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Thiazolobenzimidazole: biological and biochemical anti-retroviral activity of a new nonnucleoside reverse transcriptase inhibitor

Robert W. Buckheit, Jr.[a], Melinda G. Hollingshead[a], Julie Germany-Decker[a], E. Lucile White[a], James B. McMahon[c], Lois B. Allen[a], Larry J. Ross[a], W. Don Decker[a], Louise Westbrook[a], William M. Shannon[a], Owen Weislow[d], John P. Bader[b] and Michael R. Boyd[c]

[a]*Microbiology Research Department, Southern Research Institute, Birmingham, AL, USA,* [b]*Antiviral Evaluations Branch, Developmental Therapeutics Program, Division of Cancer Treatment, National Cancer Institute, Bethesda, MD, USA,* [c]*Laboratory of Drug Discovery Research and Development, Developmental Therapeutics Program, Frederick, MD, USA and* [d]*Program Resources, Inc., Frederick Cancer Research and Development Center, Frederick, MD, USA*

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Summary

Thiazolobenzimidazole (NSC 625487) was a highly potent inhibitor of human immunodeficiency virus-induced cell killing and viral replication in a variety of human cell lines, as well as fresh human peripheral blood lymphocytes and macrophages. The compound was active against a panel of biologically diverse laboratory and clinical strains of HIV-1, including the AZT-resistant strain G910-6. However, the agent was inactive against HIV-2 and a pyridinone-resistant strain (A17) of HIV-1, a strain which is cross-resistant to several structurally diverse members of a common pharmacologic class of nonnucleoside reverse transcriptase inhibitors. The compound selectively inhibited HIV-1 reverse transcriptase but not HIV-2 reverse transcriptase. Combinations of thiazolobenzimidazole with either AZT or ddI synergistically inhibited HIV-1 induced cell killing in vitro. Thiazolobenzimidazole also inhibited the replication of the Rauscher murine leukemia retrovirus. Thus, thiazolobenzimidazole is a new active anti-HIV-1 chemotype and may represent a subclass of nonnucleoside reverse transcriptase inhibitors with an enhanced range of anti-retroviral activity.

Correspondence to: R.W. Buckheit, Frederick Research Center, 431 Aviation Way, Frederick, Maryland 21701-4756, USA. Telefax 301-694-7223.

Thiazolobenzimidazole; Nonnucleoside reverse transcriptase inhibitor; Mechanism of action; Combination therapy

Introduction

Human immunodeficiency viruses (HIV) are generally accepted as causes of acquired immunodeficiency syndrome, or AIDS (Barré-Sinoussi et al., 1983; Gallo et al., 1984; Levy et al., 1984). Despite vigorous efforts to develop antivirals which would effectively inhibit key steps of HIV replication, the only agents found to be clinically useful to date have been reverse transcriptase (RT) inhibitors (Connolly and Hammer, 1992). The HIV RT enzyme copies the viral RNA genome into a DNA form which is integrated into the host cell DNA (Varmus and Swanstrom, 1984; Gendelman et al., 1987; Goff, 1990). The nucleoside analog 3′-azido-3′-deoxythymidine (AZT), currently in clinical use, inhibits the action of this key HIV enzyme following conversion to the active metabolite, AZT triphosphate (AZT-TP) (Furman et al, 1986). AZT-TP prevents HIV reverse transcription and HIV replication by acting as a chain terminator (Mitsuya et al., 1985; Yarchoan et al., 1986; St. Clair et al., 1987; Matthes et al., 1987; Reardon and Miller, 1990; Parker et al, 1991). A second nucleoside analog, 2′,3′-dideoxyinosine (ddI), was approved for use in infected individuals (Ahluwalia et al., 1987). However, the serious toxicities associated with administration of these inhibitors (Richman et al., 1987; Yarchoan et al., 1988) and the increasingly frequent appearance of drug resistant virus strains (Larder et al., 1989; St. Clair et al., 1991) emphasizes the urgent need to identify new chemical and pharmacologic classes of anti-HIV agents for consideration for drug development and possible clinical evaluation alone or in combination with other active anti-HIV agents.

A new pharmacologic class comprised of structurally diverse nonnucleoside RT inhibitors recently has emerged. In early 1990, Pauwels et al. published the results of antiviral studies with a series of tetrahydroimidazo[4,5,1-jk][1,4]benzodiazepin-2(1H)-one and -thione (TIBO) derivatives that inhibited the replication of HIV-1 but not HIV-2 or other mammalian retroviruses. Merluzzi et al. (1990) discovered a series of dipyridodiazepinones which appeared to have the same type of specificity for HIV-1 as the TIBO compounds. Goldman et al. (1991) described derivatives of pyridinone that inhibited the growth of HIV-1 in cell culture and inhibited HIV-1 RT but not the RT from HIV-2 and Romero et al. (1991) have described a series of bis(heteroaryl)piperazines (BHAPs) which inhibit HIV-1 RT and HIV-1 replication, but not the replication of HIV-2, simian or feline immunodeficiency virus, or Rauscher murine leukemia virus (MuLV) in cell culture. Most recently, Klunder et al. (1992) have described a series of HIV-1 specific oxazepinones. Thus, each of these very different chemotypes is highly potent and selectively inhibits HIV-1 replication and cell killing, but has no activity

against those isolates of HIV-2 or other retroviruses which were tested. Moreover, viral mutants cross-resistant to these nonnucleoside RT inhibitors have been obtained in tissue culture (Nunberg et al., 1991; Richman et al., 1991; Mellors et al., 1992). Sequence analysis of the mutant viruses has identified two specific amino acid changes at positions 181 and 188, corresponding to a common site on the RT enzyme at which the different inhibitors interact (Nunberg et al., 1991; Cohen et al., 1991; Condra et al., 1992; Grob et al., 1992).

The National Cancer Institute (NCI) operates a high capacity screening program in which synthetic and natural products submitted from various sources throughout the world are tested for in vitro anti-HIV activity. The subject compound, 1-(2′,6′-difluorophenyl)-1H,3H-thiazolo[3,4-a]benzimidazole, or thiazolobenzimidazole, was submitted to the NCI program by chemists from the University of Messina (Messina, Italy) and was found active in the anti-HIV screen. The compound was one of a series synthesized from o-phenylenediamine, disubstituted aromatic aldehydes, and 2-mercaptoacetic acid; the 1-(2′,6′-difluorophenyl) derivative (Fig. 1) was the most potent of a series of derivatives synthesized (Chimirri et al., 1991a, 1991b). The further characterization of the antiviral properties of this compound, thiazolobenzimidazole (NSC 625487), are described.

Materials and Methods

Cells and viruses. The CEM-SS cell line (Nara and Fischinger, 1988) was maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum, 2 mM glutamine, penicillin (100 U/ml), and streptomycin (100 μg/ml). Other tissue culture cell lines utilized include H9, AA5, MT2, 174 × CEM, and U937. These cells were obtained from the NIAID AIDS Research and Reference Reagent Program and were cultured in the same medium. Fresh human cells

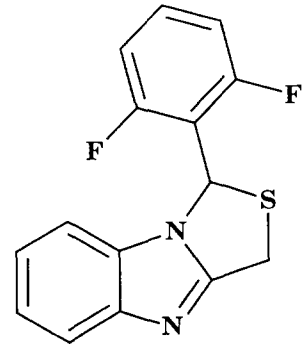

Fig. 1. Structure of 1-(2′,6′-difluorophenyl)-1H,3H-thiazolo[3,4-a]benzimidazole (thiazolobenzimidazole, NSC625487)

were obtained from the American Red Cross (Birmingham, AL, USA). Peripheral blood lymphocytes and macrophages were isolated following Ficoll-Hypaque centrifugation as described (Gartner and Popovic, 1990). The HIV-1 isolates included the common laboratory HIV-1 strains III_B, LAV, MN, and RF, as well as a panel of laboratory-derived and clinical HIV-1 isolates cultured from peripheral blood lymphocytes of patients at Duke University Medical Center (Durham, NC) and the University of Alabama at Birmingham (Birmingham, AL). The biological and biochemical properties of the panel of laboratory-derived isolates have been previously described (Cloyd and Moore, 1989; Buckheit and Swanstrom, 1991). The HIV-2 isolate ROD (Nr.I-532) was obtained from Dr. Luc Montagnier. The HIV-2 isolate MS and the AZT-resistant isolate G910-6 were obtained from the AIDS Research and Reference Reagent Program, AIDS Program, NIAID, NIH (Kanki et al., 1989; Larder et al., 1989). The pyridinone-resistant isolate A17 was obtained from Dr. M. Goldman at Merck, Sharp and Dohme Laboratories (West Point, PA) (Nunberg et al., 1991). Cells and viruses utilized in the performance of Rauscher MuLV assays have been previously described (Shannon et al., 1974).

Materials. All experimental antiviral agents were obtained from the Drug Synthesis and Chemistry Branch, Developmental Therapeutics Program, Division of Cancer Treatment, National Cancer Institute. Crystalline stock materials were stored at −70°C and solubilized in 100% dimethylsulfoxide (DMSO). The reference anti-HIV compounds used in these studies were 3′-azido-3′-deoxythymidine (AZT, NSC 602670) and 2′,3′-dideoxycytidine (ddC, NSC 606170). Biscarboxyethyl-5(6)-carboxy-fluorescein acetoxymethyl ester (BCECF) was purchased from Molecular Probes, Inc. (Eugene, OR) and dissolved immediately before use in DMSO (1 mg/ml). A working solution of 2 μg/ml was prepared in Dulbecco's phosphate-buffered saline (PBS) (GIBCO, Grand Island, New York). 4′,6-Diamidino-2-phenylindole (DAPI) was purchased from Sigma Chemical Co. (St. Louis, MO). Stock solutions of DAPI were prepared at 100 μg/ml in distilled water by sonication, passed through a 0.45-μm filter and stored at −20°C. Working solutions of DAPI were prepared at 10 μg/ml in PBS containing 0.5% nonidet P-40 (NP-40) (Sigma). Poly (rA):$p(dT)_{12-18}$, poly(rC):$p(dG)_{12-18}$, poly(rA), oligo(dT), dATP, dGTP, dCTP, and dTTP were purchased from Pharmacia LKB Biotechnology (Piscataway, NJ). [Methyl,1′2′-^{3}H]dTTP (100 Ci/mmol) and [8,5′-^{3}H]dGTP (31.9 Ci/mmol) in Tricine buffer were obtained from New England Nuclear Research Products (Wilmington, DE). The sodium salt of lauryl sulfate (SDS) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were obtained from Sigma Chemical Co. (St. Louis, MO). A solution of 16S and 23S *E. coli* ribosomal RNA was obtained from Boehringer–Mannheim Biochemicals (Indianapolis, IN). The 15 base primer, complementary to a sequence in the 16S *E. coli,* was purchased from Genosys Biotechnologies, Inc. (The Woodlands, TX). The sequence of the 15 base primer was 5′-TAACCTTGCGGCCGT-3′. DE81 and GF/A chromatography paper were

from Whatman International Ltd (Maidstone, England). Tissue culture medium and additives, were obtained from GIBCO. Fetal bovine serum was obtained from HyClone (Logan, UT). Human serum was obtained from volunteer donors and was stored at −70°C prior to use. Rat serum was obtained from Fisher rats housed at Southern Research Institute. ELISA plates were obtained from Coulter Cytometry (Hialeah, FL). 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide inner salt, XTT, was obtained from the National Cancer Institute.

Anti-HIV assays. Cells were placed in each well of a 96-well microtiter plate to a density of 5×10^3 cells per well. The cells were infected with virus at a multiplicity of infection (MOI) previously determined to give complete cell killing (CEM-SS, AA5, MT2, 174 × CEM) or maximal levels of virus production (H9, U937, PBL, macrophage) at 6 days post-infection (MOI of 0.01–0.05). The panel of virus isolates was pretitered to induce equivalent infections (based on cell killing or virus production) in these assays. Serial half-log dilutions of test compounds were added to appropriate wells in triplicate to evaluate their ability to inhibit HIV infection. Controls for each assay included drug colorimetric control wells (drug only), drug cytotoxicity control wells (cells plus drug), virus control wells (cells plus virus), and cell viability control wells (cells only). AZT and ddC were run in parallel as positive control drugs. Following 6 days of incubation at 37°C, the viability of the cells in each well was determined spectrophotometrically for cells which were cytopathically infected with HIV. XTT was added to each well as previously described (Weislow et al., 1989), the plates incubated for 4 h at 37°C, and the viability assay performed. In noncytopathic infections the results were analyzed by quantitation of RT activity or p24 core protein in cell free supernatants derived from each well of the microtiter plate. In some experiments the MOI or the time of drug addition were varied to measure the effect on the antiviral activity of the compound. Antiviral and toxicity data is reported as the quantity of drug required to inhibit 50% of virus induced cell killing or virus production (EC_{50}) and the quantity of drug required to reduce cell viability by 25% (IC_{25}).

Assay of virus growth in supernatant samples. Samples of virus-containing supernatants were removed from each well of the microtiter plate prior to staining with XTT. These samples were analyzed for their content of virus by RT activity assay, p24 ELISA, and CEM-SS infectivity assay. The RT and CEM-SS infectivity assays were performed as previously described (Nara and Fischinger, 1988; Buckheit and Swanstrom, 1991). The p24 ELISA was performed according to the manufacturers recommendations.

Multiparameter antiviral assays. The activity and toxicity of an active compound were further analyzed in a microtiter assay plate by quantitation of virus production by RT activity assay, p24 ELISA, and infectious particle assay and by quantitation of cellular growth kinetics by XTT, BCECF, and

DAPI. The protocols utilized for simultaneous measurement of these parameters has been described previously (Gulakowski et al., 1991).

Effect of compounds on chronically infected cells. Chronically infected cell lines (H9-III_B) were obtained from the outgrowth of HIV-infected, virus-producing cells following acute infection of H9 cells. These cells were cultured in the presence of serial one-log dilutions of antiviral compounds. Cell-free supernatant samples were obtained on a daily basis and analyzed for virus content by RT assay, p24 ELISA and CEM-SS infectivity assay as described above.

Combination antiviral analysis. Analysis of drug combination assays was performed utilizing the anti-HIV assay methodology described above with statistical evaluations performed according to the method of Prichard and Shipman (1990). Combination antiviral XTT assays were performed with CEM-SS cells utilizing the III_B strain of virus. The standard anti-HIV assay was altered for combination analysis by increasing the multiplicity of infection 3-fold, allowing greater statistical consistency in these assays.

Reverse transcriptase enzyme inhibition assays. Purified recombinant RT ($HIV1_{BH10}$) prepared in *E. coli* was obtained from the University of Alabama at Birmingham, Center For AIDS Research, Gene Expression Core Facility. This RT is now available from the AIDS Research and Reference Reagent Program. Upon SDS-PAGE analysis the purified recombinant RT contained approximately 60% full length p66 and 40% processed p51 polypeptides. The method utilized to purify RT enzyme from virus pools has been described (White et al., 1991). The assays utilizing various template:primer systems to evaluate the inhibition of HIV-1 and HIV-2 RT were based on the following modifications of previously described methods (White et al., 1991; Parker et al., 1991). The assay utilizing HIV-1 and the ribosomal RNA template contained 50 mM Tris-HCl (pH 7.4), 50 mM KCl, 10 mM $MgCl_2$, 4 mM β-mercaptoethanol, 1 mg/ml bovine serum albumin, 2% DMSO, 0.5% glycerol, 6.66 μg/ml of ribosomal RNA:primer (equivalent to 15 nM 3′-hydroxyl primers annealed to RNA), 10 μM dATP, 10 μM dCTP, 10 μM dGTP, and 2 μM dTTP (101.9 mCi $^3H/\mu$mol dTTP). For HIV-2 RT, the assay on the ribosomal RNA template contained 50 mM MOPS (pH 7.2), 75 mM KCl, 4 mM $MgCl_2$, 4 mM β-mercaptoethanol, 1 mg/ml bovine serum albumin, 2% DMSO, 3% glycerol, 6.66 μg/ml of ribosomal RNA:primer (equivalent to 15 nM 3′-hydroxyl primers annealed to RNA), 10 μM dATP, 10 μM dCTP, 10 μM dGTP, and 2 μM dTTP (101.9 mCi $^3H/\mu$mol dTTP). The assay utilizing the gapped duplex DNA template contained 50 mM Tris-HCl (pH 7.4), 125 mM KCl, 2 mM $MgCl_2$, 4 mM β-mercaptoethanol, 1 mg/ml bovine serum albumin, 0.5% glycerol, 2% DMSO, 10 μg/ml of gapped duplex DNA, 10 μM dATP, 10 μM dCTP, 10 μM dGTP, and 2 μM dTTP (101.9 mCi $_3H/\mu$mol dTTP). The assay on the poly(rA):$p(dT)_{12-18}$ template contained 38 mM Tris-HCl (pH 7.9),

180 mM KCl, 8 mM $MgCl_2$, 4 mM β-mercaptoethanol, 1 mg/ml bovine serum albumin, 0.5% glycerol, 2% DMSO, 20 μg/ml poly(rA):p$(dT)_{12\ 18}$, and 60 μM dTTP (4 mCi $^3H/\mu$mol dTTP). The assay utilizing the poly(rC):p$(dG)_{12-18}$ template contained 38 mM Tris-HCl (pH 7.9), 180 mM KCl, 8 mM $MgCl_2$, 4 mM β-mercaptoethanol, 1 mg/ml bovine serum albumin, 0.5% glycerol, 2% DMSO, 20 μg/ml poly(rC):p$(dG)_{12\ 18}$, and 50 μM dGTP (4 mCi $^3H/\mu$mol dGTP). The amount of recombinant RT was adjusted for each template so that equivalent number of units were present. A unit of enzyme activity is defined in terms of pmol of ^{3}H-dNMP incorporated into the template per hour.

Rauscher MuLV UV-XC plaque reduction and yield reduction assays. The UV-XC plaque reduction assay has been described previously (Shannon et al., 1974). Antiviral activity is expressed as the reduction in the mean number of plaques counted in the drug-treated, virus-infected cultures compared with the mean number of plaques counted in the untreated, virus-infected cultures. The drug concentration which decreased the mean number of plaques by 50% (EC_{50}) was calculated using regression analysis for semi-log curve fitting. Cytotoxicity determinations were performed in parallel by the MTT dye reduction assay (Mosmann, 1983). Samples collected for virus yield reduction were assayed for infectious virus by the UV-XC plaque assay. For this, serial 10-fold dilutions of pooled samples from each treatment group were assayed in triplicate for Rauscher MuLV by plaque assay. The assays included AZT as a positive control compound.

Results

Biological activity of thiazolobenzimidazole

A microtiter antiviral assay was performed to determine the effect of thiazolobenzimidazole (NSC 625487) on the ability of HIV-1 to initiate a productive, cytopathic infection in CEM-SS cells. Thiazolobenzimidazole reproducibly protected CEM-SS cells from HIV-1 induced cytopathicity with 50% cellular protection (EC_{50}) achieved with approximately 0.5 μM (Fig. 2C). Microscopic examination of the cells in wells protected by effective doses of the compound confirmed the complete absence of giant-cell formation and other HIV-1-induced cytopathicity. Thiazolobenzimidazole, at effective concentrations up to 10-fold greater than the antiviral EC_{50} values, was not cytotoxic to CEM-SS cells. Toxicity values (IC_{25}) in CEM-SS cells ranged from approximately 15–20 μM (Fig. 2C). Measures of in vitro therapeutic index for thiazolobenzimidazole performed with the BCECF (Fig. 2A) and DAPI (Fig. 2B) assays (see Methods and Gulakowski et al., 1991) were fully consistent with the XTT assay results. In cell-free supernatants from infected cells profound decreases in supernatant RT activity, p24 core protein, and infectious virus particles were found after incubation with cytoprotective doses of thiazolobenzimidazole (Fig. 2D). The EC_{50} values for the effects of the drug

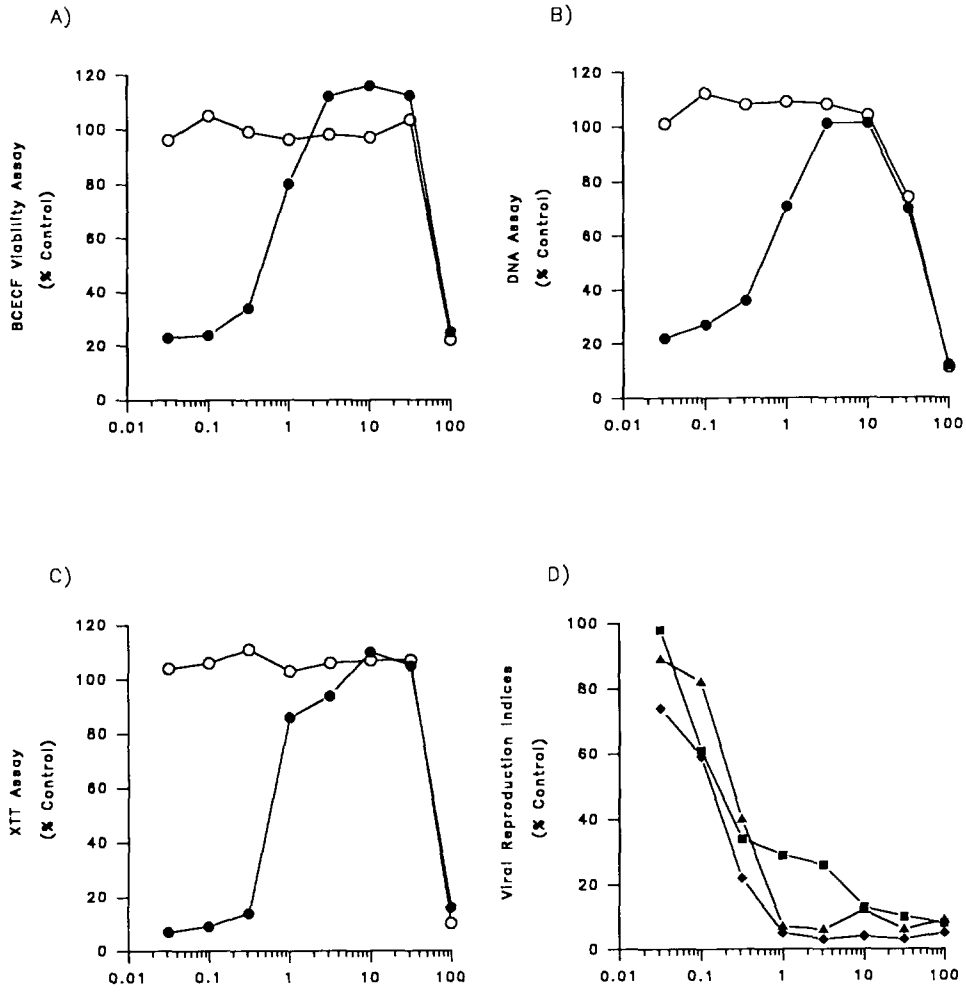

Fig. 2. Antiviral and cytotoxic effects of thiazolobenzimidazole in CEM-SS cells as measured by a multiparameter microtiter assay. (A) Effects of thiazolobenzimidizole on cell viability measured by BCECF assay. (B) Effects of thiazolobenzimidazole on relative DNA contents of cells measured by DAPI assay. (C) Effects of thiazolobenzimidazole on total cell number and cell viability measured by XTT assay. (D) Effect of thiazolobenzimidazole upon indices of virus replication, including supernatant RT activity, supernatant core protein, and infectious virus particles. In graphs A, B, and C the data are reported as the percent of uninfected, non-drug treated control values. In graph D, the data are reported as the percent of infected, non-drug treated control values.

on these indices of virus replication were similar to those obtained in the cytopathicity assays.

TABLE 1

Range of activity of thiazolobenzimidazole against HIV isolates in cell culture-based assays

Virus/strain	EC_{50} (μM)[a]	
	Thiazolobenzimidazole	AZT
HIV-1 III_B	2.33	0.012
HIV-1 RF	1.22	0.037
HIV-1 MN	1.50	0.003
HIV-1 LAV	0.52	0.002
HIV-1 SK-1	0.20	0.004
HIV-1 214	1.11	0.002
HIV-1 205	0.90	0.002
HIV-1 G	1.15	0.004
HIV-1 TP-1	2.19	0.006
HIV-1 KELL	2.33	0.003
HIV-1 ED	0.98	0.002
HIV-1 PM16	2.19	0.006
HIV-1 McK	0.66	0.002
HIV-1 C	1.14	0.008
HIV-2 ROD	>20.0	0.002
HIV-2 MS	>20.0	0.008

[a]EC_{50} values determined by XTT-based cytopathicity assay in CEM-SS cells.

Range of activity of thiazolobenzimidazole

Thiazolobenzimidazole and AZT were tested in CEM-SS cells against 14 isolates of HIV-1 and two isolates of HIV-2 (Table 1). These HIV-1 isolates included common laboratory strains as well as a group of clinical isolates obtained from patients. All of these isolates have been passaged in tissue culture cell lines and are cytopathic upon infection of human T cell lines. Thiazolobenzimidazole was active against all of the HIV-1 isolates. However, no activity against HIV-2 was detected. The EC_{50} values obtained for thiazolobenzimidazole for these various HIV-1 strains fell into a narrow range; concentrations of thiazolobenzimidazole ranging from 0.20–2.33 μM were required to inhibit virus-induced cell killing by 50%. AZT was equally active against both HIV-1 and HIV-2 isolates (EC_{50} values of 0.002–0.008 μM for HIV-2 and 0.002–0.037 μM for HIV-1) (Table 1).

Further biological experiments were performed to determine the range of action of the compound. Thiazolobenzimidazole was active against HIV-1 in all cultured cells tested including human T cells, B cells, and macrophages (Table 2). Antiviral activity in fresh human peripheral blood lymphocytes and macrophages was obtained at concentrations similar to those which were active in the continuous cell lines (Table 2). Assays in fresh human cells also confirmed the ability of the compound to inhibit the replication of clinical strains of HIV-1, which have only been passaged in fresh human cells. Further, thiazolobenzimidazole was fully active when solubilized in fresh human or rat serum (data not shown).

Limited pretreatment experiments showed that the compound had to be

TABLE 2

Range of activity of thiazolobenzimidazole in different human cell lines

Cell line	Phenotype[a]	Assay[b]	$EC_{50}(\mu M)$[c]	$IC_{25}(\mu M)$
CEM-SS	T	XTT	1.28	16.3
MT2	T (HTLV-I+)	XTT	1.32	13.2
H9	T	RT	2.26	8.7
AA5	B (EBV+)	XTT	0.90	5.8
U937	M	RT	0.52	7.0
174xCEM	BxT	XTT	1.74	19.2
PBL	lymphocyte	RT	0.69	>10.4
Macrophage	macrophage	p24	0.69	>10.4
H9-III_B	T (HIV-1+)	RT	>20.0	12.4

[a]T = T cell; B = B cell; M = macrophage; HTLV-I = human T cell leukemia virus type-I; EBV = Epstein-Barr virus.
[b]Drug-induced inhibition of virus replication was quantitated by XTT assay in cytopathically-infected cells and by RT activity assay or p24 ELISA in noncytopathically-infected cells.
[c]$HIV\text{-}1_{RF}$ was utilized in all antiviral testing involving cultured cell lines; fresh PBL and macrophage cells were infected with the clinical HIV-1 isolates WEJO and Ba-L.

present continuously to be maximally active in cell based assays (data not shown). Further, in assays in which the compound was removed from treated cells by extensive washing prior to HIV infection, no protection from HIV-1 induced cytopathic effects was detected. Experiments involving variations in the MOI or the time of drug addition suggest that the compound acts similarly to other RT inhibitors such as AZT and TIBO (data not shown). In these experiments thiazolobenzimidazole remained active when challenged at the same MOI and for the same amount of time post-infection as both AZT and TIBO. In addition, the compound had no effect on virus replication from chronically infected cells (Table 2).

HIV-1 isolates resistant to AZT or other RT inhibitors were tested for susceptibility to thiazolobenzimidazole in order to further investigate the range of action and to obtain preliminary information regarding the mechanism of action of the compound. Thiazolobenzimidazole was fully active against the AZT-resistant strain G910-6, exhibiting an EC_{50} of 1.32 μM (Table 3). The pyridinone-resistant virus strain, A17, however was able to replicate in the presence of high concentrations of thiazolobenzimidazole (Table 3). Control data demonstrating the inhibition of replication of drug sensitive HIV-1 (III_B) is presented in Table 3 for comparison in both CEM-SS and MT2 cells.

TABLE 3

Antiviral activity of thiazolobenzimidazole against drug-resistant HIV-1 strains in cell culture-based assays

Virus isolate	Resistance phenotype	Cell line	EC_{50} (μM)	IC_{25} (μM)
G910-6	AZT	MT2	1.32	7.3
A17	pyridinone	CEM-SS	>20.0	19.6
III_B	control	CEM-SS	1.28	16.3
III_B	control	MT2	1.32	13.2

TABLE 4

Inhibition of HIV-1 reverse transcriptase by thiazolobenzimidazole

Template	ID_{50} (μM)[a]
Ribosomal RNA	0.52 + 0.21
Gapped duplex DNA	4.62 + 0.73
Poly(rA):p$(dT)_{12-18}$	>347
Poly(rc):p$(dG)_{12-18}$	12.3 + 6.0

[a]Values are the mean + S.D. of three separate determinations.

Biochemical activity of thiazolobenzimidazole

The ability of thiazolobenzimidazole to inhibit recombinant HIV-1 RT was examined (Table 4) based on the cell culture data involving the resistant virus strains that suggested the compound might be a nonnucleoside RT inhibitor. When ribosomal RNA was used in the assay to mimic negative strand synthesis, thiazolobenzimidazole inhibited the activity of the enzyme by 50% (ID_{50}) at 0.520 μM. When gapped duplex DNA was used to mimic positive strand synthesis the ID_{50} was an order of magnitude higher (4.62 μM). Interestingly, if either of the synthetic homopolymer RNA templates poly(rA):p$(dT)_{12-18}$ or poly(rC):p$(dG)_{12-18}$ were used as the template the ID_{50} was much higher than that seen with the ribosomal RNA template. Similar results were obtained with the ribosomal RNA template using HIV-1 RT from partially purified detergent disrupted virions (data not shown).

The reversible inhibition of HIV-1 RT by thiazolobenzimidazole was examined further. The compound did not compete with either the ribosomal RNA template or the deoxynucleoside triphosphates for their binding sites. The type of inhibition for thiazolobenzimidazole with respect to the ribosomal RNA template was mixed with a K_i (dissociation constant for the enzyme-thiazolobenzimidazole complex) of 0.69 μM and a K'_i (equilibrium constant for the dissociation of thiazolobenzimidazole from the enzyme-thiazolobenzimidazole-substrate complex) of 0.28 μM (data not shown). The K_i with respect to dGTP was 0.49 μM and the mode of inhibition was non-competitive (data not shown).

As noted above thiazolobenzimidazole was inactive against HIV-2 in cell based assays (see Table 1). Consistent with this finding, thiazolobenzimidazole did not inhibit the partially purified RT from HIV-2 obtained from detergent disrupted virions. The ID_{50} value with the ribosomal RNA template was greater than 347 μM.

Activity of thiazolobenzimidazole versus Rauscher MuLV

Thiazolbenzimidazole was also tested and found active against the Rauscher MuLV (Table 5). The compound significantly inhibited plaque formation in the UV-XC assay (EC_{50}=4.44 μM). The activity of the compound was further confirmed in yield reduction assays. Yield reduction experiments are performed to quantitate the amount of virus produced by the infected SC-1 cells in the

TABLE 5

Antiviral activity of thiazolobenzimidazole against Rauscher MuLV

Drug concentration (μM)	SC-1 Plaque reduction (%)[a]	Viability (%)[b]	Virus production (%)[c]
52.1	100.0	64.6	2.1
16.6	81.2	83.1	4.6
5.21	48.3	91.9	18.2
1.66	35.6	97.4	23.6
0.52	16.8	97.1	31.4
0.16	6.0	100.0	50.0
None	0	100.0	100.0

[a]Percent drug-induced reduction in plaque formation measured by UV-XC assay.
[b]Viability of SC-1 cells cultured in presence of drug quantitated by MTT assay.
[c]Virus production from SC-1 cells cultured in the presence of drug compared to virus production from infected untreated SC-1 cells.

UV-XC assay at each drug concentration prior to exposure to ultraviolet light and overlay with XC cells. In this assay, significant reductions in virus production were detected with increasing concentrations of the drug. Virus production from the SC-1 cells was decreased by 50% (EC_{50}) at approximately 0.16 μM. Cytotoxicity was observed only at higher concentrations of the compound (>16.6 μM).

Combination antiviral activity with AZT and ddI

Thiazolobenzimidazole was tested for anti-HIV activity in combination with AZT or ddI using the in vitro XTT anti-HIV assay. Five concentrations of thiazolobenzimidazole were tested in all combinations with eight concentrations of AZT or ddI. Data analyses were performed using the three-dimensional model of Prichard and Shipman (36). Effects of the drug combination were calculated based on the activity of the two compounds when tested alone. The calculated additive antiviral protection was subtracted from the experimentally determined antiviral activity at each combination concentration resulting in a positive value (synergy), a negative value (antagonism), or zero (additivity). Data were analyzed by the most stringent statistical means by assuming the compounds inhibited HIV replication by action at the same site (mutually exclusive). We decided to use the same site assumption although it is known that the nucleoside and nonnucleoside RT inhibitors bind at different sites on the RT enzyme and do not compete with one another for binding to the RT enzyme. The results of these assays demonstrated that the combined antiviral activity was much greater than that predicted based on additivity, indicating significant synergy. The three dimensional plot of the data demonstrated a surface extending above the plane of additivity (Fig. 3A). The peak above the plane represented a maximal antiviral activity (protection from HIV-induced cell killing) from a combination of thiazolobenzimidazole and AZT which was nearly 60% greater than would have been expected if the antiviral effects were merely additive. Use of

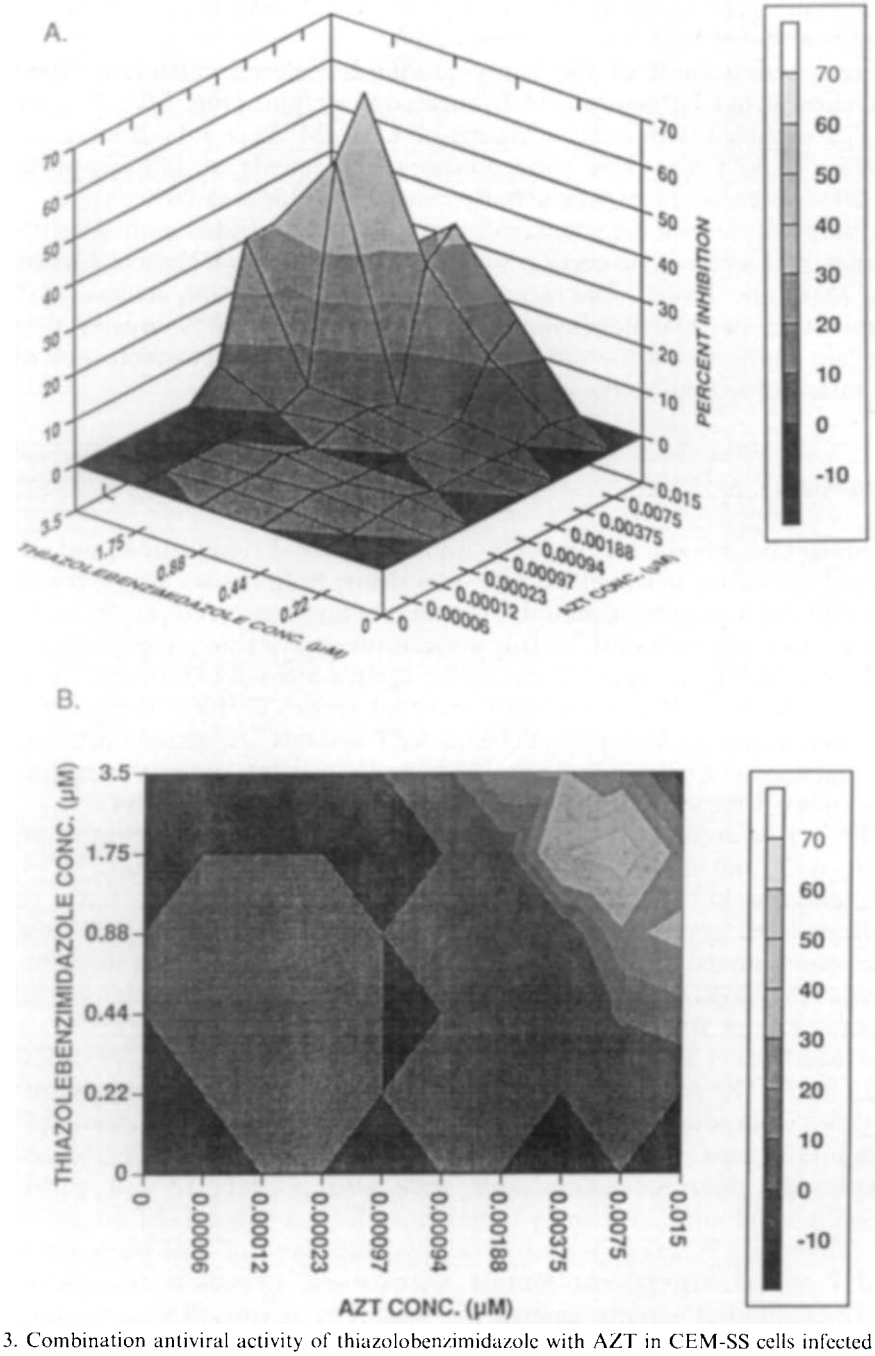

Fig. 3. Combination antiviral activity of thiazolobenzimidazole with AZT in CEM-SS cells infected with the III_B strain of HIV-1. (A) The three dimensional synergy plot demonstrating the synergistic activity of the two compounds. (B) The contour plot version of the synergy plot allowing visualization of the compound concentrations yielding maximal synergistic anti-HIV activity.

the less stringent different site assumption would have increased the level of synergy reported here.

The concentrations of the two compounds yielding maximum synergistic protection could be determined from a contour plot (Fig. 3B). The greatest synergy occurred with concentrations of 1.75 μM thiazolobenzimidazole and 0.0075 μM AZT; for these concentrations, the calculated additive protection was 30% whereas the experimentally measured value was 90%.

Synergy of thiazolobenzimidazole was also demonstrated with ddI although the maximal levels of protection were somewhat less than those achieved with AZT (data not shown). The measured maximum protection achieved with the combination of thiazolobenzimidazole and ddI was 40% greater than the calculated additivity value and occurred at a combination concentration of 1.75 μM thiazolobenzimidazole and 0.5 μM ddI.

Discussion

Thiazolobenzimidazole strongly inhibited HIV-1 replication and HIV-1-induced cell killing in cell based assays including both tissue culture-derived cell lines and fresh peripheral blood lymphocytes and macrophages. The antiviral activity was fully manifest at drug concentrations not detectably cytotoxic to the host cells. The compound was active against a panel of biologically diverse HIV-1 isolates, including the AZT-resistant strain, G910-6. Range of action and mechanistic studies in parallel with AZT and ddC suggested the compound was acting as a RT inhibitor. Maximal antiviral activity required the continuous presence of effective concentrations of the compound.

The lack of antiviral activity against HIV-2 or the pyridinone-resistant virus strain, A17, and the inhibitory activity of the compound against HIV-1 RT preferentially in the heteropolymer RNA template assay, have further implicated the compound as a nonnucleoside HIV-1 specific RT inhibitor. Other compounds of this general pharmacologic class have been described and include the TIBO compounds, the dipyridodiazepinones, the pyridinone derivatives, the BHAP compounds, and the oxazepinones (Pauwels et al., 1990; Merluzzi et al., 1990; Goldman et al., 1991; Romero et al., 1991; Klunder et al., 1992). The A17 virus strain (Nunberg et al., 1991) is cross-resistant to all of those compounds in this structurally diverse class of nonnucleoside inhibitors against which it has been tested.

Although thiazolobenzimidazole now also appears to fall within the emerging common class it may represent a distinct subclass of nonnucleoside RT inhibitors. Whereas the other known members of this class reportedly exhibit no activity against murine retroviruses, thiazolobenzimidazole has nearly equipotent activity against the murine retrovirus Rauscher MuLV as with HIV-1. This may indicate that its sites of interaction on the RT enzyme are not entirely the same as those recognized by the other nonnucleoside RT inhibitors. However, it has been recently recognized that the exquisite HIV-1

specificity of the TIBO type nonnucleoside RT inhibitors is not absolute. Debeyser et al. (1992) have reported on the anti-simian immunodeficiency virus (SIV) activity of certain TIBO derivatives. We have also determined that the TIBO analog R82913 inhibited the replication of Rauscher MuLV (Buckheit et al., 1992). It should be noted that most of the nonnucleoside RT inhibitors have not been tested against Rauscher MuLV. Our results with derivatives of TIBO and with thiazolobenzimidazole suggest that the Rauscher MuLV model system may be an effective means of examining the in vivo activity of these compounds.

Biochemical assays with thiazolobenzimidazole indicate that the compound inhibits HIV-1 RT with template specificities similar to other members of this inhibitor class. Inhibition of RT was not detected in assays utilizing the homopolymer template poly(rA):p$(dT)_{12-18}$, whereas inhibition was observed when assayed with the poly(rC):p$(dG)_{12-18}$ homopolymer template. The greatest inhibition of RT activity was observed when utilizing a heteropolymer RNA template, consistent with the pattern observed with other nonnucleoside RT inhibitors. The compound did not compete reversibly with either ribosomal RNA template or deoxynucleoside triphosphates for their binding sites. As a class, the nonnucleoside RT inhibitors are characterized by inhibition kinetics that are consistent with binding at some site other than those utilized by the template or the deoxynucleotide triphosphate substrates.

Other known members of the nonnucleoside class of RT inhibitors have been extensively examined. The binding sites of nevirapine (a dipyridodiazepinone) to the RT enzyme of HIV-1 have been defined (Cohen et al., 1991; Wu et al., 1991; Grob et al., 1992; Condra et al., 1992). Sequence analyses of the resistant mutant A17 have indicated that a mutation at amino acid 181, which is adjacent to a conserved YG/MDD motif, was responsible for the resistant phenotype (Nunberg et al., 1991). Other studies have implicated interaction of tyrosine residues at 181 and 188 in the binding of nevirapine to the RT molecule. Condra et al. (1992) recently reported on molecular interactions between the nonnucleoside RT inhibitors and a region of the RT molecule defined by amino acid residues 176 to 190 with specific contributions by the tyrosines at position 181 and 188. They also determined that nevirapine, TIBO, and the pyridinone inhibitors were functionally equivalent in terms of their binding interactions with the RT molecule. The antiviral activity of thiazolobenzimidazole against the Rauscher MuLV suggests that other RT interaction sites may be important and more detailed structure activity relationships may help define the critical residues recognized by thiazolobenzimidazole in the RT enzyme.

The rapid emergence of virus strains resistant to the antiviral action of nonnucleoside RT inhibitors in vitro and in vivo suggests that their clinical potential could be relatively limited or at least short-lived. However, thiazolobenzimidazole is highly active in combination with both AZT and ddI, synergistically inhibiting HIV replication and HIV-induced cell killing. Given the continuing problems of toxicity, acquired resistance, and other

limitations of the only approved drugs currently available for the treatment of HIV infection, continuing research and careful consideration should be focussed upon new potential anti-HIV drug development candidates with different mechanisms of action, different mechanisms of resistance, different toxicity profiles, or favorable physicochemical properties. The diverse structures represented in the emerging class of nonnucleoside RT inhibitors, which now appears to include thiazolobenzimidazole, share key mechanistic commonalities, yet may vary widely or subtly in other important attributes critical to their potential usefulness as therapeutic agents for HIV infection.

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