, a virus that has homology with HIV-2, and preliminary data from our laboratories have shown that BI-RG-587 did not inhibit HIV-2 RT. The selectivity for HIV-1 and its polymerase in contrast to the polymerases of human cells and of other viruses, including HIV-2, is remarkable. For example, the proliferation and viability of CEM cells were unaffected by BI-RG-587 at 32,000 nM.

The absence of antagonism between BI-RG-587 and AZT and, in fact, the synergism observed were encouraging. Combination chemotherapy for HIV infection has been contemplated to increase efficacy and permit lower doses to reduce toxicity, as well as to reduce the likelihood of the emergence of drug resistance (17). It is not known whether resistance to BI-RG-587 will develop. In vitro experiments to address this question are in progress, although the assessment of isolates during therapy would provide a more rigorous and relevant test.

BI-RG-587 has a profile of activity similar to those of the compounds reported by Pauwels et al. (16), for which activity was seen against HIV-1 but not HIV-2. Recent experiments have shown that BI-RG-587 is not toxic to bone marrow progenitors (15), and initial studies directed toward the metabolism, bioavailability, distribution, and pharmacological profile are favorable and indicate no adverse side effects. Levels in plasma 100-fold higher than the in vitro IC_{50} were observed in cynomologous monkeys after a single oral dose of 20 mg/kg (data not shown). These results and those of the activity shown in the present report are encouraging for the development of BI-RG-587 as an antiviral drug for the treatment of HIV-1 infection in patients.

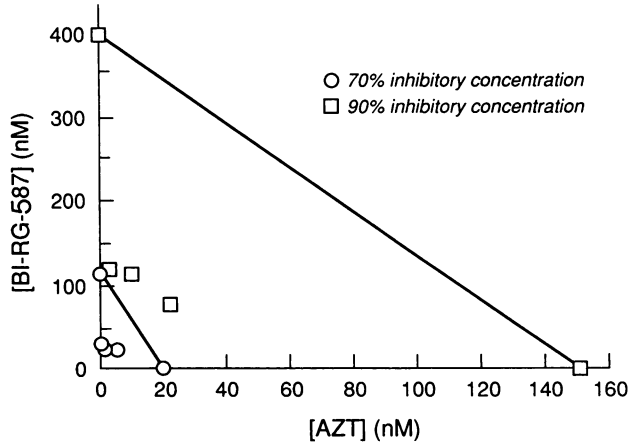

FIG. 3. Isobologram plot of inhibition, by combinations of AZT and BI-RG-587, of plaque production by $HIV\text{-}1_{BRU}$ in HT4-6C HeLa cells. The line between the values for single drugs indicates the values at which additive effects would occur. Values below and to the left of the lines indicate synergy.

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REFERENCES

1. **Chesebro, B., and K. Wehrly.** 1988. Development of a sensitive quantitative focal assay for human immunodeficiency virus infectivity. J. Virol. **62**:3779–3788.
2. **Chou, J., and T.-C. Chou.** 1987. Dose-effect analysis with microcomputers: quantitation of ED50, LD50, synergism, antagonism, low-dose risk, receptor-ligand binding and enzyme kinetics, p. 1–93. Elsevier-Biosoft, Cambridge.
3. **Chou, T.-C., and P. Talalay.** 1984. Quantitative analysis of dose-effect relationships: the combined effect of multiple drugs of enzyme inhibitors. Adv. Enzyme Regul. **22**:27–55.
4. **Fischl, M. A., D. D. Richman, M. H. Grieco, M. S. Gottlieb, P. A. Volberding, O. L. Laskin, J. M. Leedom, J. E. Groopman, D. Mildvan, R. T. Schooley, G. G. Jackson, D. T. Durack, D. King, and AZT Collaborative Working Group.** 1987. The efficacy of azidothymidine (AZT) in the treatment of patients with AIDS and AIDS-related complex. A double-blind, placebo-controlled trial. N. Engl. J. Med. **317**:185–191.
5. **Fischl, M. A., D. D. Richman, N. Hansen, A. C. Collier, J. T. Carey, M. F. Para, W. D. Hardy, R. Dolin, W. G. Powderly, J. D. Allan, B. Wong, T. C. Merigan, V. J. McAuliffe, N. E. Hyslop, F. S. Rhame, P. A. Volberding, C. Pettinelli, J. Ander-**

son, and AIDS Clinical Trials Group. 1990. The safety and efficacy of zidovudine (AZT) in the treatment of patients with mildly symptomatic HIV infection: a double-blind, placebo-controlled trial. Ann. Intern. Med. **112**:727–737.

6. **Gilboa, E., S. W. Mitra, S. P. Goff, and D. Baltimore.** 1979. A detailed model of reverse transcription and tests of crucial aspects. Cell **18**:93–100.
7. **Ho, D. D., T. Moudgil, and M. Alam.** 1989. Quantitation of human immunodeficiency virus type 1 in the blood of infected persons. N. Engl. J. Med. **321**:1621–1625.
8. **Holford, N. H. C., and L. B. Sheiner.** 1982. Kinetics of pharmacologic response. Pharmacol. Ther. **16**:143–166.
9. **Kornbluth, R. S., P. S. Oh, J. R. Munis, P. H. Cleveland, and D. D. Richman.** 1989. Interferons and bacterial lipopolysaccharide protect macrophages from productive infection by HIV in vitro. J. Exp. Med. **169**:1137–1151.
10. **Larder, B. A., B. Chesebro, and D. D. Richman.** 1990. Susceptibilities of zidovudine-susceptible and -resistant human immunodeficiency virus isolates to antiviral agents determined by using a quantitative plaque reduction assay. Antimicrob. Agents Chemother. **34**:436–441.
11. **Larder, B. A., G. Darby, and D. D. Richman.** 1989. HIV with reduced sensitivity to zidovudine isolated during prolonged therapy. Science **243**:1731–1734.
12. **Marlin, S. D., D. E. Staunton, T. A. Springer, C. Stratowa, W. Sommergruber, and V. J. Merluzzi.** 1990. A soluble form of intercellular adhesion molecule-1 inhibits rhinovirus infection. Nature (London) **344**:70–72.
13. **Merluzzi, V. J., K. D. Hargrave, M. Labadia, K. Grozinger, M. Skoog, J. Wu, C.-K. Shih, K. Eckner, S. Hattox, J. Adams, A. S. Rosenthal, R. Faanes, R. J. Eckner, R. A. Koup, and J. L. Sullivan.** 1990. Inhibition of HIV-1 replication by a nonnucleoside reverse transcriptase inhibitor. Science **250**:1411–1413.
14. **Mitsuya, H., K. J. Weinhold, P. A. Furman, M. H. St. Clair, S. Nusinoff-Lehrman, R. C. Gallo, D. Bolognesi, D. W. Barry, and S. Broder.** 1985. 3′-Azido-3′-deoxythymidine (BW A509U): an antiviral agent that inhibits the infectivity and cytopathic effect of human T-lymphotropic virus type III/lymphadenopathy-associated virus in vitro. Proc. Natl. Acad. Sci. USA **82**:7096–7100.
15. **Moore, M. A. S.** Personal communication.
16. **Pauwels, R., K. Andires, J. Desmyter, D. Schols, M. J. Kukla, H. J. Bresline, A. Raeymaeckers, J. Van Gelder, R. Woestenborghs, J. Heykants, K. Schellekens, M. A. C. Janssen, E. De Clercq, and P. A. J. Janssen.** 1990. Potent and selective inhibition of HIV-1 replication in vitro by a novel series of TIBO derivatives. Nature (London) **343**:470–474.
17. **Richman, D. D.** 1990. Zidovudine resistance of human immunodeficiency virus. Rev. Infect. Dis. **12**:S507–S512.
18. **Richman, D. D., M. A. Fischl, M. H. Grieco, M. S. Gottlieb, P. A. Volberding, O. L. Laskin, J. M. Leedom, J. E. Groopman, D. Mildvan, M. S. Hirsch, G. G. Jackson, D. T. Durack, S. Nusinoff-Lehrman, and AZT Collaborative Working Group.** 1987. The toxicity of azidothymidine (AZT) in the treatment of patients with AIDS and AIDS-related complex. A double-blind, placebo-controlled trial. N. Engl. J. Med. **317**:192–197.
19. **Richman, D. D., J. M. Grimes, and S. W. Lagakos.** 1990. Effect of stage of disease and drug dose on zidovudine susceptibilities of isolates of human immunodeficiency virus. J. AIDS **3**:743–746.
20. **Richman, D. D., R. S. Kornbluth, and D. A. Carson.** 1987. Failure of dideoxynucleosides to inhibit human immunodeficiency virus replication in cultured human macrophages. J. Exp. Med. **166**:1144–1149.
21. **SAS Institute, Inc.** 1989. SAS/STAT(R) user's guide, p. 1135–1193. SAS Institute, Inc, Cary, N.C.
22. **Staunton, D. E., V. J. Merluzzi, R. Rothlein, R. Barton, S. D. Marlin, and T. A. Springer.** 1989. A cell adhesion molecule, ICAM-1, is the major surface receptor for rhinoviruses. Cell **56**:849–853.
23. **Volberding, P. A., S. W. Lagakos, M. A. Koch, C. Pettinelli, M. W. Myers, D. K. Booth, D. H. Balfour, R. C. Reichman, J. A. Bartlett, M. S. Hirsch, R. L. Murphy, W. D. Hardy, R. Soiero, M. A. Fischl, J. G. Bartlett, T. C. Merigan, N. E. Hyslop, D. D. Richman, F. T. Valentine, L. Corey, and AIDS Clinical Trials Group.** 1990. Zidovudine in asymptomatic HIV infection. A controlled trial in persons with fewer than 500 CD4+ cells/mm^3. N. Engl. J. Med. **322**:941–949.