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A TIBO derivative, R82913, is a potent inhibitor of HIV-1 reverse transcriptase with heteropolymer templates

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Summary

R82913, (+)-*S*-4,5,6,7-tetrahydro-9-chloro-5-methyl-6-(3-methyl-2-butenyl)-imidazo[4,5,1-jk][1,4]-benzodiazepin-2(1H)-thione (a TIBO derivative), inhibited the replication of thirteen different strains of HIV-1 in CEM cells with a median IC_{50} of 0.15 μM. The concentration of compound that killed 50% of the cells was much higher (46 μM), indicating that R82913 has a high selectivity index. R82913 was 20-fold more potent than AZT-TP in the inhibition of HIV-1 reverse transcriptase in an assay using a naturally occurring template (ribosomal RNA) that more accurately resembles native viral RNA than a synthetic homopolymer. With this template, R82913 inhibited HIV-1 reverse transcriptase with an ID_{50} (0.01 μM) that is equal to, or lower than, the IC_{50} for this compound in all of our cell culture assays (0.01–0.65 μM). R82913 has no effect on the replication of HIV-2 in CEM cells and does not inhibit the reverse transcriptase from this virus.

TIBO; HIV-1; HIV-2; Reverse transcriptase; Novel RNA template

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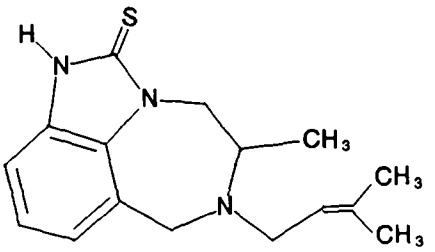
Introduction

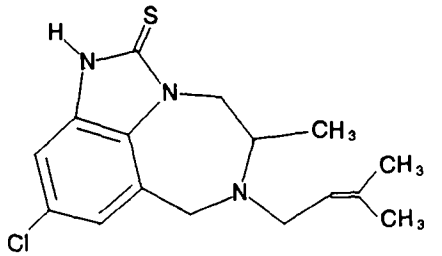
Recently, a novel series of non-nucleoside compounds, derivatives of (+)-*S*-4,5,6,7-tetrahydro-imidazo[4,5,1-jk][1,4]-benzodiazepin-2(1H)-thione (TIBO), have been shown to have potent and selective activity against human immunodeficiency virus type 1 (HIV-1) in cell culture (Pauwels et al., 1990). The prototype TIBO derivative, R82150 (Fig. 1), inhibited the replication of the HTLV-III_B strain of HIV-1 in three human cell lines and in fresh peripheral blood lymphocyte cultures. The compound also inhibited the replication of a monocytotropic HIV-1 strain and two other strains of virus isolated from patients with acquired immunodeficiency syndrome (AIDS). This compound showed the highest selectivity index (6000–90 000) of any known antiviral agent against HIV-1 in all of these assays.

Pauwels et al. (1990) concluded that their TIBO derivatives were acting by a 'reverse transcriptase-associated process.' They found that the addition of R82150 to cell cultures could be delayed up to 5 h after infection with virus with only a slight loss in its antiviral activity. Similar results were observed with AZT and ddC, two compounds that are potent inhibitors of HIV-1 reverse



R82150

(+)-S-4,5,6,7-tetrahydro-5-methyl-6-(3-methyl-2-butenyl)-imidazo [4,5,1-jk][1,4]-benzodiazepin-2(1H)-thione



R82913

(+)-S-4,5,6,7-tetrahydro-9-chloro-5-methyl-6-(3-methyl-2-butenyl)-imidazo [4,5,1-jk][1,4]-benzodiazepin-2(1H)-thione

Fig. 1. Structure of the TIBO derivatives R82150 and R82913.

transcriptase (EC 2.7.7.49). The TIBO derivatives inhibited HIV-1 reverse transcriptase when poly(rA)-p(dT)$_{12\ 18}$, poly(rI)-p(dC)$_{12\ 18}$ or poly(rC)-p(dG)$_{12-18}$ was used as the template-primer. In fact there was a very close correlation found between the TIBO derivatives' ability to inhibit viral replication and their ability to inhibit reverse transcriptase activity. However, the discrepancy between the magnitude of the ID_{50} values reported for inhibition of reverse transcriptase (5–6 μM on the poly(rA)-p(dT)$_{12\ 18}$ and poly(rI)-p(dC)$_{12-18}$ templates and 0.3 μM on the poly(rC)-p(dG)$_{12\ 18}$ template) and the magnitude of the IC_{50} found in cell culture (0.01–0.03 μM), combined with the novel structure of these compounds, has led to questions as to whether inhibition of reverse transcriptase is the primary mechanism by which these compounds act. Unfortunately, direct assessment of activity against HIV-1 reverse transcriptase is clouded by the use of a highly artificial assay system for the enzyme that employs a synthetic homopolymer as the template and a single deoxynucleotide substrate. We (Parker et al., 1991) have recently reported on an assay that uses ribosomal RNA as the template for HIV-1 reverse transcriptase. Assays using a naturally occurring ribosomal RNA template offer a number of advantages for this type of study over the homopolymers that are normally used in the assay of HIV-1 reverse transcriptase and may more closely resemble the in vivo activity of this enzyme. Ideally, the best template to use would be the native viral RNA genome complexed with the appropriate tRNA primer and nucleoproteins. Unfortunately, this template is generally not available in sufficient quantities to use in routine or extensive enzyme inhibition studies. When the endogenous template has been used to characterize the enzyme (St. Clair et al., 1987; Yong et al., 1990), the kinetic parameters are closer to those that we obtained with the ribosomal RNA than those reported for synthetic homopolymer templates.

We have studied a second TIBO derivative, R82913 (Fig. 1), which was reported by Pauwels et al. (1990) to have a lower IC_{50} in MT-4 cells than R82150 and have determined that this compound selectively inhibits the replication of HIV-1. In addition, the inhibition by R82913 of HIV-1 reverse transcriptase has been determined with respect to its activity on both an RNA template (negative strand synthesis) and a DNA template (positive strand synthesis) in assays that contain all four deoxynucleotides.

Materials and Methods

Materials

Poly(rA)-p(dT)$_{12\ 18}$, poly(rA), oligo(dT), dATP, dGTP, dCTP, dTTP, and Blue Sepharose CL-6B were purchased from Pharmacia LKB Biotechnology (Piscataway, NJ). [Methyl, 1′,2′-^{3}H]dTTP (100 Ci/mmol) in Tricine buffer was obtained from New England Nuclear Research Products (Wilmington, DE). Single-strand DNA-cellulose, calf thymus DNA, 3-(4,5-dimethylthiazol-2-yl)-

2,5-diphenyltetrazolium bromide (MTT), and the sodium salt of lauryl sulfate (SDS) were obtained from Sigma Chemical Co. (St. Louis, MO). Affi-Gel Heparin was purchased from Bio-Rad (Richmond, CA). A solution containing 16 S and 23 S *E. coli* ribosomal RNA was obtained from Boehringer Mannheim Biochemicals (Indianapolis, IN). The 15-base primer, complementary to a sequence in the 16 S *E. coli* RNA, was purchased from Genetic Designs, Inc. (Houston, TX). The sequence of the 15 base primer was 5'-TAACCTTGCGGCCGT-3'. Tissue culture medium (RPMI 1640) and additives (glutamine, pen-strep, fetal bovine serum) were obtained from Irvine Scientific (Santa Ana, CA). DE81 and GF/A chromatography paper were from Whatman International Ltd. (Maidstone, U.K.).

Cells and viruses

The CEM-SS cell line (Nara and Fischinger, 1988) was used in all the cell-based assays. These human T-lymphoblastic cells were maintained in RPMI 1640 medium with 10% fetal bovine serum, glutamine, and antibiotics, and routinely screened for mycoplasma contamination. The HIV-1 isolates were the common laboratory strains HTLV-III$_B$, LAV, RF, and MN, as well as a panel of HIV-1 clinical isolates obtained from patients at Duke University Medical Center (Durham, North Carolina). The biological and biochemical properties of these isolates have been previously described (Cloyd and Moore, 1989). The HIV-2 isolate ROD (Nr. I-532) was obtained from Dr. Luc Montagnier (Pasteur Institute, Paris, France).

Antiviral assay

CEM cells were added to each well of a 96-well microtiter plate at 5×10^3 cells per well. The cells were infected with virus at a multiplicity of infection previously determined to give complete cell killing at 6 days post-infection. The multiplicity of infection varied over a range of approximately 0.01–0.50. The panel of virus isolates was pretitered to induce equivalent infections in these assays. Serial half-log dilutions of R82913 were added to appropriate wells in triplicate to evaluate its ability to inhibit HIV infection. Controls for each assay included drug colorimetric control cultures (drug only), drug cytotoxicity control cultures (cells plus drug), virus control cultures (cells plus virus), and cell viability control cultures (cells only). AZT was run in parallel as a positive control drug. Following 6 days of incubation at 37 C, the viability of the cells in each well was determined spectrophotometrically. MTT was added to each well at a concentration of 450 μg/ml and the plates were incubated for 4 h at 37°C. Viable cells metabolize the tetrazolium salt to an insoluble, colored formazan product. The cells and MTT-formazan were solubilized by the addition of 20% SDS in 0.01 N HCl and the results determined by reading the optical density of each well at 590 nm using a Molecular Devices Vmax plate reader.

Assay of reverse transcriptase activity in supernatant samples

Samples of virus-containing supernatants were removed from each well of the microtiter plate before staining with MTT. These samples were analyzed for their content of virus by a rapid reverse transcriptase assay. Fifteen μl of supernatant was incubated with 10 μl of reverse transcriptase assay buffer which contains 1 μCi of ^{3}H-dTTP, 50 ng/ml poly(rA), 10 ng/ml oligo(dT), 1 mM ethyleneglycol-bis-(β-aminoethyl ether) N,N,N',N'-tetraacetic acid (EGTA), 8 mM $MgCl_2$, 0.5% Triton X-100, 10 mM dithiothreitol, and 10 mM Tris-HCl (pH 8.0) for 45 min at 37°C. Following this incubation, the entire reaction volume was spotted onto DE81 chromatography paper, washed six times with 5% sodium phosphate buffer (pH 8), twice with distilled water, and twice with ethanol. Following drying, the samples were assayed for the amount of radioactivity on the filters by standard liquid scintillation counting techniques.

Preparation of reverse transcriptases from HIV-1 and HIV-2

CEM cells were infected with either a strain of HIV-1 that produces high extracellular levels of reverse transcriptase (SK1 strain) or the ROD isolate of HIV-2. The virus was pelleted from cell-free supernatant by centrifugation and resuspended in 10 mM Tris-HCl (pH 7.8), 1 mM ethylenediaminetetraacetic acid (EDTA), 200 mM KCl, 10 mM β-mercaptoethanol, 1 μM pepstatin, 3 μM leupeptin, and 0.5% Triton X-100. The HIV-1 reverse transcriptase was purified on a single-strand DNA cellulose column equilibrated in 25 mM potassium phosphate (pH 7.4), 10% glycerol, 1 mM phenylmethylsulfonyl fluoride (PMSF), and 10 mM β-mercaptoethanol. Reverse transcriptase was eluted from the column with a 0–1 N KCl gradient. The column was monitored by measuring reverse transcriptase activity, single strand DNA nuclease activity, and terminal transferase activity. (Protein could not be detected by either measuring absorbance at 280 nm or by Bio-Rad's microprotein method.) Fractions from the ascending portion of the reverse transcriptase activity curve (which were essentially free of nuclease activity and free of terminal transferase activity) were pooled. The HIV-2 reverse transcriptase was purified by a two step procedure. The cell-free viral pellet was applied to a Blue-Sepharose column equilibrated with buffer containing 25 mM Tris-HCl (pH 7.4), 10% glycerol, 1 mM PMSF, and 10 mM β-mercaptoethanol. The reverse transcriptase was eluted by a gradient of 0.2–1.0 N KCl. Fractions containing HIV-2 reverse transcriptase activity greater than 500 000 dpm/10 μl were pooled and, after dialysis to remove salt, applied to a Affi-Gel Heparin column equilibrated in 25 mM potassium phosphate buffer (pH 7.4), 10% glycerol, 1 mM PMSF, and 10 mM β-mercaptoethanol. The reverse transcriptase was eluted with a gradient from 0–0.8 N KCl. This purification procedure gives a pool of HIV-2 reverse transcriptase free of contaminating nuclease and terminal transferase activity. Bovine serum albumin was added to the final pool

of each reverse transcriptase at a final concentration of 200 μg/ml. Each enzyme stock was dialyzed against 25 mM Tris (pH 7.4) buffer containing 10 mM β-mercaptoethanol, 30% glycerol, and 0.01% Triton X-100, aliquoted, and stored at −70°C until needed.

Preparation of template-primer

The poly(rA)-p(dT) template-primer was prepared by dissolving 1 mg of poly(rA)-p$(dT)_{12-18}$ in 1 ml of H_2O, heating at 42°C for 5 min and cooling slowly to room temperature. The ribosomal RNA template-primer was prepared by annealing the DNA primer to the 16 S ribosomal RNA at a 3:1 molar ratio. Two hundred μg of 16 S/23 S ribosomal RNA and 2 μg of DNA primer were mixed in 0.05 N NaCl, 0.2 M Tris-HCl, pH 8.0 (final volume 200 μl). The mixture was placed in boiling H_2O and then allowed to cool slowly (overnight) to room temperature. Assuming that this 3:1 molar ratio of primer to template is sufficient to anneal one DNA primer to each 16 S ribosomal RNA molecule, then the concentration in the stock of 3′-hydroxyl's available for extension by the enzyme is 0.73 μM. The gapped duplex DNA was prepared from calf thymus DNA by the method of Baril et al. (1977). Stock solutions of each template-primer were divided into small aliquots and stored at −20°C until needed.

Enzyme inhibition assays

To determine the effect of an inhibitor on HIV-1 and HIV-2 reverse transcriptase, 10 μl of a solution containing the test compound was added to one well of a 96-well flat bottom microtest plate with lid, followed by 30 μl of a mixture containing the assay buffer, template-primer, and substrates. The reaction was started by the addition of 10 μl of a solution containing the enzyme. 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, 3% glycerol, 0.2% DMSO, 1 mM EGTA, 20 μg/ml poly(rA)-p$(dT)_{12-18}$, and 60 μM dTTP (4 mCi ^3H/μmol dTTP). The assay on 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, 0.2% DMSO, 3% glycerol, 1 mM EGTA, 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/μmol dTTP). The assay on a 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, 0.2% DMSO, 1 mM EGTA, 10 μg/ml of gapped duplex DNA, 10 μM dATP, 10 μM dCTP, 10 μM dGTP, and 2 μM dTTP (101.9 mCi ^3H/μmol dTTP). EGTA was added to the assays to chelate Ca^{++}, the preferred divalent cation for nucleases. Each assay was run for 1 h at 37°C, and terminated by the addition of 65 μl of 0.2 M sodium pyrophosphate. One

hundred μl from each well was spotted on a GF/A glass microfibre filter and processed by the method described by White et al. (1982).

Results and Discussion

A microtiter antiviral assay was performed to determine the effect of R82913 on the ability of HIV-1 and HIV-2 to initiate productive infection in CEM cells. R82913 and AZT were tested against thirteen HIV-1 strains and one HIV-2 strain. These HIV-1 viral isolates included common laboratory isolates as well as a panel of isolates obtained from ARC patients being treated at Duke University. All of these isolates have been extensively passaged in tissue culture and are cytopathic upon infection of human T-cell lines. Preliminary experiments were performed to adjust the input dose of virus such that all the isolates induced total cell killing by day 6 post-infection. In this way we have controlled for differences in the activity of the drug that occur due to the biological heterogeneity of the various isolates. We have determined that the kinetics of virus growth in cell culture, as measured by supernatant reverse transcriptase activity, p24 ELISA, and titration of infectious particles, is directly related to viral cytopathicity. Peak levels of virus production vary depending on the virus isolate but always occur as virus induced cell killing falls through the 50% viability range. Thus, the multiplicity of infection used for each virus isolate ($TCID_{50}$) is different but the kinetics of the infection has been

TABLE 1

Inhibition of HIV-1 infectivity and productivity by R82913

Isolate	IC_{50} (μM)	
	Infectivity[a]	Productivity[b]
ED	0.009 (0.001)[c]	0.060
SK1	0.034 (0.004)	0.031
RF	0.043 (0.004)	0.009
McK	0.064 (0.005)	0.078
HTLV-III_B	0.067 (0.004)	0.051
MN	0.124 (0.002)	0.092
LAV	0.148 (0.005)	0.149
214	0.161 (0.010)	0.058
KELL	0.183 (0.007)	0.143
G	0.188 (0.006)	0.075
205	0.205 (0.006)	0.065
C	0.222 (0.007)	0.095
PM16	0.652 (0.014)	0.161
HIV-2_{ROD}	> 310 (0.018)	> 310

[a] Infectivity is measured by MTT quantitation of cytopathic effects in culture.

[b] Productivity is measured by reverse transcriptase assay of cell free supernatant samples.

[c] The number in the parentheses is the IC_{50} value for AZT which was determined in parallel with R82913.

standardized by the ability of the isolate to kill CEM cells in 6 days. The results of these antiviral assays indicate that R82913 has significant activity against all the isolates of HIV-1 and no activity against HIV-2 (Table 1). The IC_{50} values obtained for R82913, when assayed against a variety of HIV-1 isolates, fell into a narrow range of concentrations. For most isolates, 0.03–0.22 μM R82913 was required to inhibit virus induced cell killing by 50%. Toxicity was observed with R82913 at much higher concentrations (TC_{50} = 46 μM), indicating that R82913 has a high selectivity index. R82913 was inactive against the isolate $HIV\text{-}2_{ROD}$ whereas AZT was as active against this isolate (IC_{50} of 0.018 μM) as it was against HIV-1. Our experiments are consistent with those of Pauwels et al. (1990) in showing that TIBO derivatives are able to inhibit productive infection by a number of isolates of HIV-1 but not by HIV-2. However, we report an IC_{50} for R82913 that is at least one order of magnitude higher than expected from their publication. The reason for this difference is not clear, but is probably due to differences in the details of our respective assays.

Confirmation of the activity of R82913 was obtained by performing reverse transcriptase assays on cell free supernatant samples derived from the acute infection assays described above (Table 1). In preliminary experiments we determined that the carry-over from the microtiter wells of R82913 at all concentrations used in the antiviral studies did not inhibit the reverse transcriptase activity of a control virus sample (data not presented). Thus, the observed reduction in supernatant reverse transcriptase activity was the result of the antiviral activity of the compound and not an effect on the activity of supernatant reverse transcriptase. The reverse transcriptase activity present in samples removed from each well was used to calculate an IC_{50} value and this value was compared with the value obtained from the cell culture data. This comparison demonstrates that the ability of R82913 to protect cells is directly comparable to its ability to inhibit virus replication and is not merely inhibiting virus-induced cytopathic effects.

Having confirmed that R82913 was a potent inhibitor of HIV-1 infection with a number of viral isolates, including SK1, we examined its ability to inhibit HIV-1 reverse transcriptase partially purified from a virus pool of SK1. R82913 was 20-fold more potent than AZT-TP in the inhibition of HIV-1 reverse transcriptase using ribosomal RNA as the template (Table 2). When gapped duplex DNA was the template, R82913 was equal to AZT-TP in its ability to inhibit HIV-1 reverse transcriptase. These two assays should mimic negative

TABLE 2

Inhibition of HIV-1 reverse transcriptase by R82913 and AZT-TP

Template	ID_{50} (μM)	
	R82913	AZT-TP
Ribosomal RNA	0.006, 0.015, 0.006	0.35
Gapped duplex DNA	1.5, 1.1	1.0
Poly(rA)-p(dT)$_{12-18}$	8.7, 7.3	0.03

and positive strand synthesis of the double stranded DNA copy of the RNA virus. Consistent with the findings published by Pauwels et al. (1990), we found that R82913 was only a moderate inhibitor of HIV-1 reverse transcriptase when poly(rA)-p$(dT)_{12-18}$ was used as the template. Data for AZT-TP are included in the summary table for ease of comparison.

In evaluating these data, the unknowns about the mechanism by which R82913 is inhibiting the enzyme need to be considered. Ideally, in an ID_{50} screen all the substrates would be at saturating concentrations except the one with which the inhibitor competes. That substrate should be at a concentration equal to its K_m value. For the ribosomal RNA and gapped duplex DNA assays, all the reagents were at saturating concentrations except dTTP which was present at about twice its K_m value. This optimizes the assays for compounds that compete with dTTP, such as AZT-TP. Theoretically, these compounds would have lower ID_{50} values in our screen than compounds that inhibit the enzyme by some other mechanism.

As expected from the lack of antiviral activity in cell culture on HIV-2, R82913 failed to inhibit the reverse transcriptase from HIV-2. When identical assay conditions to those for HIV-1 reverse transcriptase were used, the enzyme from HIV-2 was unaffected by R82913 at concentrations up to 310 μM in studies with either the ribosomal RNA or gapped duplex DNA templates.

Both AZT-TP and R82913 appear to be more inhibitory to the HIV-1 reverse transcriptase when RNA is the template instead of DNA. With more detailed kinetic studies, Reardon and Miller (1990) have reported a K_i for AZT-TP on a defined sequence RNA template containing all four bases of 0.16 μM and a K_i of 7.1 μM on a defined sequence DNA template. St. Clair et al. (1987) have reported a 30-fold difference in the K_is for AZT-TP on the native viral RNA template vs. gapped duplex DNA. In our hands R82913 appeared to have an even more enhanced differential than AZT-TP in its ability to inhibit HIV-1 reverse transcriptase on the RNA template. There was about a 150-fold difference in the ID_{50} values on the two templates. Interestingly, not only does R82913 appear to be highly selective for HIV-1 reverse transcriptase, but we would also infer that it is most effective against the RNA-directed DNA synthesis of this enzyme.

In conclusion, we have confirmed that the TIBO derivative R82913 has antiviral activity against several strains of HIV-1. This antiviral activity correlates well with the known reverse transcriptase inhibitor AZT in acute infection assays. When ribosomal RNA is used as the template, R82913 is a potent inhibitor of HIV-1 reverse transcriptase with an ID_{50} (0.01 μM) that is equal to, or lower than, the IC_{50} for this compound in all of our cell culture assays (0.01–0.65 μM). The high ID_{50} values (6–0.3 μM) reported by Pauwels et al. (1990) seem to be a result of the artificial, homopolymer templates that were used. Our data substantially strengthen the conclusion that TIBO derivatives prevent HIV-1 infection by the same process as AZT, ddC, or ddI, i.e., inhibition of the viral reverse transcriptase. However in their interaction with this enzyme TIBO derivatives differ from nucleoside analogs in several

important respects. These benzodiazepine derivatives probably do not act as chain terminators of DNA synthesis since they should not be able to substitute as substrates for HIV-1 reverse transcriptase. TIBO derivatives are more specific in that they do not inhibit HIV-2 infection or inhibit the reverse transcriptase from HIV-2. Their failure to exhibit much cellular toxicity also implies that unlike nucleosides they would not inhibit human DNA polymerases. Work is in progress to explore in more detail the mode by which these novel non-nucleoside derivatives inhibit HIV-1 reverse transcriptase.

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References

Baril, E., Mitchener, J., Lee, L. and Baril, B. (1977) Action of pancreatic DNase: requirements for activation of DNA as a template-primer for DNA polymerase γ. Nucleic Acids Res. 4, 2641 2653.

Cloyd, M.W. and Moore, B.E. (1989) Spectrum of biological properties of human immunodeficiency virus (HIV-1) isolates. Virology 174, 103 116.

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

Parker, W.B., White, E.L., Shaddix, S.C., Ross, L.J., Buckheit, Jr., R.W., Germany, J.M., Secrist III, J.A., Vince, R. and Shannon, W.M. (1991) Mechanism of inhibition of human immunodeficiency virus type 1 reverse transcriptase and human DNA polymerases α, β, and γ by the 5'-triphosphates of carbovir, 3'-azido-3'-deoxythymidine, 2',3'-dideoxyguanosine, and 3'-deoxythymidine. A novel RNA template for the evaluation of antiretroviral drugs. J. Biol. Chem. 266, 1754 1762.

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.

Reardon, J.E. and Miller, W.H. (1990) Human immunodeficiency virus reverse transcriptase. Substrate and inhibitor kinetics with thymidine 5'-triphosphate and 3'-azido-3'-deoxythymidine 5'-triphosphate. J. Biol. Chem. 265, 20302 20307.

St.Clair, M.H., Richards, C.A., Spector, T., Weinhold, K.J., Miller, W.H., Langlois, A.J. and Furman, P.A. (1987) 3'-Azido-3'-deoxythymidine triphosphate as an inhibitor and substrate of purified human immunodeficiency virus reverse transcriptase. Antimicrob. Agents Chemother. 31, 1972 1977.

White, E.L., Shaddix, S.C., Brockman, R.W. and Bennett, L.L., Jr. (1982) Comparison of the actions of 9-β-D-arabinofuranosyl-2-fluoroadenine and 9-β-D-arabinofuranosyladenine on target enzymes from mouse tumor cells. Can. Res. 42, 2260 2264.

Yong, W.H., Wyman, S. and Levy, J.A. (1990) Optimal conditions for synthesizing complementary DNA in the HIV-1 endogenous reverse transcriptase reaction. AIDS 4, 199 206.