ELSEVIER

Antiviral Research 26 (1995) 117–132

Antiviral Research

# Comparative anti-HIV evaluation of diverse HIV-1-specific reverse transcriptase inhibitor-resistant virus isolates demonstrates the existence of distinct phenotypic subgroups

Robert W. Buckheit Jr. [a,*], Valerie Fliakas-Boltz [a], William Don Decker [a], Joseph L. Roberson [a], Tracy L. Stup [a], Cathi A. Pyle [a], E. Lucile White [b], James B. McMahon [c], Michael J. Currens [c], Michael R. Boyd [c], John P. Bader [d]

[a] *Virology Research Group, Southern Research Institute-Frederick Research Center, 431 Aviation Way, Frederick, MD 21701-4766, USA*

[b] *Microbiology Research Department, Southern Research Institute, Birmingham, AL 35255, USA*

[c] *Laboratory of Drug Discovery Research and Development, Developmental Therapeutics Program, Division of Cancer Treatment, National Cancer Institute, Frederick, MD 21702, USA*

[d] *Antiviral Evaluations Branch, Developmental Therapeutics Program, Division of Cancer Treatment, National Cancer Institute, Bethesda, MD 20892, USA*

Received 21 July 1994; accepted 4 October 1994

**Abstract**

We have biologically and biochemically evaluated a structurally diverse group of HIV-1-specific reverse transcriptase (RT) inhibitors and determined that the members of this class share many common properties. These include reproducible and selective antiviral activity against a panel of biologically distinct laboratory and clinical strains of HIV-1, activity against HIV-1 in a wide variety of cultured and fresh human cells, and potent inhibition of HIV-1 RT when evaluated using a heteropolymeric ribosomal RNA template assay. Each of the HIV-1-specific compounds was capable of inhibiting HIV replication when challenged at high m.o.i., further distinguishing them from the nucleoside analogs 3′-azido-3′-deoxythymidine (AZT) and 2′,3′-dideoxycytidine (ddC). When tested in combination with AZT, each of the HIV-1-specific compounds synergistically inhibited the replication of HIV-1. HIV-1 isolates resistant to different HIV-1-specific inhibitors exhibited heterogeneous patterns of cross-resistance to other members of this pharmaco-

* Corresponding author. Fax: +1 (301) 694 7223.


*SSDI* 0166-3542(94)00069-7

logic class. Four distinct phenotypic classes have been defined through the use of drug-resistant virus isolates which derive from distinct mutations in the RT. These results indicate that the various subgroups of HIV-1-specific inhibitors interact differently with HIV-1 RT, suggesting important potential implications for drug combination therapeutic strategies.

*Keywords:* HIV-1-specific inhibitor; Anti-HIV activity; Combination therapy; Drug resistance; Phenotypic subgroups

## 1. Introduction

Despite vigorous efforts to develop antivirals which effectively inhibit key steps of HIV replication, the only agents which are proven clinically useful to date are reverse transcriptase (RT) inhibitors (Connolly and Hammer, 1992). The nucleoside analogs 3′-azido-3′-deoxythymidine (AZT), 2′,3′-dideoxyinosine (ddI), 2′,3′-dideoxycytidine (ddC) and 2′,3′-dideoxythymine (d4T) have been approved for use in infected individuals (Ahluwalia et al., 1987; Yarchoan et al., 1988, 1989; Mansuri et al., 1989). The serious toxicities associated with administration of these inhibitors (Richman et al., 1987; Yarchoan et al., 1988) and the emergence of drug-resistant virus strains (Larder et al., 1989; St. Clair et al., 1991) emphasize the urgent need to identify and develop new chemical and pharmacologic classes of anti-HIV agents for clinical evaluation alone or in combination with other active anti-HIV agents.

A new general pharmacologic class of structurally diverse non-nucleoside RT inhibitors has been recognized. Compounds which have been characterized as non-nucleoside RT inhibitors include a series of tetrahydro-imidazo[4,5,1-*jk*][1,4]-benzodiazepin-2(1*H*)-one and -thione (TIBO) derivatives (Pauwels et al., 1990), a series of dipyridodiazepinones (Merluzzi et al., 1990), derivatives of pyridinone (Goldman et al., 1991), a series of bis(heteroaryl)piperazines (BHAPs)(Romero et al., 1991), a series of oxazepinones (Klunder et al., 1992), and the thiazolo-iso-indolinones (Maass et al., 1993). Each of these structurally distinct chemotypes are potent and selective inhibitors of HIV-1 replication and cell killing, but have relatively little or no activity against all tested isolates of HIV-2 or other retroviruses. Viral isolates or RT cross-resistant to these non-nucleoside RT inhibitors have been obtained in tissue culture or by site-directed mutagenesis (Nunberg et al., 1991; Richman et al., 1991a; Wu et al., 1991; De Vreese et al., 1992; Mellors et al., 1992; Balzarini et al., 1993a,b) as well as in patients (Saag et al., 1993; Richman et al., 1994). Sequence analysis of these viruses has identified resistance-engendering mutations at amino acid positions 98, 100, 103, 106, 108, 138, 181, 188 and 236. In the crystal structure of the RT (Kohlstaedt et al., 1992; Jacobo-Molina et al., 1993) these amino acid changes correspond to a deep hydrophobic pocket near the polymerase active site in the p66 palm subdomain of the RT at which the different HIV-1-specific inhibitors interact (Cohen et al., 1991; Nunberg et al., 1991; Condra et al., 1992; Grob et al., 1992; Smerdon et al., 1994). The effects of some of these resistance-engendering mutations on the efficacy of both HIV-1-specific and nucleoside RT inhibitors have been recently reported (Byrnes et al., 1993; Byrnes et al., 1994).

A number of additional diverse HIV-1-specific compounds have been identified through the National Cancer Institute (NCI) high capacity screening program (Boyd, 1988; Weislow et al., 1989). These compounds include the synthetic compounds oxathiin carboxanilide (Bader et al., 1991), the diarylsulfones (McMahon et al., 1993), thiazolobenzimidazole (Buckheit et al., 1993a), the benzothiadiazines (Buckheit et al., 1994b), and the natural product calanolide A (Kashman et al., 1992). A comparative analysis of the antiviral activities which are common to the group of non-nucleoside inhibitors as well as properties distinctive to the individual compounds are presented.

## 2. Materials and methods

### *2.1. Cells and viruses*

The established human cells used in these evaluations have been previously described in detail (Buckheit et al., 1994b). These cells were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum, 2 mM glutamine, penicillin (100 U/ml), and streptomycin (100 $\mu$g/ml). Established cell lines were obtained from the NIAID AIDS Research and Reference Reagent Program. Fresh human cells were obtained from the American Red Cross (Baltimore, MD, USA) and were cultured as previously described (Buckheit et al., 1994b). The laboratory-derived and low-passage clinical isolates of HIV-1, HIV-2 and SIV have also been previously described (Buckheit et al., 1994b). The nevirapine-resistant isolate N119 was obtained from the NIAID AIDS Research and Reference Reagent Program (Richman et al., 1991a) while the pyridinone-resistant isolate A17 was obtained from E. Emini at Merck, Sharp and Dohme Laboratories (West Point, PA) (Nunberg et al., 1991).

### *2.2. Materials*

All experimental antiviral agents were obtained from the Developmental Therapeutics Program, Division of Cancer Treatment, National Cancer Institute or from the NIAID AIDS Research and Reference Reagent Program. 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). ELISA plates were obtained from Coulter Cytometry (Hialeah, FL). Materials required for the performance of RT inhibition assays, anti-HIV assays, and for the growth and maintenance of established and fresh human cells have been previously described (Buckheit et al., 1994b).

### *2.3. Generation of drug-resistant virus strains and analysis of cross-resistance*

Resistant virus isolates were generated in duplicate in cell culture by serial passage of the $III_B$ strain of HIV-1 in CEM-SS cells in the presence of increasing concentrations of antiviral compound. The initial selection was performed with a drug concentration of two times the $EC_{50}$ concentration of the compound as determined in the microtiter

anti-HIV assay. With successive passages the drug concentration was increased two-fold to enhance the selective pressure on the virus. The final concentrations used in the selection of the resistant virus isolates was 13.9 $\mu$M for thiazolobenzimidazole, 2.8 $\mu$M for oxathiin carboxanilide, 4.2 $\mu$M for TIBO (R 82150), 2.7 $\mu$M for calanolide A, 76 $\mu$M for the diphenylsulfone, and 64.9 $\mu$M for HEPT. Each resistant virus isolate was obtained in 3–5 passages. Upon attainment of a drug-resistant virus strain, cross-resistance testing was performed according to the methods described for the performance of antiviral assays. Resistance has been defined in this study as a greater than 5-fold increase in $EC_{50}$ concentration when compared to the wild type ($III_B$) isolate.

### *2.4. Anti-HIV assays*

The HIV inhibitory activity of the compounds was evaluated as described (Buckheit et al., 1994b) in a microtiter anti-HIV assay which quantitates the ability of a compound to inhibit HIV-induced cell killing or HIV replication. Antiviral and toxicity data are 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 50% ($IC_{50}$). Confirmation of antiviral XTT data was obtained by a multiparameter analysis performed as previously described (Gulakowski et al., 1991). The reproducibility of the $EC_{50}$ values obtained in the anti-HIV assay between replicate experiments has been determined to be such that the standard error averaged less than 10% of the respective mean value (Kashman et al., 1992; McMahon et al., 1993).

### *2.5. Quantitative assays of virus reproduction in supernatant samples*

Supernatant samples from each well of the microtiter plate 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 et al., 1991). The p24 ELISA was performed according to the manufacturer's recommendations.

### *2.6. 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 as previously described (Buckheit et al., 1994a).

### *2.7. Reverse transcriptase enzyme inhibition assays*

Evaluation of the activity of compounds using homopolymer and heteropolymer templates was performed as previously described (Buckheit et al., 1994b).

## 3. Results

### 3.1. Range of action of the HIV-1-specific antiviral compounds

The efficacy ($EC_{50}$) of the HIV-1-specific nucleoside and non-nucleoside inhibitors was evaluated in a microtiter antiviral assay. The results of these experiments are summarized in Table 1. Cytotoxic concentrations ($IC_{50}$) were routinely at least 50–100 times greater than the $EC_{50}$ concentrations determined for each compound and for some compounds was at least 350 times greater (i.e. TIBO, oxathiin carboxanilide, nevirapine). All of the compounds tested were inactive against the ROD and MS isolates of HIV-2. The HIV-1-specific compounds also inhibited HIV-1 replication in phenotypically diverse human cell lines (Table 1), including the HTLV-I-infected T-cell line MT2, the macrophage-monocyte cell line U937, and the EBV-infected B-cell line AA5. Finally, the compounds were equally effective when tested in fresh human peripheral blood lymphocytes and in fresh human macrophages infected with a range of diverse, low passage clinical HIV-1 strains, including both syncytium-inducing and non-syncytium-inducing virus isolates (data not shown).

The ability of the HIV-1-specific compounds to inhibit the replication of HIV-1 in cells infected at various m.o.i. was also evaluated. In contrast to results obtained with AZT and ddC, the HIV-1-specific compounds were able to suppress virus replication when challenged at high m.o.i. with a relatively small increase in $EC_{50}$ concentration (Table 2). At over 125-times the standard m.o.i. used in the antiviral assay (standard m.o.i. = 0.01, high m.o.i. = 1.28), each of these compounds inhibited HIV-1 at an $EC_{50}$ concentration approximately 10–20 times the concentration obtained when challenged at the standard m.o.i. AZT and ddC, however, were not able to inhibit HIV-1 at greater than 250-times their normal $EC_{50}$ concentration when challenged at the same high m.o.i.

Table 1
Range of antiviral activity of HIV-1-specific compounds

| Compound | $EC_{50}$ ($\mu$M) [a] | | | | | |
|---|---|---|---|---|---|---|
| Cell line: | CEM | CEM | U937 | AA5 | PBMC | Macrophage |
| Virus: | HIV-1 | HIV-2 | HIV-1 | HIV-1 | HIV-1 | HIV-1 |
| Oxathiin carboxanilide | 0.084 | > 28.1 | 1.7 | 0.53 | 0.10 | 0.26 |
| Benzothiadiazine [b] | 1.39 | > 73 | 5.04 | 2.88 | 0.40 | 0.66 |
| Thiazolobenzimidazole | 0.35 | > 69 | 0.59 | 1.35 | 0.83 | 1.80 |
| TIBO (R 82150) | 0.10 | > 35 | 0.17 | 0.10 | 0.19 | 0.10 |
| Diphenylsulfone [c] | 1.94 | > 114 | 7.98 | 2.13 | 0.24 | 0.91 |
| Calanolide A | 0.08 | > 2.7 | 0.20 | 0.16 | 0.22 | 0.05 |
| Nevirapine | 0.11 | > 38 | 0.34 | 0.07 | 0.056 | 0.39 |
| HEPT [d] | 0.008 | > 2.7 | 0.022 | 0.038 | 0.046 | 0.007 |
| AZT | 0.003 | 0.005 | 0.0026 | 0.013 | 0.022 | 0.026 |

[a] Values shown are representative of results obtained from at least two replicate determinations; standard error measurements of replicate $EC_{50}$ determinations typically averaged less than 10%.
[b] The particular benzothiadiazine derivative studied was NSC 287474 (Buckheit et al., 1994b).
[c] The particular diphenylsulfone derivative studied was NSC 624231 (McMahon et al., 1993).
[d] The particular HEPT derivative studied was NSC 648400.

Table 2
Efficacy of HIV-1-specific compounds when challenged at increased m.o.i.

| Compound | $EC_{50}$ (fold-increase) [a] | | |
|---|---|---|---|
| m.o.i.: | 0.08 | 0.32 | 1.28 |
| Oxathiin carboxanilide | 3.0 | 3.5 | 9.5 |
| TIBO (R 82150) | 2.3 | 5.2 | 19.5 |
| Diphenylsulfone (NSC 624231) | 3.4 | 12.5 | 23.7 |
| Calanolide A | 7.7 | 16.4 | 26.4 |
| Nevirapine | 1.8 | 3.6 | 11.2 |
| HEPT (NSC 648400) | 11.6 | 15.1 | 47.7 |
| ddC | 32.5 | 63.8 | > 250 |
| AZT | 22.4 | 368.0 | > 370 |

[a] Fold-increase values are based upon at least two replicate determinations of the respective $EC_{50}$s at each of the different m.o.i.; standard error measurements of replicate $EC_{50}$ determinations typically averaged less than 10%.

Greater fold increases in $EC_{50}$ concentrations were also observed for AZT and ddC when compared with the HIV-1-specific inhibitors when challenged at 8-times (m.o.i. = 0.08) and 32-times (m.o.i. = 0.32) the standard m.o.i.

### 3.2. *Inhibition of reverse transcriptase by the HIV-1-specific antiviral compounds*

The ability of the HIV-1-specific compounds to inhibit the RT of HIV-1 was evaluated using three different template primer assay systems and recombinant HIV-1 and HIV-2 RT (Table 3). When assayed using a heteropolymeric ribosomal RNA template : primer system, all of the HIV-1-specific inhibitors potently inhibited the RT of HIV-1. The compounds exhibited equipotent or slightly lower activity when evaluated using the homopolymer poly rC : oligo dG system and were relatively inactive when evaluated using the homopolymer poly rA : oligo dT system. Consistent with HIV-2 cell

Table 3
Inhibition of RT activity by HIV-1-specific compounds with different template primers

| Compound | $IC_{50}$ ($\mu$M) [a] | | |
|---|---|---|---|
| | Poly(rA): oligo $dT_{12-18}$ | Poly(rC): oligo $dG_{12-18}$ | Ribosomal RNA |
| Oxathiin carboxanilide | > 282 | 0.79 | 0.062 |
| Benzothiadiazine (NSC 287474) | > 365 | 10.2 | 0.73 |
| Thiazolobenzimidazole | > 347 | 12.5 | 0.52 |
| TIBO (R 82150) | 30.3 | 0.09 | 0.021 |
| Diphenylsulfone (NSC 624231) | > 380 | 106.5 | 2.43 |
| Calanolide A | 1.6 | 0.025 | 0.070 |
| Nevirapine | 10.5 | 0.038 | 1.16 |
| HEPT (NSC 648400) | 42.5 | 0.014 | 0.008 |
| AZT-TP | 0.02 | 0.006 | 0.34 |

[a] Values shown are representative of results obtained from at least 2 replicate determinations; standard error measurements of replicate $EC_{50}$ determinations typically averaged less than 10%.

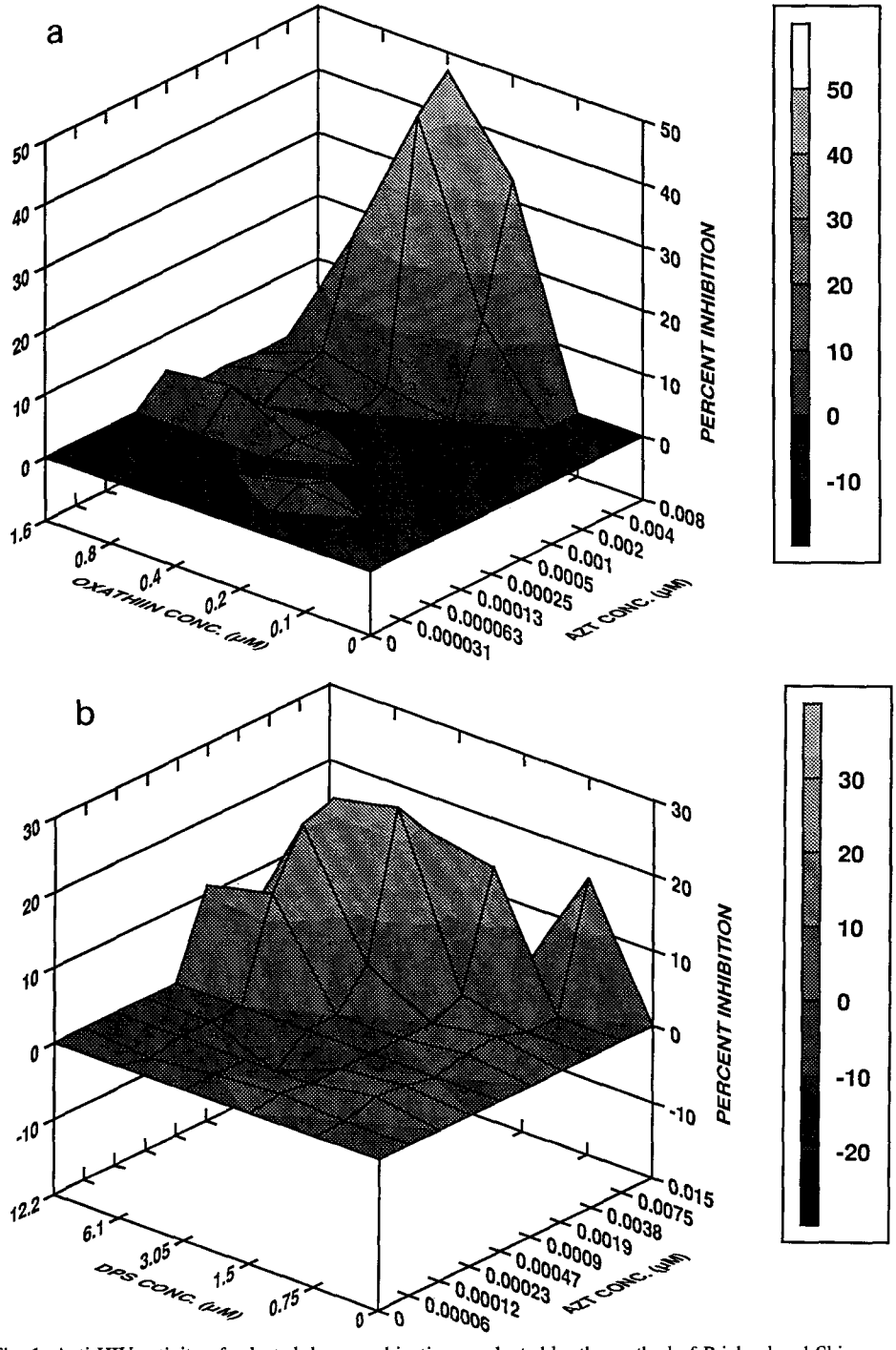
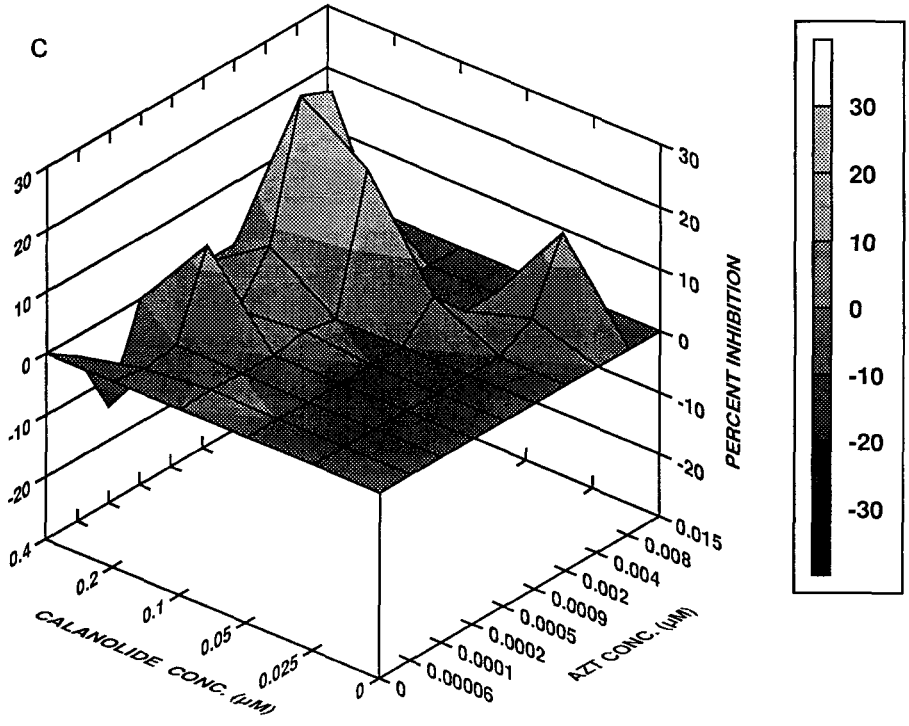



Fig. 1. Anti-HIV activity of selected drug combinations evaluated by the method of Prichard and Shipman at the 95% confidence interval. (a) Antiviral synergy plot of oxathiin carboxanilide plus AZT. (b) Antiviral synergy plot of diphenylsulfone plus AZT. (c) Antiviral synergy plot of calanolide A plus AZT. (d) Antiviral synergy plot of HEPT plus AZT.



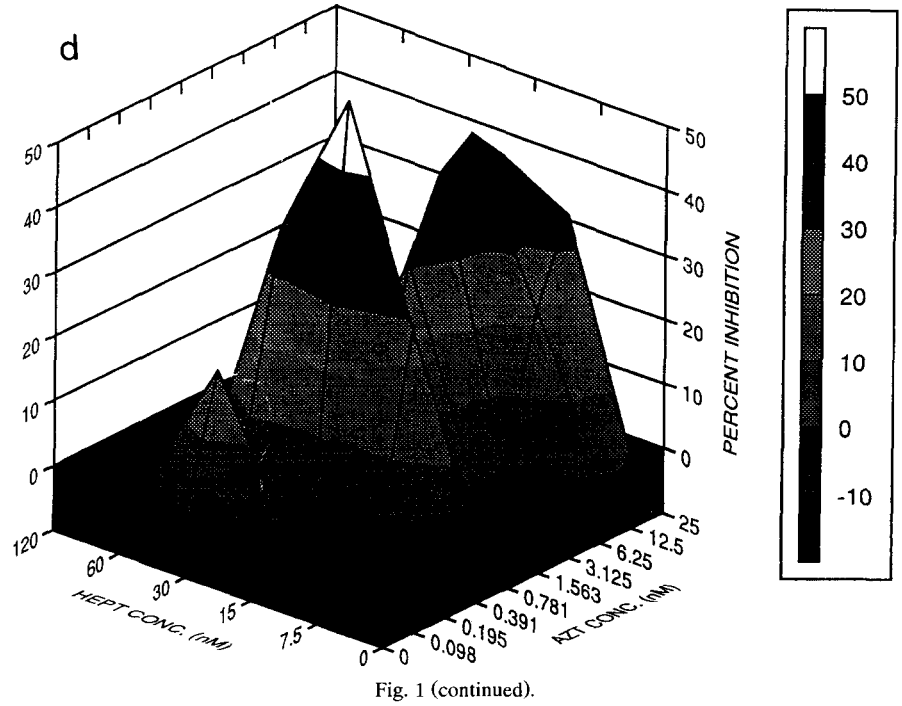




Fig. 1 (continued).

culture data, HIV-2 RT remained fully active in the presence of the various inhibitors, irrespective of the template–primer employed (data not shown).

### 3.3. Combination anti-HIV activity of the HIV-1-specific antiviral compounds when used with AZT

The HIV-1-specific compounds were tested for anti-HIV activity in combination with AZT using the microtiter XTT assay. Five concentrations of each HIV-1-specific inhibitor were tested in all possible combinations with 8 concentrations of AZT. The results of these assays demonstrated that the combined antiviral activity of the HIV-1-specific RT inhibitors used with AZT was much greater than that predicted based on additivity, indicating significant synergy. The three-dimensional plots of data obtained for calanolide A, the representative diphenylsulfone (NSC 624231), oxathiin carboxanilide and HEPT exhibit a surface extending above the plane of additivity (Fig. 1). Results for the remaining compounds (thiazolobenzimidazole, the representative benzothiadiazine (NSC 287474), and TIBO) have been previously reported (Buckheit et al., 1993a, 1994a,b).

### 3.4. Cross-resistance phenotype of the viruses resistant to HIV-1-specific antiviral compounds

Viruses resistant to each of the HIV-1-specific RT inhibitors were generated in cell culture. The 6 drug-resistant virus isolates as well as isolates resistant to nevirapine (N119) and pyridinone (A17) were evaluated for their sensitivity to the panel of HIV-1-specific anti-HIV agents. The results of these assays (Table 4) suggest that the drug-resistant virus isolates may be divided into 4 distinct subgroups. The subgroups are defined by the presence of distinct mutations in the RT of each isolate. These mutations are defined in Table 4. A complete description of identification and confirmation of the mutations responsible for the cross-resistance phenotype will be reported elsewhere (Buckheit et al., 1994c).

The first subgroup (group I) includes the viruses resistant to the compounds thiazolobenzimidazole, oxathiin carboxanilide and TIBO. The RT of these virus isolates contained the mutations V108I, L100I, and A98G/V108I, respectively. These viruses were cross-resistant to all of the HIV-1-specific RT inhibitors tested. Each of the three compounds remained inhibitory to calanolide A-resistant virus and had enhanced efficacy against the HEPT-resistant virus.

The second group of drug-resistant viruses (group II) presently includes only the calanolide A-resistant isolate. The RT of this isolate contained three previously undescribed mutations (V90I, T139I, P225S) which are currently being confirmed. This virus isolate remained sensitive to all of the other compounds tested. Calanolide A was active against the other drug-resistant virus isolates with the exception of the group I-resistant viruses (thiazolobenzimidazole-resistant, oxathiin carboxanilide-resistant and TIBO-resistant), which were cross-resistant to calanolide A.

Group III includes the viruses resistant to the representative diphenylsulfone compound, as well as the nevirapine- and pyridinone-resistant isolates. The mutation

Table 4
Activity of HIV-1-specific compounds against various drug-resistant HIV-1 isolates

| Compound | Resistant strain (subgroup and mutation)/fold-resistant [a,b] | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | THZ-R (I) V108I | OC-R (I) L100I | TIBO-R (I) A98G/V108I | CALO-R (II) V90I, T139I, P225S | DPS-R (III) Y181C | N119 (III) Y181C | A17 (III) Y181C/K103N | HEPT-R (IV) P236L |
| Calanolide A | 16 | > 22 | 17.4 | > 19 | 0.1 | 0.5 | 1.5 | 2.4 |
| Thiazolobenzimidazole | > 200 | > 200 | > 200 | 2.0 | > 200 | > 200 | > 200 | 0.2 |
| Diphenylsulfone (NSC 624231) | > 60 | 6 | > 60 | 2.3 | > 60 | > 60 | 60 | 0.8 |
| Oxathiin carboxanilide | 15 | > 333 | > 333 | 1.3 | 11 | > 333 | > 333 | 0.3 |
| HEPT | 47 | 15 | 57 | 1.0 | 84 | > 333 | > 333 | 62 |
| TIBO (R 82150) | 51 | > 1000 | > 1000 | 2.2 | 70 | > 1000 | 166 | 1.0 |
| Nevirapine | 33 | 18 | 15.2 | 1.0 | 593 | > 333 | > 333 | 2.3 |
| Benzothiadiazine (NSC 287474) | 42 | 11 | 44.5 | 1.9 | > 250 | > 250 | 52 | 1.0 |
| AZT | 2.5 | 0.7 | 1.8 | 1.0 | 1.1 | 2.7 | 0.4 | 0.7 |

[a] Fold-resistant values are based upon at least two determinations of the respective $EC_{50}$s against each of the resistant virus isolates; standard error measurements of replicate $EC_{50}$ determinations typically averaged less than 10%.

[b] Fold-resistant values defined as "greater than" are due to toxicity of the compound at the highest concentration used or the high test concentration was not exceeded due to limited compound supply.

detected in the RT of these isolates was uniformly Y181C (Richman et al., 1991a; Buckheit et al., 1994c); the pyridinone-resistant strain also contained the K103N amino acid change (Nunberg et al., 1991). These viruses were cross-resistant to all of the compounds tested, except for calanolide A. The diphenylsulfone- and nevirapine-resistant virus isolates exhibited enhanced sensitivity to calanolide A, while A17 remained sensitive to calanolide A. The data reported in Table 4 appear to indicate a loss of sensitivity to AZT with the N119 isolate. This result is related to the highly efficient replication and cytopathicity of this isolate. Pretreatment of CEM-SS cells with AZT prior to infection demonstrates N119 remains sensitive to AZT (data not shown). The compounds of group III were only able to inhibit the replication of the calanolide A-resistant and the HEPT-resistant viruses.

Finally, group IV contains only the virus isolate resistant to the HIV-1-specific nucleoside, HEPT. The RT of this virus contained a P236L amino acid change. The HEPT-resistant virus isolate was not cross-resistant to any of the non-nucleoside RT inhibitors. The HEPT compound, however, inhibited only the replication of the calanolide A-resistant isolate.

## 4. Discussion

Comparative analysis of a panel of structurally diverse HIV-1-specific nucleoside and non-nucleoside RT inhibitors has suggested that this pharmacologic class of inhibitors shares many common properties as well as significant differences in anti-HIV activity. These common properties include reproducible and selective antiviral activity against a panel of biologically distinct laboratory and low passage clinical strains of HIV-1, activity in a wide variety of established and fresh human cells, and potent inhibition of HIV-1 RT when evaluated using a heteropolymeric ribosomal RNA template assay. These properties are typical of numerous other members of the HIV-1-specific RT inhibitor class (Merluzzi et al., 1990; Pauwels et al., 1990; Bader et al., 1991; Goldman et al., 1991; Romero et al., 1991; Kashman et al., 1992; Klunder et al., 1992; Buckheit et al., 1993a, 1994b; Maass et al., 1993; McMahon et al., 1993).

We report here that the HIV-1-specific compounds were much less sensitive to increasing m.o.i. than were the nucleoside analogs AZT and ddC. These results may hold important implications for the clinical use of these compounds because of the broad therapeutic index of the HIV-1-specific compounds. Similar studies reporting drug concentrations which totally inhibited HIV replication, or "knocking-out" concentrations (Balzarini et al., 1993c), similarly suggest the potent inhibition of HIV replication by the HIV-1-specific compounds.

All of the HIV-1-specific RT inhibitors evaluated exhibited synergistic anti-HIV activity when tested in combination with AZT. We have also determined that the combination of calanolide A and AZT will synergistically inhibit the pyridinone-resistant strain A17 in cell culture assays (R.W.B., unpublished results). These results are consistent with the view that clinical combination therapy investigations with AZT and one or more HIV-1-specific RT inhibitor(s) should be considered. Synergistic anti-HIV

activity with the combination use of nucleoside and non-nucleoside RT inhibitors has been previously reported (Richman et al., 1991b; Buckheit et al., 1993a,b, 1994a).

The generation of drug resistance to each of the HIV-1-specific inhibitors occurs rapidly in cell culture as well as in patients (Emini et al., 1992; Saag et al., 1993; Richman et al., 1994). Many investigators initially feared that this common property of the non-nucleoside inhibitors would be the Achilles heel of the entire class. Further analysis of drug-resistant viruses, however, suggests that the HIV-1-specific inhibitors actually may be more diverse than initially thought (Dueweke et al., 1993) and that this diversity might be therapeutically exploitable. Our results suggest that at least 4 subclasses of HIV-1-specific RT inhibitors may be defined based on the respective patterns of generation of virus strains cross-resistant to other members of the class. These subgroups are defined by the specific changes induced in the RT of the respective drug-resistant virus isolates. The group I viruses exhibit the mutations V108I, L100I, A98G/V108I, group II exhibits several newly described and unconfirmed mutations (V90I, T139I, P225S) (Buckheit et al., 1994c), group III isolates exhibit the well-defined Y181C mutation and the group IV isolate has the previously described P236L mutation. Our results indicate that mutations at 98, 100 and 108 may be more important therapeutically than the Y181C mutation since these mutations, present in the group I viruses, yield cross-resistance to all of the HIV-1-specific inhibitors studied.

We have determined that the phenotypic classes defined by the use of drug-resistant virus isolates derive from distinct mutations in the RT. Moreover, literature precedents suggest that structural differences among analogs of a given HIV-1-specific compound may result in the generation of mutations which would place the compound in a subgroup different from that defined by the original prototype compound. For example, it has been recently reported that various structurally distinct HEPT compounds may induce different mutations in the RT (Balzarini et al., 1993b). We have generated a resistant virus isolate to a second, structurally different, HEPT derivative which by cross-resistance phenotyping would belong to group III rather than group IV (Buckheit et al., 1994c). This isolate contains the Y181C mutation diagnostic of the group III compounds. This resistant isolate is cross-resistant to all of the HIV-1-specific inhibitors, but exhibits enhanced sensitivity to calanolide A.

The results of this study suggest that investigational therapeutic combinations of non-nucleoside and/or nucleoside HIV-1-specific RT inhibitors might be selected from different known subclasses. For example, investigational therapy with calanolide A (group II) and either HEPT (group IV) and/or diphenylsulfone (group III) would be supported by the present in vitro cross-resistance data. In these scenarios, viruses resistant to one of the two compounds presumably might remain sensitive or super-sensitive to the second compound. However, the possible generation of resistance to the simultaneous presence of two or more compounds should also be considered. Variation in mutation has been documented when one or two drugs have been used to select for resistant virus in cell culture (Balzarini et al., 1993a). Amino acid changes at position 181 (Y181C) have been identified as resulting in abrogation of activity of all of the reported HIV-1-specific compounds. The group II compound calanolide A remains active against viruses with the Y181C mutation (Boyer et al., 1993), suggesting that

calanolide A may be useful in combination with agents which exhibit enhanced sensitivity to the calanolide A-resistant isolate.

In summary, the HIV-1-specific RT inhibitor class of compounds is more heterogeneous than initially appreciated. The relative potency and lack of cytotoxicity of the compounds, as well as the differences in antiviral activities toward resistant HIV-1 isolates, supports that further clinical investigations are warranted, especially in combination with each other and/or with AZT.

## Acknowledgements

The authors acknowledge the efforts of Ms. Barbara Toyer in the preparation of compounds for this study and Dr. Lois Allen for the Prichard-Shipman-based analysis of the combination anti-HIV data. Molecular analysis of drug-resistant virus isolates was provided by Dr. Douglas Mayers and Dr. Owen Weislow. Several reagents utilized in these studies were obtained through the NIAID AIDS Research and Reference Reagent Program. The preparation of purified RT by the Gene Expression Core Facility, Center For AIDS Research, University of Alabama at Birmingham, was supported in part by the NIH Centers For AIDS Research program Grant P30 AI27767. This work was supported in part by Contract NO1-AI-05087 from the National Institute of Allergy and Infectious Diseases and in part by Contract NO1-CM-37818 from the National Cancer Institute to Southern Research Institute.

## References

Ahluwalia, G., Conney, D.A., Mitsuya, H., Fridland, A., Flora, K.P., Hao, Z., Dalal, M., Broder, S. and Johns, D.G. (1987) Initial studies on the cellular pharmacology of 2-3-dideoxyinosine, an inhibitor of HIV infectivity. Biochem. Pharmacol. 36, 3797–3800.

Bader, J.P., McMahon, J.B., Schultz, R.J., Narayanan, V.L., Pierce, J.B., Harrison, W.A., Weislow, O.S., Midelfort, C.F. Stinson, S.F. and Boyd, M.R. (1991) Oxathiin carboxanilide, a potent inhibitor of human immunodeficiency virus reproduction. Proc. Natl. Acad. Sci. U.S.A. 88, 6740–6744.

Balzarini, J., Karlsson, A., Perez-Perez, M.-J., Camarasa, M.-J., Tarpley, W.G. and DeClercq, E. (1993a) Treatment of human immunodeficiency virus type 1 (HIV-1)-infected cells with combinations of HIV-1-specific inhibitors results in a different resistance pattern than does treatment with single drug therapy. J. Virol. 67, 5353–5359.

Balzarini, J., Karlsson, A. and DeClercq, E. (1993b) Human immunodeficiency virus type 1 drug-resistance patterns with different 1-[(2-hydroxyethoxy)methyl]-6-(phenylthio)thymine derivatives. Mol. Pharmacol. 44, 694–701.

Balzarini, J., Karlsson, A., Perez-Perez, M.-J., Camarasa, M.-J. and DeClercq, E. (1993c) Knocking-out concentrations of HIV-1-specific inhibitors completely suppress HIV-1 infection and prevent the emergence of drug-resistant virus. Virology 196, 576–585.

Boyd, M.R. (1988) Strategies for the identification of new agents for the treatment of AIDS: a national program to facilitate the discovery and preclinical development of new drug candidates for clinical evaluation. In: V.T. DeVita, Jr., S. Hellman and S.A. Rosenberg (Eds.), AIDS Etiology, Diagnosis, Treatment, and Prevention, pp. 305–317, Lippincott, Philadelphia.

Boyer, P.L., Currens, M.J., McMahon, J.B., Boyd, M.R. and Hughes, S.H. (1993) Analysis of nonnucleoside drug-resistant variants of human immunodeficiency virus type 1 reverse transcriptase. J. Virol. 67, 2412–2420.

Buckheit, R.W., Jr. and Swanstrom, R.I. (1991) HIV-heterogeneity: Characterization of HIV-1 isolates displaying an apparent absence of virion-associated reverse transcriptase activity. AIDS Res. Hum. Retroviruses 7, 295–302.

Buckheit, R.W., Jr., Hollingshead, M.G., Germany-Decker, J., White, E.L., McMahon, J.B., Allen, L.B., Ross, L.J., Decker, W.D., Westbrook, L., Shannon, W.M., Weislow, O., Bader, J.P. and Boyd, M.R. (1993a) Thiazolobenzimidazole: biological and biochemical anti-retroviral activity of a new nonnucleoside reverse transcriptase inhibitor. Antiviral Res. 21, 247–265.

Buckheit, R.W., Jr., Germany-Decker, J., Hollingshead, M.G., Allen, L.B., Shannon, W.M., Janssen, P.A.J. and Chirigos, M.A. (1993b) Differential antiviral activity of two TIBO derivatives against the human immunodeficiency and murine leukemia viruses alone and in combination with other anti-HIV agents. AIDS Res. Hum. Retroviruses 9, 1097–1106.

Buckheit, R.W., Jr., White, E.L., Germany-Decker, J., Allen, L.B., Ross, L.J., Shannon, W.M., Janssen, P.A.J. and Chirigos, M.A. (1994a) Cell based and biochemical analysis of the anti-HIV activity of combinations of 3′-azido-3′-deoxythymidine and analogs of TIBO. Antiviral Chem. Chemother. 5, 35–42.

Buckheit, R.W., Jr., Decker, W.D., Roberson, J.L., Pyle, C.A., White, E.L., Bowdon, B.J., McMahon, J.B., Boyd, M.R., Bader, J.P., Nickell, D.G., Barth, H. and Antonucci, T.K. (1994b) Biological and biochemical anti-HIV activity of the benzothiadiazine class of nonnucleoside reverse transcriptase inhibitors. Antiviral Res. 25, 43–56.

Buckheit, R.W., Jr., Fliakas-Boltz, V., Weislow, O. and Mayer, D. (1994c) Unpublished data.

Byrnes, V., Sardana, V.V., Schleif, W.A., Condra, J.H., Waterbury, J.A., Wolfgang, J.A., Long, W.J., Schneider, C.L., Schlabach, A.J., Wolanski, B.S., Graham, D.J., Gotlib, L., Rhodes, A., Titus, D.L., Roth, E., Blahy, O.M., Quintero, J.C., Staszewski, S. and Emini, E.A. (1993) Comprehensive mutant enzyme and viral variant assessment of human immunodeficiency virus type 1 reverse transcriptase resistance to nonnucleoside inhibitors. Antimicrob. Agents Chemother. 37, 1576–1579.

Byrnes, V., Emini, E.A., Schleif, W.A., Condra, J.H., Schneider, C.L., Long, W.J., Wolfgang, J.A., Graham, D.J., Gotlib, L., Schlabach, A.J., Wolanski, B.S., Blahy, O.M., Quintero, J.C., Rhodes, A., Roth, E., Titus, D.L. and Sardana, V.V. (1994) Susceptibilities of human immunodeficiency virus type 1 enzyme and viral variants expressing multiple resistance-engendering amino acid substitutions to reverse transcriptase inhibitors. Antimicrob. Agents Chemother. 38, 1404–1407.

Cohen, K.A., Hopkins, J., Ingraham, R.H., Pargellis, C., Wu, J.C., Palladino, D.E.H., Kinkade, P., Warren, T.C., Rogers, S., Adams, J., Farina, P.R. and Grob, P.M. (1991) Characterization of the binding site for nevirapine (BI-RG-587), a nonnucleoside inhibitor of human immunodeficiency virus type-I reverse transcriptase. J. Biol. Chem. 266, 14671–14674.

Condra, J.H., Emini, E.A., Gotlib, L., Graham, D.J., Schlabach, A.J., Wolfgang, J.A., Colonno, R.J. and Sardana, V.V. (1992) Identification of the human immunodeficiency virus reverse transcriptase residues that contribute to the activity of diverse nonnucleoside inhibitors. Antimicrob. Agents Chemother. 36, 1441–1446.

Connolly, K.J. and Hammer, S.M. (1992) Antiretroviral therapy: Reverse transcriptase inhibition. Antimicrob. Agents Chemother. 36, 245–254.

De Vreese, K., Debyser, Z., Vandamme, A.-M., Pauwels, R., Desmyter, J., De Clercq, E. and Anne, J. (1992) Resistance of human immunodeficiency virus type I reverse transcriptase to TIBO derivatives induced by site-directed mutagenesis. Virology 188, 900–904.

Dueweke, T.J., Pushkarskaya, T., Poppe, S.M., Swaney, S.M., Zhao, J.Q., Chen, I.S.Y., Stevenson, M. and Tarpley, W.G. (1993) A mutation in reverse transcriptase of bis(heteroaryl)piperazine-resistant human immunodeficiency virus type I that confers increased sensitivity to other nonnucleoside inhibitors. Proc. Natl. Acad. Sci. U.S.A. 90, 4713–4717.

Emini, E.A., Schleif, W.A., Byrnes, V.W., Condra, J.H., Sardanal, V.V., Kappes, J.C., Saag, M. and Shaw, G. (1992) In vitro and in vivo derivation of HIV-1 variants resistant to inhibition by the nonnucleoside reverse transcriptase inhibitor L-697, 661. Abstracts of the HIV Drug-Resistance Workshop, Noordwijk, The Netherlands, 16–18 July 1992, p. 14.

Goldman, M.E., Nunberg, J.H., O'Brien, J.A., Quintero, J.C., Schleif, W.A., Freund, K.F., Gaul, S.L., Saari, W.S., Wai, J.S., Hoffman, J.M., Anderson, P.S., Hupe, D.J., Emini, E.A. and Stern, A.M. (1991) Pyridinone derivatives: specific human immunodeficiency virus type 1 reverse transcriptase inhibitors with antiviral activity. Proc. Natl. Acad. Sci. U.S.A. 88, 6863–6867.

Grob, P.M., Wu, J.C., Cohen, K.A., Ingraham, R.H., Shih, C.-K., Hargrave, K.D., T.L. Mctague, and Merluzzi, V.J. (1992) Nonnucleoside inhibitors of HIV-1 reverse transcriptase: nevirapine as a prototype drug. AIDS Res. Hum. Retroviruses. 8, 145–152.

Gulakowski, R.J., McMahon, J.B., Staley, P.G., Moran, R.A. and Boyd, M.R. (1991) A semiautomated multiparameter approach for anti-HIV drug screening. J. Virol. Methods 33, 87–100.

Jacobo-Molina, A., Ding, J., Nanni, R., Clark, A.D., Jr., Lu, X., Tantillo, C., Williams, R.L., Kamer, G., Ferris, A.L., Clark, P., Hizi, A., Hughes, S.H. and Arnold, E. (1993) Crystal structure of human immunodeficiency virus type 1 reverse transcriptase complexed with double-stranded DNA at 3.0 A resolution shows bent DNA. Proc. Natl. Acad. Sci. U.S.A. 90, 6320–6324.

Kashman, Y., Gustafson, K.R., Fuller, R.W., Cardellina, J.H., II, McMahon, J.B., Currens, M.J., Buckheit, R.W., Jr., Hughes, S.H., Cragg G.M., and Boyd, M.R. (1992) The calanolides, a novel HIV-inhibitory class of coumarin derivatives from the tropical rainforest tree *Calophyllum lanigerum*. J. Med. Chem. 35, 2735–2743.

Klunder, J.M., Hargrave, K.D., West, M., Cullen, E., Pal, K., Behnke, M.L., Kapadia, S.R., McNeil, D.W., Wu, J.C., Chow G.C. and Adams, J. (1992) Novel non-nucleoside inhibitors of HIV-1 reverse transcriptase. 2. Tricyclic pyridobenzoxazepinones and dibenzoxazepinones. J. Med. Chem. 35, 1887–1897.

Kohlstaedt, L.A., Wang, J., Friedman, J.M., Rice, P.A. and Steitz, T.A. (1992) Crystal structure at 3.5 A resolution of HIV-1 reverse transcriptase complexed with an inhibitor. Science 256, 1783–1790.

Larder, B.A., Darby. G. and Richman, D.D. (1989) HIV with reduced sensitivity to zidovudine (AZT) isolated during prolonged therapy. Science 243, 1731–1734.

Maass, G., Immendoerfer, U., Koenig, B., Leser, U., Mueller, B., Goody R. and Pfaff, E. (1993) Viral resistance to the thiazolo-iso-indolinones, a new class of nonnucleoside inhibitors of human immunodeficiency virus type I reverse transcriptase. Antimicrob. Agents Chemother. 37, 2612–2617.

Mansuri, M.M., Starrett, J.E., Jr., Ghazzouli, I., Hitchcock, M.J., Sterzycki, R.Z., Brankovan, V., Lin, T.S., August, E.M., Prusoff, W.M. and Sommadossi, J.P. (1989) 1-(2,3-dideoxy-beta-D-glycero-pent-2-enofuranosyl)thymine. A highly potent and selective anti-HIV agent. J. Med. Chem. 32, 461–466.

McMahon, J.B., Gulakowski, R.J., Weislow, O.S., Schultz, R.J., Narayanan, V.L., Clanton, D.J., Pedemonte, R., Wassmundt, F.W., Buckheit, R.W., Jr., Decker, W.D., White, E.L., Bader, J.P. and Boyd, M.R. (1993) Diarylsulfones, a new chemical class of nonnucleoside antiviral inhibitors of human immunodeficieny virus type 1 reverse transcriptase. Antimicrob. Agents Chemother. 37, 754–760.

Mellors, J.W., Dutschman, G.E., Im, G.-J., Tramontano, E., Winkler, S.R. and Cheng, Y.-C. (1992) In vitro selection and molecular characterization of human immunodeficiency virus-1 resistant to non-nucleoside inhibitors of reverse transcriptase. Mol. Pharmacol. 41, 446–451.

Merluzzi, V.J., Hargrave, K.D., Labadia, M., Grozinger, K., Skoog, M., Wu, J.C., Shih, C.-K., Eckner, K., Hattox, S., Adams, J., Roshthal, A.S., Faanes, R., Eckner, R.J., Koup, R.A. and Sullivan, J.L. (1990) Inhibition of HIV-1 replication by a non-nucleoside reverse transcriptase inhibitor. Science 250, 1411–1413.

Nara, P.L. and Fischinger, P.J. (1988) Quantitative infectivity assay for HIV-1 and HIV-2. Nature 332, 469–470.

Nunberg, J.H., Schleif, W.A., Boots, E.J., O'Brien, J.A., Quintero, J.C., Hoffman, J.M., Emini, E.A. and Goldman, M.E. (1991) Viral resistance to human immunodeficiency virus type 1-specific pyridinone reverse transcriptase inhibitors. J. Virol. 65, 4887–4892.

Pauwels, R., Andries, K., Desmyter, J., Schols, D., Kukla, M.J., Breslin, H.J., Raeymaeckers, A., Van Gelder, J., Woestenborghs, R., Heykants, J., Schellekens, K., Janssen, M.A.C., De Clercq, E. and Janssen, P.A.J. (1990) Potent and selective inhibition of HIV-1 replication in vitro by a novel series of TIBO derivatives. Nature 343, 470–474.

Prichard, M.N. and Shipman, C., Jr. (1990) A three-dimensional model to analyze drug–drug interactions. Antiviral Res. 14, 181–206.

Richman, D.D., Fischl, M.A., Grieco, M.H., Gottlieb, M.S., Jackson, G.G., Durack, D.T. and Lehrman-Nusinoff, S. (1987) The toxicity of azidothymidine (AZT) in the treatment of patients with AIDS and AIDS-related complex. A double-blind, placebo-controlled trial. New Engl. J. Med. 371, 192–197.

Richman, D., Shih, C.-K., Lowy, I., Rose, J., Prodanovich, P., Goff S., and Griffin, J. (1991a) Human immunodeficiency virus type 1 mutants resistant to nonnucleoside inhibitors of reverse transcriptase arise in tissue culture. Proc. Natl. Acad. Sci. U.S.A. 88, 11241–11245.

Richman, D., Rosenthal, A.S., Skoog, M., Eckner, R.J., Chou, T.-C., Sabo, J.P. and Merluzzi, V.J. (1991b) BI-RG-587 is active against zidovudine-resistant human immunodeficiency virus type 1 and synergistic with zidovudine. Antimicrob. Agents Chemother. 35, 305–308.

Richman, D.D., Havlir, D., Corbeil, J., Looney, D., Ignacio, C., Spector, S.A., Sullivan, J., Cheeseman, S., Barringer, K., Pauletti, D., Sihh, C.-K., Myers, M. and Griffin, J. (1994) Nevirapine resistance mutations of human immunodeficiency virus type I selected during therapy. J. Virol. 68, 1660–1666.

Romero, D.L., Busso, M., Tan, C.-K., Reusser, F., Palmer, J.R., Poppe, S.M., Aristoff, P.A., Downey, K.M., So, A.G., Resnick, L. and Tarpley, W.G. (1991) Nonnucleoside reverse transcriptase inhibitors that potently and specifically block human immunodeficiency virus type 1 replication. Proc. Natl. Acad. Sci. U.S.A. 88, 8806–8810.

Saag, M.S., Emini, E.A., Laskin, O.L., Douglas, J., Lapidis, W.I., Schleif, W.A., Whitley, R.J., Hildebrand, C., Byrnes, V.W., Kappes, J.C., Anderson, K.W., Massari, F.E. and Shaw, G.M. (1993) A short-term clinical evaluation of l-697, 661, a non-nucleoside inhibitor of HIV-1 reverse transcriptase. New Engl. J. Med. 329, 1065–1072.

Smerdon, S.J., Jager, J., Wang, J., Kohlstaedt, L.A., Chirino, A.J., Friedman, J.M., Rice, P.A. and Steitz, T.A. (1994) Structure of the binding site for nonnucleoside inhibitors of the reverse transcriptase of the human immunodeficiency virus type 1. Proc. Natl. Acad. Sci. U.S.A. 91, 3911–3915.

St. Clair, M.H., Martin, J.L., Tudor-Williams, G., Bach, M.C., Vavro, C.L., King, D.M., Kellam, P., Kemp, S.D. and Larder, B.A. (1991) Resistance to ddI and sensitivity to AZT induced by a mutation in HIV-1 reverse transcriptase, Science 245, 1557–1559.

Weislow, O.S., Kiser, R., Fine, D.L., Bader, J.P., Shoemaker, R.H. and Boyd, M.R. (1989) New soluble formazan assay for HIV-1 cytopathic effects: application to high flux screening of synthetic and natural products for AIDS antiviral activity. J. Natl. Cancer Inst. 81, 577–586.

Wu, J.C., Warren, T.C., Adams, J., Proudfoot, J., Skiles, J., Raghavan, P., Perry, C., Potocki, I., Farina, P.R. and Grob, P.M. (1991) A novel dipyridodiazepinone inhibitor of HIV-1 reverse transcriptase acts through a nonsubstrate binding site. Biochemistry 30, 2022–2026.

Yarchoan, R., Perno, C.F., Thomas, R.V., Klecker, R.W., Allain, J.P., Wills, R.J., McAtee, N., Fischl, M.A., Dubinsky, R., McNeely, M.C., Mitsuya, H., Pluda, J.M., Lawley, T.J., Leuther, M., Safai, B., Collins, J.M., Myers, C.E. and Broder, S. (1988) Phase I studies of 2,3-dideoxycytodine in severe human immunodeficiency virus infection as a single agent and alternating with zidovudine (AZT). Lancet 1, 76–81.

Yarchoan, R., Mitsuya, H., Thomas, R.V., Pluda, J.M., Hartman, N.R., Perno, C.-F., Marczyk, K.S., Allain, J.-P., Johns D.G. and Broder, S. (1989) In vivo activity against HIV and favorable toxicity profile of 2′,3′-dideoxycytidine. Science 245, 412–415.