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MECHANISTIC EVALUATION OF NEW PLANT-DERIVED COMPOUNDS THAT INHIBIT HIV-1 REVERSE TRANSCRIPTASE

THITIMA PENGSUPARP, LINING CAI, HOWARD CONSTANT, HARRY H.S. FONG, LONG-ZE LIN, A. DOUGLAS KINGHORN, JOHN M. PEZZUTO,* GEOFFREY A. CORDELL,

Program for Collaborative Research in the Pharmaceutical Sciences, Department of Medicinal Chemistry and Pharmacognosy, College of Pharmacy, University of Illinois at Chicago, Chicago, Illinois 60612

KRISTÍN INGOLFSDÓTTIR,

Department of Pharmacy, University of Iceland, 101 Reykjavik, Iceland

HILDEBERT WAGNER,

Institute of Pharmaceutical Biology, University of Munich, 80333 Munich, Germany

and STEPHEN H. HUGHES

ABL-Basic Research Program, NCI-Frederick Cancer Research and Development Center, Frederick, Maryland 21702

ABSTRACT.—Swertifrancheside [**1**], a new flavonone-xanthone glucoside isolated from *Swertia franchetiana*, 1β-hydroxyaleuritolic acid 3-*p*-hydroxybenzoate [**2**], a triterpene isolated from the roots of *Maprounea africana*, and protolichesterinic acid [**3**], an aliphatic α-methylene-γ-lactone isolated from the lichen *Cetraria islandica*, were found to be potent inhibitors of the DNA polymerase activity of human immunodeficiency virus-1 reverse transcriptase (HIV-1 RT), with 50% inhibitory doses (IC_{50} values) of 43, 3.7, and 24 μM, respectively. They were not cytotoxic with cultured mammalian cells. The kinetic mechanisms by which compounds **1–3** inhibited HIV-1 RT were studied as was their potential to inhibit other nucleic acid polymerases. Swertifrancheside [**1**] bound to DNA and was shown to be a competitive inhibitor with respect to template-primer, but a mixed-type competitive inhibitor with respect to TTP. On the other hand, 1β-hydroxyaleuritolic acid 3-*p*-hydroxybenzoate [**2**] and protolichesterinic acid [**3**] were mixed-type competitive inhibitors with respect to template-primer and noncompetitive inhibitors with respect to TTP. Therefore, the mechanism of action of 1β-hydroxyaleuritolic acid 3-*p*-hydroxybenzoate [**2**] and protolichesterinic acid [**3**] as HIV-1 RT inhibitors involves nonspecific binding to the enzyme at nonsubstrate binding sites, whereas swertifrancheside [**1**] inhibits enzyme activity by binding to the template-primer.

Over the past decade, substantial progress has been made in defining strategies for the treatment of human immunodeficiency virus (HIV) infection, the cause of acquired immunodeficiency syndrome (AIDS). Since reverse transcriptase (RT) is required for early proviral DNA synthesis (1), inhibition of the RT-catalyzed polymerization of DNA from viral RNA inhibits virus replication. RTs may be viral specific and are thus considered viable chemotherapeutic targets. The most potent inhibitors of HIV-1 RT are nucleoside analogues that are converted to triphosphates by cellular enzymes and act as chain terminators (2,3). Azidothymidine (AZT), didanosine (dideoxyinosine or ddI), zalcitabine (dideoxycytidine or ddC), and stavudine (didehydrodideoxythymidine or d4T) are all examples of HIV-1 RT inhibitors and are the only drugs currently approved for clinical use in HIV-1 infection (4,5). Although these compounds have been shown to benefit HIV-infected individuals, there are substantial toxic side-effects associated with their use (5), and complete inhibition of viral replication is not achieved (5–7). In addition, the emergence of nucleotide-resistant HIV strains may complicate long-term therapy (8).

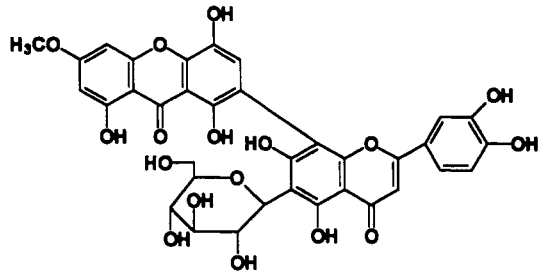
Most reverse transcriptase inhibitors, such as antimonitungstate (9) and suramin (10) also inhibit cellular DNA or RNA polymerases, and the non-selectivity of such agents can contribute to in vivo toxicity (11,12). Hence, the discovery and characterization of agents capable of specifically inhibiting HIV RT without mediating a toxic

response remain of high priority. Natural products serve as one source of structurally novel chemicals that are worth investigating as specific inhibitors of HIV RT. Previous screening of various natural products for HIV-1 RT inhibitory activity showed stringent structural requirements (13), inasmuch as few compounds demonstrated potent activity. Natural product RT inhibitors, such as benzophenanthridine (14) and protoberberine (15) alkaloids, flavonoids (16,17), a variety of other compounds with phenolic hydroxy groups (18,19), and certain antibiotics (20), were found to inhibit the HIV-1 RT (as well as the HIV-2 RT) with similar potency (12). We have also shown that *O*-methylpsychotrine is a selective inhibitor of HIV-1 RT (21), and even greater activity was observed with HIV-2 RT by this alkaloid (22).

In our continuing study of natural product-mediated inhibition of human immunodeficiency virus-1 reverse transcriptase (HIV-1 RT), three new active inhibitors were identified, as follows: swertifrancheside [**1**], a new flavonone-xanthone glucoside isolated from *Swertia franchetiana* (23), 1β-hydroxyaleuritolic acid 3-*p*-hydroxybenzoate [**2**], a triterpene isolated from the roots of *Maprounea africana* (24,25), and protolichesterinic acid [**3**], an aliphatic α-methylene-γ-lactone from the lichen *Cetraria islandica* (26). We currently describe the inhibition mediated by these compounds with a variety of nucleic acid polymerases, as well as an analysis of the mechanism by which the inhibition is mediated.

RESULTS AND DISCUSSION

As summarized in Table 1, compounds **1–3** were evaluated for their potential to inhibit nucleic acid polymerases. In each case, RNA polymerase was least affected by the test compounds. Compound **1** inhibited each of the remaining polymerases with similar efficacy, whereas **2** demonstrated greater inhibition with HIV-1 RT and DNA poly-



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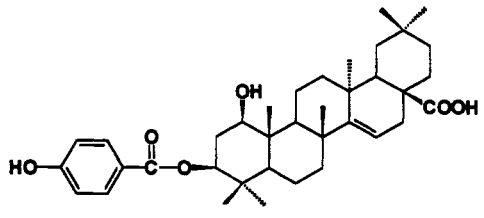

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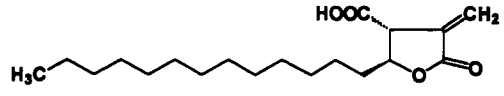

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TABLE 1. Nucleic Acid Polymerase Inhibitory Effects of Compounds **1–3** and AZT Triphosphate.[a]

Compound	HIV-1 RT	HIV-2 RT	AMV RT	DNA Polymerase	RNA Polymerase
1	42.9	56.6	81.0	57.7	272
2	3.7	59.0	15.1	2.2	>338
3	24.3	29.6	21.9	7.4	92.8
AZT..... triphosphate	0.05	0.20	0.43	>20	>20

[a]Results are given as IC_{50} values (concentration which inhibits enzyme activity by 50%) in μM.

merase β. Compound **3** inhibited DNA polymerase β most effectively, with the remaining polymerases being inhibited with IC_{50} values in the 20–30 μM range.

Relative to AZT triphosphate, these test compounds were not potent inhibitors of HIV-1 RT. However, relative to the most potent secondary natural products tested in our laboratory previously [e.g., fagaronine chloride, IC_{50} value of 13 μM (13); *O*-methylpsychotrine sulfate heptahydrate, IC_{50} value of 14 μM (21)], and in the laboratory of others using the same template-primer system [e.g., calanolide A, IC_{50} value of 1 μM (27); halocynthiaxanthin, IC_{50} value of 7.2 μM (28)], the inhibitory activity observed with HIV-1 RT was considered significant and worth exploring in greater detail. In addition, these inhibitors were judged to be of interest because no appreciable cytotoxic activity was observed with a variety of cultured mammalian cells [e.g., ED_{50} values of >20 μg/ml were obtained with human oral epidermoid carcinoma (KB); human lung cancer (Lu1); hormone-dependent human prostate cancer (LNCaP); hormone-dependent human breast cancer (ZR-75-1)]. Thus, kinetic analyses were undertaken to investigate the mode of interaction of the test compounds and HIV-1 RT.

As shown in Figures 1A and 2A, respectively, swertifrancheside [**1**] demonstrated competitive inhibition with respect to the template-primer [oligo $(dT)_{12-18}$+poly (rA)] (K_i=6.7 μM), while it was a mixed-type competitive inhibitor with respect to the substrate (TTP) (K_i=7.1 μM). On the other hand, 1β-hydroxyaleuritolic acid 3-*p*-hydroxybenzoate [**2**] and protolichesterinic acid [**3**] demonstrated mixed-type competitive inhibition (K_i=6.7 and 10.0 μM, respectively) with respect to template-primer (Figures 1B and C, respectively), and non-competitive inhibition (K_i=8.3 and 7.1 μM, respectively) with respect to the substrate (TTP) (Figures 2B and C, respectively).

These data suggest that compound **1** binds to DNA to mediate inhibition, whereas compounds **2** and **3** function by non-specific mechanisms. Thus, binding to DNA was initially assessed utilizing a hplc method devised in our laboratory (29). As shown in Table 2, these data support the notion that compound **1** binds to DNA, whereas no effect was observed with compounds **2** and **3**. Fagaronine chloride, an agent known to bind to DNA (30), was used as a positive control.

Lastly, the effect of bovine serum albumin (BSA) on the inhibitory activity of compounds **1–3** was investigated. As summarized in Table 3, the inhibitory activity mediated by compounds **2** and **3** was greatly reduced when the BSA concentration in the incubation mixture was increased. In contrast, increasing BSA concentration had no effect on the HIV-1 RT inhibitory activity mediated by compound **1**. This is consistent with its mechanism of action involving interaction with the nucleic acid template-primer and not the enzyme.

To conclude, relative to other secondary metabolites isolated from plants, swertifrancheside [**1**], 1β-hydroxyaleuritolic acid 3-*p*-hydroxybenzoate [**2**], and protolichesterinic acid [**3**] are potent inhibitors of HIV-1 RT. Compound **1** was a competitive inhibitor with respect to template-primer and a mixed-type competitive

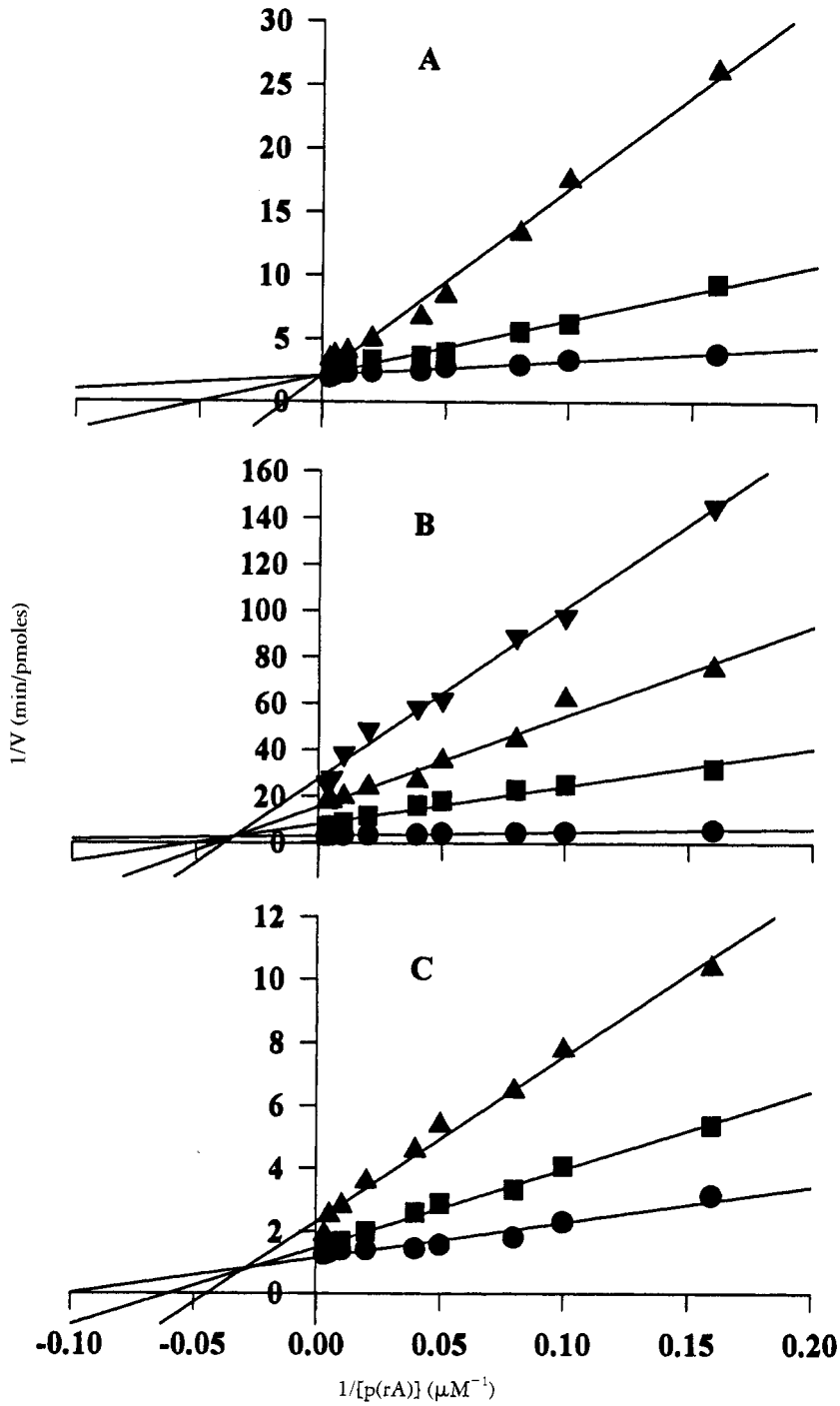

FIGURE 1. HIV-1 RT inhibition kinetics of **1–3** with respect to template-primer [oligo $(dT)_{12-18}$+poly (rA)]. Details of experimental conditions are described in the HIV-1 RT assay in the Experimental section. (1A) Competitive inhibition of HIV-1 RT by varying the concentration of **1** as follows: (●) 0 μM, (■) 30 μM, (△) 60 μM. (1B) Mixed-competitive inhibition of HIV-1 RT by varying the concentration of **2** as follows: (●) 0 μM, (■) 6.7 μM, (▲) 13.5 μM, (▼) 20.2 μM. (1C) Mixed-competitive inhibition of HIV-1 RT by varying the concentration of **3** as follows: (●) 0 μM, (■) 30.4 μM, (▲) 36.5 μM. All experiments used 10% DMSO as a control.

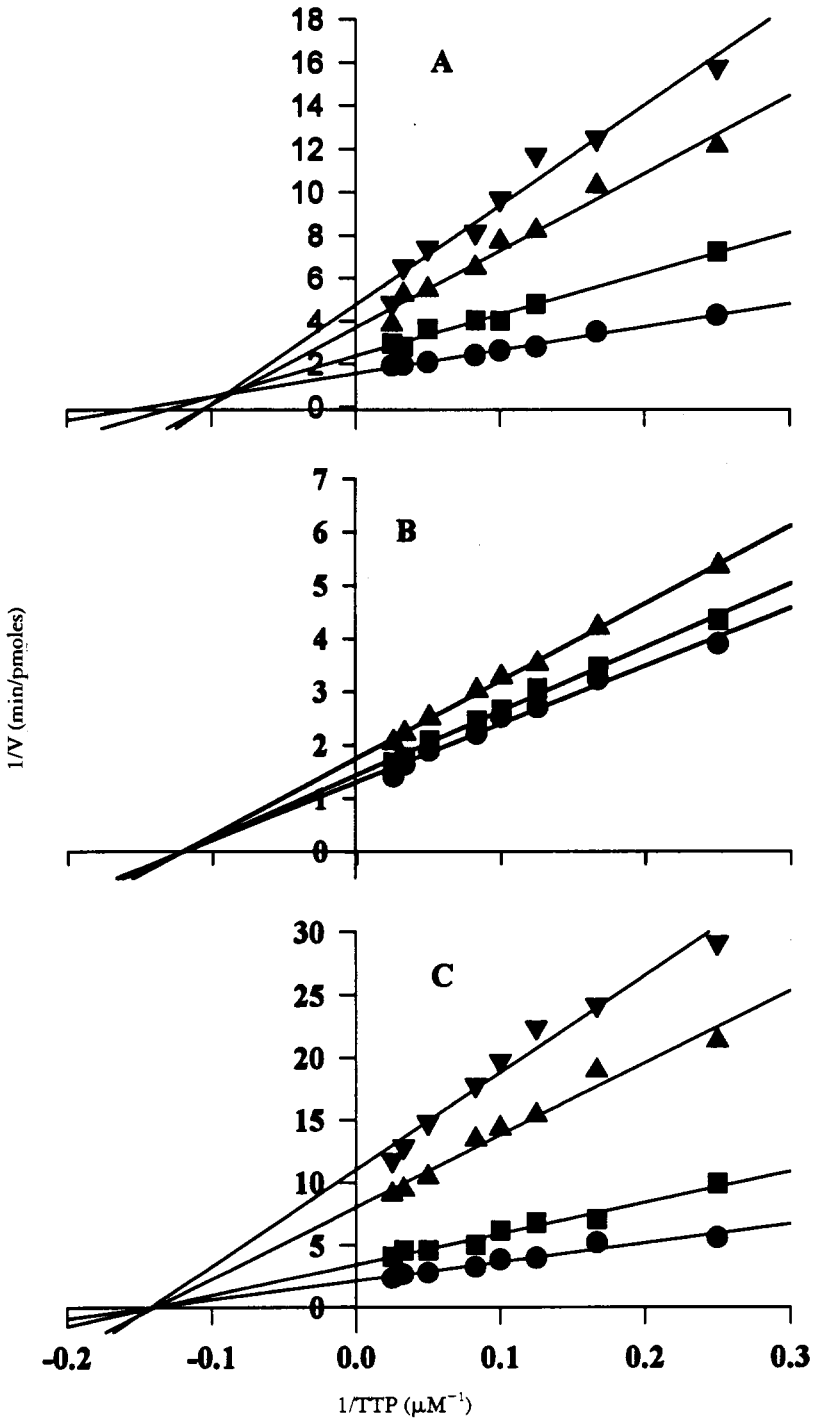

FIGURE 2. HIV-1 RT inhibition kinetics of **1**–**3** with respect to substrate (TTP). Details of experimental conditions are described in the HIV-1 RT assay in the Experimental section. (2A) Mixed-competitive inhibition of HIV-1 RT by varying the concentration of **1** as follows: (●) 0 μM, (■) 30 μM, (▲) 60 μM (▼) 80 μM. (2B) Noncompetitive inhibition of HIV-1 RT by varying the concentration of **2** as follows: (●) 0 μM, (■) 3.4 μM, (▲) 6.7 μM. (2C) Mixed-competitive inhibition of HIV-1 RT by varying the concentration of **3** as follows: (●) 0 μM, (■) 30.4 μM, (▲) 36.5 μM, (▼) 45.6 μM. All experiments used 10% DMSO as a control.

TABLE 2. Evaluation of DNA-Binding Potential of Compounds **1–3** and Fagaronine Chloride Using Hplc

Compound[a]	% Inhibition of DNA Peak at 260 nm
1	71
2	0
3	1.3
Fagaronine chloride	100

[a]Sample concentration=250 μg/ml; DNA concentration=125 μg/ml.

TABLE 3. Effect of Bovine Serum Albumin (BSA) Concentration on the HIV-1 RT Inhibitory Activity of **1–3**.

BSA Concentration (μg/ml)	% HIV-1RT Inhibition		
	1[b]	**2**[b]	**3**[b]
2.5[a]	98	94	99
250	89	19	52
1000	82	0.8	19

[a]This BSA concentration is used in the standard nucleic acid polymerase assays.

[b]Compound **1** was tested at 200 μg/ml; compounds **2** and **3** were tested at 50 μg/ml.

inhibitor with respect to substrate (TTP). It binds to DNA and this explains its mechanism of action. On the other hand, because compounds **2** and **3** are mixed-type competitive inhibitors with respect to template-primer and noncompetitive inhibitors with respect to substrate (TTP), they have no specific effect on the substrate binding site (nucleotide binding site) or the template-primer binding site. Further, because the inhibitory activity was greatly reduced by increasing the concentration of BSA, it is likely that these two compounds interact with the enzyme non-specifically to exert their inhibitory activity. Nevertheless, it is possible that one or more of these test substances could selectively inhibit viral proliferation without mediating dose-limiting toxicity. In support of this supposition, for example, the compounds are not overtly toxic with cultured mammalian cells. Due to these considerations, additional tests are currently underway to monitor the potential of compounds **1–3** to protect cultured cells from HIV infection, and future development will be considered on the basis of these data.

EXPERIMENTAL

GENERAL EXPERIMENTAL PROCEDURES.—[^{3}H]TTP (15 Ci/mmol) and [*methyl*-^{3}H]UTP (6.5 Ci/mmol) were obtained from ICN Radiochemicals (Irvine, CA). Activated calf thymus DNA, native DNA, TTP, poly A, oligo $(dT)_{12-18}$, dithiothreitol, and glutathione were purchased from Sigma Chemical (St. Louis, MO). DEAE-cellulose filter discs (Whatman DE 81) were obtained from VWR Scientific (Batavia, IL). All other reagents were of analytical grade. Reverse transcriptase from avian myeloblastosis virus (AMV RT) was purchased from Sigma Chemical (St. Louis, MO). DNA polymerase β was purchased from Molecular Biology Resources, Inc. (Milwaukee, WI). RNA polymerase (*E. coli*) was purchased from Pharmacia (Piscataway, NJ).

PLANT MATERIAL.—The collection locations and dates, and the provisions made for the identification and herbarium deposit of *Swertia franchetiana* (23) and *Maprounea africana* (24,25) have been published previously. The lichen, *Cetraria islandica* (L.) Ach. (family Parmeliaceae) (26) was collected in Jökuldalsheidi, Iceland. A voucher specimen is deposited at the Department of Pharmacy, University of Iceland.

EXTRACTION AND ISOLATION.—Swertifrancheside [**1**] was isolated from *S. franchetiana* (whole plants) and structurally characterized as described previously (23). 1β-Hydroxyaleuritolic acid 3-*p*-hydroxybenzoate [**2**] was isolated from *M. africana* roots and structurally characterized as previously described (24,25). Protolichesterinic acid [**3**] was isolated from the lichen, *Cetraria islandica* (L.) Ach., and structurally characterized as described previously (26).

HIV-1 (p66/p51) AND HIV-2 (p66/p51) REVERSE TRANSCRIPTASES (HIV-1 AND HIV-2 RTs).—Dimeric HIV-1 RT was purified by modification of the procedures of Clark *et al.* (31). HIV-2 RT is a recombinant enzyme consisting of two polypeptide subunits (68 and 55 kD). It was synthesized in an *Escherichia coli* expression system using a genetically engineered plasmid (32). The enzyme possesses both RNA-dependent DNA polymerase and ribonuclease H activities typical of retroviral RTs.

HIV-1 AND HIV-2 RT AND AMV RT ASSAYS.—Assays were conducted as described previously (12).

Identical conditions were utilized for assays performed with HIV-1 and HIV-2 RTs (33), and AMV RT. The assay mixture (final volume 100 μl) contained the following: 50 mM Tris•HCl buffer (pH 8.0), 150 mM KCl, 5 mM $MgCl_2$, 0.5 mM ethylene glyco-*bis*-(β-amino-ethylether) *N,N'*-tetraacetic acid (EGTA), 5 mM dithiothreitol, 0.3 mM glutathione, 2.5 μg/ml bovine serum albumin, 41 μM poly (rA) [ϵ_{260} (mM)=7.8], 9.5 μM oligo $(dT)_{12-18}$ [ϵ_{260} (mM)=5.6], 20 μM TTP, and 0.5 μCi [^{3}H]TTP. The reaction was started by the addition of 10 μl of HIV-1 RT or HIV-2 RT or 1 unit of AMV RT [1 unit being defined as the amount of enzyme which incorporates 7.84 pmol of TMP into acid-insoluble product in 10 min using poly (rA) and oligo $(dT)_{12-18}$ as template-primer], and the mixture (final volume, 100 μl) was permitted to incubate at 37° for 1 h. Reactions were terminated by the addition of 20 μl of 0.1 M EGTA followed by chilling in ice. Aliquots of each reaction mixture (100 μl) were then spotted uniformly onto circular 2.5 cm DE-81 (Whatman) filters, kept at room temperature for 15 min, and washed four times with 5% aqueous Na_2HPO_4. This was followed by two more washings with distilled H_2O. Finally, the filters, which retained radiolabeled polynucleotide products, were thoroughly dried and subjected to scintillation counting. With these reaction conditions, the uninhibited rates of incorporation were 2.5 and 1.3 μmol TMP/mg protein/10 min for HIV-1 and HIV-2 RTs, respectively.

For testing enzyme inhibition, five serial dilutions of samples in DMSO (10 μl) were added to the reaction mixtures prior to the addition of enzyme. The median inhibitory concentration (IC_{50}) was calculated from a linearly regressed dose-response plot of percent control activity versus concentration of compound, utilizing at least five concentrations of each compound. Each data-plot represents the average of duplicate tests.

DNA POLYMERASE β ASSAY.—Each assay mixture (100 μl) contained 80 mM Tris•HCl (pH 7.5), 5 mM KCl, 10 mM $MgCl_2$, 1.5 mM dithiothreitol, 25 μg/ml bovine serum albumin, 12% (v/v) glycerol, 41 μM activated calf thymus DNA, 80 μM each of dATP, dCTP, dGTP, and ATP, 1.5 μM (2 μCi) [*methyl*-^{3}H]TTP, and 0.5 units of DNA polymerase enzyme. The enzyme reaction was performed in the same way as the RT assay reaction.

RNA POLYMERASE ASSAY.—Each assay mixture (100 μl) contained 40 mM Tris•HCl (pH 7.9), 150 mM KCl, 10 mM $MgCl_2$, 0.1 mM dithiothreitol, 25 μg/ml bovine serum albumin, 41 μM native calf thymus DNA, 80 μM each of ATP, CTP, and GTP, 1.5 μM (2 μCi) [*methyl*-^{3}H]UTP, and 0.5 unit of RNA polymerase enzyme. The enzyme reaction was performed in the same way as the RT assay reaction.

DNA BINDING ASSAY.—The procedure was based on that described by Pezzuto *et al.* (29). The hplc system used was comprised of a Waters 712b WISP auto-injector, two Waters 510 high pressure pumps, a M991 photodiode array detector controlled by a PC, and a C_{18} NovaPak column (Waters) (150 ×3.9 mm). Elution was performed as follows: 0–3 min 100% H_2O; 3–20 min 100% MeOH; 20–30 min H_2O (equilibration). Calf thymus DNA was dissolved in H_2O to yield a final concentration of 125 μg/ml. The test compounds were dissolved in MeOH to yield stock solutions of 1 mg/ml. The DNA peak was measured at 260 nm in the presence and absence of test compound at a final concentration of 250 μg/ml.

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