Psychotrine and Its O-Methyl Ether Are Selective Inhibitors of Human Immunodeficiency Virus-1 Reverse Transcriptase*

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Psychotrine dihydrogen oxalate and O-methylpsychotrine sulfate heptahydrate (MP), the salts of isoquinoline alkaloids from ipecac, were found to be potent inhibitors of the DNA polymerase activity of human immunodeficiency virus-1 reverse transcriptase (HIV-1 RT). We currently report the results of additional studies designed to characterize the mechanism of inhibition facilitated by MP. The inhibition was noncompetitive with respect to TTP and uncompetitive with respect to poly(rA) and oligo(dT)12-18 (4:1) at low template-primer concentrations, but competitive at high concentrations (>200 μM). Identical non-Michaelis-type kinetics were observed when activated DNA was used as the template. The biphasic nature of the double-reciprocal plots and Hill coefficients of less than 1 indicate that MP functions as an allosteric inhibitor of the enzyme which appears to possess multiple active sites that interact in a cooperative (negative) fashion in the presence of the inhibitor. MP was selective for the recombinant HIV-1 RT (p66) utilizing poly(rA) and oligo(dT)12-18 (4:1) as template-primer. Greater inhibition was observed with this template primer as compared with other natural and synthetic template-primers tested. MP had significantly less effect on avian myeloblastosis virus RT as well as mammalian or bacterial DNA and RNA polymerases. Other members of the ipecac class of alkaloids, e.g. emetine hydrochloride, were inactive against all of these enzymes, including HIV-1 RT. Conversely, MP did not inhibit in vitro protein synthesis, a property manifested by all the other ipecac alkaloids tested. Studies conducted with structural analogs revealed that 2'-deoxyguanosine oxalate; HEPES, and other nucleoside analogs (4,5) in addition to the emergence of viral resistance to azidothymidine (3). Most reverse transcriptase inhibitors (e.g. antitumoragentstatin (4) and suramin (5)) also inhibit cellular DNA polymerases, and the nondiscriminatory nature of such agents accounts for their in vivo toxicity (6,7). Hence, the discovery and characterization of agents capable of specifically inhibiting the HIV RT without mediating a toxic response remain a high priority.

Natural products represent a rich and largely untapped source of structurally novel chemicals which are worth investigating as specific inhibitors of the HIV RT. Previous efforts in the screening of diverse structural classes of natural products for HIV-1 RT inhibitory activity revealed stringent structural requirements (8), since few compounds demonstrated potent activity. Nevertheless, these results enabled the delineation of activity cutoffs for natural products in general. Natural product RT inhibitors include the benzophenanthridine (9) and protoberberine (10) alkaloids, flavonoids (11), certain antibiotics (12), and a variety of compounds with phenolic hydroxy groups (13). These classes of compounds were also found to inhibit the HIV-1 RT. In addition to these, the tricyclic fulvoplumierin and an acid salt of the isoquinoline alkaloid O-methylpsychotrine (Fig. 1) were found to be potent HIV-1 RT inhibitors when compared with known active substances such as the protoberberine alkaloid fagaronine chloride (14). Relative to approximately 150 natural product drugs that were evaluated for inhibitory activity, O-methylpsychotrine sulfate heptahydrate (MP) and psychotrine dihydroxylate (PDO) were two of the most potent inhibitors uncovered. For example, MP had a 50% inhibitory dose (IC50) in

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† The abbreviations used are: HIV, human immunodeficiency virus; RT, reverse transcriptase; AMV, avian myeloblastosis virus; MP, O-methylpsychotrine sulfate heptahydrate; PDO, psychotrine dihydroxylate; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EOTA, [ethylendiois(cxyethylbenzenitrilo)tetraacetic acid. 23529
Selective Inhibition of HIV-1 Reverse Transcriptase

O-Methylpsychotrine is a minor alkaloid of ipecac, the dried rhizome and root of *Cephaelis ipecacuanha* (Brotero) A. Rich. relationships. In contrast, O-methylpsychotrine and isoemetine are shown as the free bases; in addition, various salt forms were tested for biological activity.

The HIV-1 RT system of 10 μg/ml (14 μM) compared with fagarinine chloride, which had an IC₅₀ value of 5 μg/ml (13 μM). These observations prompted us to investigate additional members of the ipecac alkaloids for HIV-1 RT inhibitory activity with the aim of defining relevant structure-activity relationships.

*O*-Methylpsychotrine is a minor alkaloid of ipecac, the dried rhizome and root of *Cephaelis ipecacuanha* (Brotero) A. Richard (Rubiaceae) (15). Ipecac yields more than 2% (w/w) of ether-soluble alkaloids, with the three principal ones being emetine, cephaeline, and psychotrine (16). These structurally related alkaloids are prepared as their inorganic acid salts for the HIV-1 RT system of 10 pg/ml (14 μM) by incubating the preparation at 70 °C for 5 min. Nucleic acid synthesis is selective and this permitted expression of large quantities of HIV-1 RT in drug screening assays (27). The recombinant HIV-1 RT assay—The HIV-1 RT is a 66-kDa recombinant enzyme obtained in an *E. coli* expression system using a genetically engineered plasmid; the enzyme was purified to near homogeneity (27). Synthetic DNA segments were used to introduce initiation and termination codons into the HIV-1 RT coding sequence, and this permitted expression of large quantities of HIV-1 RT in *E. coli* (28). The enzyme was shown to be active in RT assays and exhibited inhibitory properties with several known retroviral inhibitors from human placenta, casein acid hydrolysate (Hy-Case amine) from bovine milk, and all other reagents of analytical grade were supplied by Sigma. Escherichia coli DNA polymerase I (Klenow fragment) was provided by Pharmacia. DEAE-cellulose filter discs (Whatman DE81) were obtained from VWR Scientific (Batavia, IL). The rabbit reticuloocyte translation kit, type II (nucleoside-treated and message-dependent) and RNA from tobacco mosaic virus were obtained from Boehringer Mannheim.

Materials and Methods

Test and Reference Compounds—MP, cephaeline hydrochloride, and dehydroemetine were purchased from or donated by Hoffmann-La Roche, Inc. (Nutley, NJ). Emetine hydrochloride was purchased from Sigma, and psychotrine free base and PDO were obtained from the natural product compound repository at the Program for Collaborative Research in the Pharmaceutical Sciences, College of Pharmacy, University of Illinois at Chicago. All compounds were tested for purity by thin layer chromatography in several different solvent systems and were found to be >96% pure in all cases. Psychotrine free base and PDO exhibited UV data (λmax and ε values) and electronic impact mass spectra consistent with literature values (24, 25). Fagarinine chloride was isolated as described previously (26).

Molecular and Cellular Methods—Primary human small cell lung carcinoma cells (HW-1) and human T-cell lymphoblastoid cells (RT-11) were maintained at the Program for Collaborative Research in the Pharmaceutical Sciences, College of Pharmacy, University of Illinois at Chicago. All compounds were tested in cell culture (20, 21). The synthesis of DNA with 50 ng of DNase/mg of DNA for 30 min at 37 °C in buffer analogous to that of the enzyme assay. DNase was inactivated by incubating the preparation at 70 °C for 5 min. Nucleic acid concentrations were determined spectrophotometrically.

**FIG. 1. Structures of selected ipecac alkaloids.** The structures are shown as the free bases; in addition, various salt forms were tested for biological activity.

The HIV-1 RT is a 66-kDa recombinant enzyme obtained in an *E. coli* expression system using a genetically engineered plasmid; the enzyme was purified to near homogeneity (27). Synthetic DNA segments were used to introduce initiation and termination codons into the HIV-1 RT coding sequence, and this permitted expression of large quantities of HIV-1 RT in *E. coli* (28). The enzyme was shown to be active in RT assays and exhibited inhibitory properties with several known retroviral inhibitors from human placenta, casein acid hydrolysate (Hy-Case amine) from bovine milk, and all other reagents of analytical grade were supplied by Sigma. Escherichia coli DNA polymerase I (Klenow fragment) was provided by Pharmacia. DEAE-cellulose filter discs (Whatman DE81) were obtained from VWR Scientific (Batavia, IL). The rabbit reticuloocyte translation kit, type II (nucleoside-treated and message-dependent) and RNA from tobacco mosaic virus were obtained from Boehringer Mannheim.

Enzymes and Reagents—Avian myeloblastosis virus (AMV) RT, dithiothreitol, glutathione, nuclease-free bovine serum albumin, ribonuclease A (RNase A, type I-AS from bovine pancreas), RNase inhibitor from human placenta, casein acid hydrolysate (Hy-Case amine) from bovine milk, and all other reagents of analytical grade were supplied by Sigma. Escherichia coli DNA polymerase I (Klenow fragment) was provided by Pharmacia. DEAE-cellulose filter discs (Whatman DE81) were obtained from VWR Scientific (Batavia, IL). The rabbit reticuloocyte translation kit, type II (nucleoside-treated and message-dependent) and RNA from tobacco mosaic virus were obtained from Boehringer Mannheim.

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in place of poly(rA) and oligo(dT)12-18, and only labeled substrate [methyl-3H]TTP (1.5 μM, 2 μCi) was used. The reaction was initiated by the addition of 10 μl (0.11 μg) of HIV-1 RT, and the mixture was incubated at 37 °C for 1 h. The time course of this reaction has been described previously (8). Reactions were terminated by the addition of 25 μl of 0.1 M EGTA followed by chilling in ice. Aliquots of the reactions were then added to ethanol circular 2 DE-81 (Whatman) filters and washed four times with 5% aqueous Na2HPO4, and twice with H2O. Finally, the filters were dried and subjected to scintillation counting.

For testing enzyme inhibition, serial dilutions of samples dissolved in H2O or dimethyl sulfoxide (10 μl) were added to the reaction mixtures prior to the addition of enzyme (10 μl). The median inhibitory concentration (IC50) was calculated from a linearly regressed dose-response plot of percent control activity versus concentration or log concentration of compound, utilizing at least five concentrations of each compound. Each data point represents the average of duplicate tests. The antiviral and antiproliferative activities of this compound were tested in a number of in vitro assays as described previously.

Polymerization Assay Conditions—Assays involving various polymerases were performed by the general procedures described above. Attempts were made to optimize all assay conditions with respect to the ratios and concentrations of template-primers, monovalent and divalent ion concentrations, and pH. Preliminary experiments were conducted to ensure that product incorporation was linear with respect to time, and each set of assay conditions was established to represent saturation levels with respect to the template, substrate, and enzyme concentrations. Wherever possible, the effects of inhibitors were evaluated at the same region on the substrate saturation curves of the various enzymes as an attempt to standardize the polymerization activity of each system and hence their susceptibility to the inhibitors.

Avian Myeloblastosis Virus RT Assay—Reaction mixtures of 100 μl consisted of 50 mM Tris- HCl (pH 8.3), 10 mM MgCl2, 40 mM KCl, 1.0 mM EGTA, 1.0 mM dithiothreitol, 41 μM poly(A), and 9.5 μM oligo(dT)12-18 in 20 μM TTP, and 2 μCi of [methyl-3H]TTP. Alternatively, 41 μM activated calf thymus DNA (Promega, Madison, WI) and oligo(dT)12-18, and the mixture was supplemented with 80 μM each of the dNTPs. Each reaction was started by the addition of 1 unit of AMV RT, 1 unit being defined as the amount of enzyme which incorporates 7.84 pmol of TTP into acid-insoluble product in 10 min using poly(rA) and oligo(dT)12-18 as template-primer.

Preparation of Partially Purified Mammalian DNA Polymerase—The isolation of nuclei and the subsequent preparations of partially purified DNA polymerase were performed as described by Sedwick et al. (33) with some modifications. Cultured human breast cancer cells (UISO-BCA-1) were harvested during the exponential growth phase and suspended in 1 M sucrose buffer (1 mM sucrose, 50 mM Tris- HCl (pH 7.5), 2.5 mM KCl, 1 mM MgCl2, 25 mM 2-mercaptoethanol) at a density of 1 × 10^7 cells/ml. The cells were homogenized, and complete nuclear clearance was monitored by light microscopy. The homogenate was then centrifuged (1,000 × g, 15 min, 4 °C), and the resulting pellet was resuspended in 10 volumes (v/w) of a sucrose buffer identical in composition to the one described above except containing a lower (0.25 M) sucrose concentration. The suspension was again centrifuged (800 × g, 5 min, 4 °C), the pellet was again washed and resuspended in 9 volumes (v/w) of detergent-containing buffer (1 mM K2HPO4, 0.3% Triton X-100, 1 mM MgCl2, and 1 M 2-mercaptoethanol). After centrifugation (800 × g, 5 min, 4 °C), the lysed nuclei were resuspended in 9 volumes (v/w) of buffer (0.2 M sucrose, 1 mM 2-mercaptoethanol, and 1 mM MgCl2), and EDTA (1 mM final concentration) and NaCl (4M final concentration) were added to an adjusted volume of 4°C, and then dialyzed (12,000-14,000 molecular weight cutoff; 2025 K2HPO4, (pH 7.5), 1 M 2-mercaptoethanol, 1 mM MgCl2) for 24 h with three changes of buffer solution. The resulting precipitate was removed by centrifugation, and the protein concentration of this resulting enzyme preparation was determined by the method of Lowry et al. (33). The preparation was stored as small aliquots at −80 °C.

Mammalian DNA Polymerase Assay—Each assay mixture (100 μl) contained the following: 80 mM Tris- HCl (pH 7.5), 10 mM MgCl2, 1.5 mM dithiothreitol, 5% (v/v) glycerol, 25 μg/ml bovine serum albumin, 5 mM NaF, 3 mM spermidine, 16% glycerol, 40 mM (NH4)2SO4, 0.1 mM GTP and CTP, 0.2 mM ATP, 5.6 μM (5′-3H)TTP (0.2 Ci/ mmol), 41 μM native calf thymus DNA, and 28.4 μg of enzyme protein. The enzyme preparation had a specific activity of 12.7 pmol of UTP incorporated per mg of protein in 10 min at 37 °C. Each assay was of 25-45 min duration; product incorporation was linear with time for up to at least 1 h. The reaction mixture further distinguished replication from DNA repair activity (data not shown). Avidin, a selective inhibitor of DNA polymerase α (34, 35), demonstrated an IC50 value of 15 μM/ml in this system. These results confirmed the presence of DNA polymerase activity and β in the preparation.

Preparations of Partially Purified Mammalian DNA Polymerase—Nuclei were prepared from cultured human breast cancer (UISO-BCA-1) cells as described for the preparation of DNA polymerase, and partially purified DNA polymerase was isolated using a modification of the procedure described by Rosse et al. (36). The nuclei were suspended in 4 volumes (v/w) of buffer (50 mM Tris- HCl (pH 8.9), 50 mM KCl, 1 mM MgCl2, 0.5 mM phenylmethylsulfonyl fluoride, 0.1 mM EDTA, and 20% glycerol) and homogenized. Ammonium sulfate was then added (0.4 g/ml, final concentration), and the suspension was incubated (35 °C for 10 min) and stirred (3 h at 4 °C). The mixture was then dialyzed (12,000-14,000 molecular weight cutoff; 2025 mM Tris- HCl (pH 7.9), 50 mM KCl, 1 mM MgCl2, 2 mM dithiothreitol, 0.1 mM EDTA, and 25% glycerol) for 24 h with three changes of buffer solution. A resulting precipitate was removed by centrifugation, and glycerol was added to a final concentration of 50% (v/v) and the mixture was incubated at 37 °C as described above.

The activities obtained with 4 mM Mn2+ in buffer of high salt concentration was approximately three times greater than that obtained with an identical amount of Mg2+ in a buffer of low ionic strength, i.e. one without (NH4)2SO4. Furthermore, 4 mM Mg2+ was inhibitory in the Mn2+ system with high ionic strength. Native DNA was preferred over denatured DNA, and spermidine was found to stimulate the reaction utilizing native DNA. The activities obtained in the presence and absence of 3 units of ribonuclease inhibitor/reaction were identical, indicating the absence of contaminating ribonucleases. α-Amanitin at a concentration of 1 μg/ml inhibited in 60% inhibition of enzyme activity, and the reactions were ribonuclease sensitive.

E. coli DNA Polymerase Assay—Each assay mixture (100 μl) contained the following: 80 mM Tris- HCl (pH 7.5), 10 mM MgCl2, 1.5 mM dithiothreitol, 5% (v/v) glycerol, 25 μg/ml bovine serum albumin, 3.3 mM NaF, 3 mM spermidine, 16% glycerol, 40 mM (NH4)2SO4, 0.1 μM GTP and CTP, 0.2 mM ATP, 5.6 μM (5′-3H)TTP (0.2 Ci/ mmol), 41 μM native calf thymus DNA, and 28.4 μg of enzyme protein. The enzyme preparation had a specific activity of 12.7 pmol of UTP incorporated per mg of protein in 10 min at 37 °C. Each assay was of 25-45 min duration; product incorporation was linear with time for up to at least 1 h. The reaction mixture further distinguished replication from DNA repair activity (data not shown). Avidin, a selective inhibitor of DNA polymerase α (34, 35), demonstrated an IC50 value of 15 μM/ml in this system. These results confirmed the presence of DNA polymerase activity and β in the preparation.

In Vitro Protein Translation Assay—Each reaction mixture (16 μl) was prepared from the components supplied in a commercially available translation kit as follows: 78.13 μM CH2COOK, 1.56 mM (CH3COO)2Mg, 0.5 μg of tobacco mosaic virus RNA, 0.35 μM (1 μCi) L-[3,4,5-3H]leucine (177 Ci/mm), and 5 μl of the translation reaction mixture provided which contained 312.5 μM of each of the 19 amino acids except leucine, 37.5 mM fructose 1,6-biphosphate, 30 mM CAMP, 12.5 mM ATP, 2.5 mM GTP, 100 mM creatine phosphate, 6.25 mM spermidine, 25 mM dithiothreitol, and 250 mM HEPES. Five μl of each test compound dissolved in H2O or dimethyl sulfoxide was added prior to the addition of 5 μl of rabbit reticulocyte lysate which had previously been incubated at 30 °C for 30 min after which a solution of 1 M NaOH containing 5% (v/v) H2O2 (500 μl) was added. The reaction mixtures were then incubated at 37 °C for an additional 10 min. Aqueous trichloroacetic acid solution (50% w/v) containing 2% sodium pyrophosphate (2%) and 0.25 M ammonium acetate, pH 8 was added to stop the reaction, and the mixtures were then filtered through presoaked GFC discs using approximately 100 ml of
aqueous trichloroacetic acid solution (5% w/v) containing 10 mM Na2P2O7. The filters were dried and subjected to scintillation counting.

Interaction with Template-primer—The potential of MP to interact with the various template-primer was determined, as described previously (37, 38), by monitoring changes in its UV absorption spectra in the presence of varying amounts of each template-primer. Mixtures (1.5 ml) were prepared consisting of buffer analogous to that used in the HIV-1 RT assay, various amounts of the template-primer (0–80 μM) and 20 μg/ml MP with water as the solvent. Difference spectra were recorded from 190 to 600 nm using mixtures containing the corresponding concentrations of template-primer (without MP) as reference solutions. Analogous studies were performed using fagaronine chloride (26) rather than MP as the ligand.

RESULTS

Enzyme Inhibitory Activities of the Ipecac Alkaloids—Ipecac alkaloids were examined for their effects on the enzymic activities of HIV-1 RT, AMV RT, mammalian DNA and RNA polymerases, and E. coli DNA polymerase I. Emetine hydrochloride, cephaeline hydrochloride, dehydroemetine, and psychotrine free base demonstrated 10–20% inhibition of these enzyme activities at a concentration of 400 μg/ml. MP strongly inhibited the HIV-1 RT system with poly(rA) and oligo(dT)12-18 (4:1) as template-primer (IC50 = 10 μg/ml (14 μM)) but proved less inhibitory when activated DNA was used as substrate (IC50 = 245 μg/ml (348 μM)) (Fig. 2). It was significantly less active toward the AMV RT, showing IC50 values of 135 μg/ml (192 μM) with poly(rA) and oligo(dT)12-18 (4:1) as template-primer and 574 μg/ml (816 μM) when activated DNA was used. Fifty percent inhibition of mammalian and E. coli DNA polymerase activities was observed at concentrations of greater than 450 μg/ml (640 μM) of MP when either activated DNA or poly(dA) and oligo(dT)12-18 was used. Mammalian RNA polymerase was essentially unaffected by up to 200 μg/ml (285 μM) of MP (data not shown).

PDO demonstrated enzyme specificities and inhibition properties almost identical to those of MP. Its IC50 values in the HIV-1 RT system were 6 μg/ml (9 μM) with poly(rA) and oligo(dT)12-18 (4:1) and 269 μg/ml (404 μM) with activated DNA. It was essentially inactive in the remaining enzyme systems up to a concentration of 200 μg/ml (310 μM).

Effect of Ipecac Alkaloids on in Vitro Protein Synthesis—The effect of the ipecac alkaloids on in vitro protein synthesis was examined using the rabbit reticulocyte lysate translation system. Emetine hydrochloride and cephaeline hydrochloride demonstrated IC50 values of 0.52 μg/ml (0.94 μM) and 0.36 μg/ml (0.67 μM), respectively. Psychotrine free base showed intermediate potency (IC50 = 56 μg/ml (120 μM)) whereas MP and PDO were very weakly active, demonstrating IC50 values of 166 μg/ml (236 μM) and 186 μg/ml (288 μM), respectively (Fig. 3).

Template Specificity of HIV-1 RT (66 kDa) and the Inhibitory Effect of O-Methylpsychotrine Sulfate Hexahydrate—The template specificities of the 66-kDa enzyme used in the present investigation is important when considering inhibition that is obtained when using these various template-primer. This 66-kDa subunit of the HIV-1 RT transcribed poly(rA) with oligo(dT)12-18 (4:1) most efficiently, but poly(rC)-oligo(dG)12-18 and the synthetic DNA duplexes poly(dA-dT)-poly(dA-dT) (heat denatured) and poly(dC) with oligo(dG)12-18 were also utilized with reasonable efficiency under the same conditions described (data presented in legend of Fig. 4). Poly(I) with oligo(dC)15, poly(dA) with oligo(dT)12-18, poly(rCm)-oligo(dG)12-18 and poly(U) with oligo(dA)6 were poor template-primers for the enzyme under the assay conditions currently employed.

The inhibitory effect of MP on the activity of HIV-1 RT was examined using activated DNA and various synthetic nucleic acids as template-primer (Fig. 4). MP was most inhibitory in systems incorporating poly(rA) with oligo(dT)12-18 (4:1) and heat-denatured poly(dA-dT)-poly(dA-dT); the resulting IC50 values were 10 μg/ml (14 μM) and 18 μg/ml (26 μM), respectively. It was a much less potent inhibitor when activated calf thymus DNA, poly(dC) with oligo(dG)12-18 or poly(I) with oligo(dC): were used (IC50 = 200–220 μg/ml (285–313 μM)). HIV-1 RT inhibition curves for these three template-primer were very similar. Least inhibition was observed in assays utilizing poly(rC)-oligo(dG)12-18, poly(dA) with oligo(dT)12-18, or poly(rCm)-oligo(dG)12-18 (IC50 > 500 μg/ml (711 μM)) as template-primer.

Kinetic Analyses of the Inhibition of HIV-1 RT Mediated by MP—To characterize further the inhibition mediated by MP, kinetic studies were conducted using poly(rA) or activated DNA, with [methyl-3H]TTP as labeled substrate. As shown in the double-reciprocal plot (Fig. 5), the Ks for TTP was 9
The template-primers used were: poly(dA-dT)·poly(dA-dT) (40.0 μg/ml) (Ο); poly(rC)·oligo(dG)12-18 (10.0 μg/ml) (V); poly(dC) (10.0 μg/ml) with oligo(dG)12-18 (17.5 μg/ml) (△); poly(dA) (40.0 μg/ml) with oligo(dT)12-18 (5.0 μg/ml) (□); poly(rA) (13.6 μg/ml) with oligo(dT)12-18 (2.9 μg/ml) (molar ratio, 4:1) (■); activated calf thymus DNA (13.3 μg/ml) (△). The total amount of radiolabeled substrate used, together with their specific activities were: [methyl-3H]TTP (20 μCi, 250 mCi/mmol) for poly(rA) with oligo(dT)12-18; [methyl-3H]TTP (1.43 μCi, 14 Ci/mmol) for poly(dA-dT)·poly(dA-dT), poly(dA) with oligo(dT)12-18, and activated DNA; [5-3H]dCTP (0.77 μCi, 26 Ci/mmol) for poly(I) with oligo(dC); [8-3H]dTTP (1.82 μCi, 11 Ci/mmol) for the remaining two template-primers. The amount of product incorporated (pmol/h) for the controls were: 2.4 (Ο); 0.16 (●); 2.9 (V); 29.3 (△); 0.05 (□); 210 (■); 0.7 (△). In cases in which the template and primer were added as separate entities, the ratio of template to primer used was optimal. The use of poly(U) (40 μg/ml) and oligo(dA)5 (17.5 μg/ml) with [8-3H]dTTP (1.43 μCi, 14 Ci/mmol) or poly(rC)·oligo(dG)12-18 (65.0 μg/ml) with [5-3H]dCTP (1.82 μCi, 11 Ci/mmol) both resulted in the incorporation of 0.03 pmol of TTP, and this rate of incorporation was too low for inhibition to be measured accurately. Additional details are described under "Materials and Methods."

μM, and the mode of inhibition was noncompetitive with respect to TTP when poly(rA) with oligo(dT)12-18 (4:1) was used as the template-primer. The K, for MP, as determined from a Dixon plot (data not shown), was 10 μM. With respect to poly(rA) with oligo(dT)12-18, the mode of inhibition was uncompetitive at lower concentrations of template-primer but approached a competitive mode as the concentration of the template-primer reached 200 μM (Fig. 6). The K, for MP as an uncompetitive inhibitor was 6 μM, and the K, for this template-primer was 10 μM. The Hill coefficient was determined to be less than one over the entire range of template-primer concentration tested (data not shown).

When activated DNA was used, it was observed that the mode of inhibition by MP was also noncompetitive with respect to TTP (Fig. 7) and uncompetitive with respect to activated DNA over most of the concentration range tested (Fig. 8). Above 200 μM DNA, however, the pattern of inhibition was competitive with respect to DNA. The K, for TTP and DNA were 0.4 and 27 μM, respectively. The K, for HIV-1 RT for MP was 140 μM with TTP as the variable substrate and 95 μM when the concentration of DNA was varied (uncompetitive inhibition).

The K, value for MP obtained with variable concentrations of poly(rA) with oligo(dT)12-18 (4:1) was less than the K, value for the template-primer itself. This indicates that the enzyme had a slightly higher affinity for the inhibitor. The above results show that the K, for TTP and the K, for MP vary.
with the template-primer used, but the mode of inhibition is the same. Also, the inhibition of HIV-1 RT by MP appears to deviate from Michaelis-type kinetics at high concentrations of templates. This pattern of inhibition suggests that MP may also be an allosteric inhibitor of the enzyme, and in its presence the HIV-1 RT demonstrated negative cooperativity toward the nucleotide substrate. The ratio of $K_i$ to $K_m$ serves to indicate the relative potency of MP in the inhibition of nucleotide incorporation; when comparing uncompetitive inhibition mediated by MP, this value was lower when poly(rA) with oligo(dT)$_{12-18}$ was used as template-primer.

Temporal Studies to Evaluate the Mechanism of Inhibition of HIV-1 RT Mediated by MP—Studies wherein MP was preincubated with the HIV-1 RT or the template-primer prior to the start of the reaction revealed almost identical time-course profiles (data not shown). These preincubation studies indicate that MP did not inactivate the enzyme by irreversible binding nor did it affect the initiation of polynucleotide synthesis, since these processes would have produced reactions of lower velocity than that which were observed without preincubation. In addition, it was observed that when the concentration of bovine serum albumin in the reaction mixture was increased from 25 to 2,500 µg/ml with a fixed concentration of MP (12 µg/ml (17 µM)), the inhibitory activity was unaffected. These results suggest that the compound does not bind to proteins in a nonspecific manner. Also, preincubation of HIV-1 RT with the template-primer and MP resulted in a higher initial reaction velocity than the control wherein only the enzyme was preincubated and the remaining reaction components (including MP) were added to initiate the reaction (data not shown). This would seem to indicate that the enzyme-template-primer complex was also unaffected by the inhibition. Additional experiments were performed wherein MP (10 µg/ml (14 µM)) was added at various time points to completed reaction mixtures in which product synthesis was already in progress. An abrupt decrease in velocity was evident in all these reactions (Fig. 9). Since polynucleotide synthesis by HIV-1 RT is reported to be ordered and progressive in nature (39), these results indicate that the elongation of the nascent polynucleotide was inhibited by MP. Incubation of the mixture for prolonged periods (e.g. 4 h) did not change the profile of product incorporation or result in degradation of the nucleic acid product. The nucleic acid product was stable in the presence of the alkaloid.

Interaction with Template-primers—Employing reaction conditions analogous to those of HIV-1 RT incubation mixtures, the addition of MP (20 µg/ml) to various concentrations of poly(rA) with oligo(dT)$_{12-18}$ (4:1), poly(dA) with oligo(dT)$_{12-18}$ (4:1), poly(rA), poly(dA), oligo(dT)$_{12-18}$ or activated DNA did not yield any spectral perturbations in the range of 190-600 nm. Conversely, fagaroxime chloride (20 µg/ml), an agent known to interact with nucleic acids and to inhibit the reaction catalyzed by RT via this interaction (31), was readily observed to interact with poly(rA) and all double-stranded nucleic acids investigated using the same methodology (data not shown). Thus, these results suggest that there is no physicochemical interaction between MP and all the investigated nucleic acids.

**DISCUSSION**

MP was investigated for its effects on purified recombinant HIV-1 RT (66 kDa), AMV RT, as well as cellular and bacterial DNA and RNA polymerases. It showed specificity only for the RNA-dependent DNA polymerizing activity of HIV-1 RT (IC$_{50}$ = 10 µg/ml (14 µM)) with poly(rA) annealed to oligo(dT)$_{12-18}$ (4:1) as template-primer under the assay conditions currently described. The DNA-dependent polymerase activity of this enzyme was weakly inhibited only at much higher concentrations of the compound. This was the case whether heteropolymeric (activated DNA) or homopolymeric (poly(dA) with oligo(dT)$_{12-18}$) template-primers were used, indicating that the action of MP on this polymerase activity of the HIV-1 RT was unaffected by the nature of the template-primer. MP was also essentially inactive in all the other viral and cellular enzyme systems tested. The mode of HIV-1 RT inhibition was complex as evident from the biphasic double-reciprocal plots obtained with poly(rA) and oligo(dT)$_{12-18}$ and activated DNA. The mode of inhibition was noncompetitive with respect to TTP in the presence of either of these template-primers. These observations, together with Hill coefficients of less than 1 for both template-primers, conform with the criteria for negative cooperativity set forth previously (40). This implies that HIV-1 RT possesses multiple binding sites for the template-primer which interact in a cooperative (negative) fashion in the presence of MP. MP can thus be viewed as an allosteric inhibitor of the enzyme.

MP showed a high degree of specificity in inhibiting the HIV-1 RT reaction with poly(rA) and poly(dA-dT)·poly(dA-dT) as templates. This template specificity suggests a preference for the A/T or dA:T base pairs (under the assay conditions employed). The IC$_{50}$ values obtained with these template-primers were about 10-fold less than the values obtained with homopolymeric template-primers containing dCdG base pairs or activated calf thymus DNA. Since the genome of the HIV-1 is A/T rich (41), the use of these sensitive template-primers may be considered of physiological relevance. The template-primer poly(c)·oligo(dG)$_{12-18}$ was essentially resistant to the inhibitory effects of MP. The poor utilization of poly(dA) with oligo(dT)$_{12-18}$ and poly(U) with oligo(dA)$_p$ prevented the accurate measurement of the inhibitory potency of MP in these systems. The alternating nature of the duplex copolymer poly(dA-dT)·poly(dA-dT) may also contribute to its effectiveness as a template-primer for the HIV-1 RT and also to its susceptibility to inhibition by MP. Even though A:T or dA:T systems were strongly inhibited, spectroscopic studies did not reveal changes in the absorption
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spectra of MP in the presence of various concentrations of poly(rA) with oligo(dT)$_{12-18}$ (4:1), poly(dA) with oligo(dT)$_{12-18}$ (4:1), poly(rA), poly(dA), oligo(dT)$_{12-18}$, or activated DNA [data not shown]. This is in contrast to results obtained with dC:dG base pairs in this heteropolymeric nucleic acid.

Previous reports suggested that the reaction pathway for DNA synthesis is ordered, with the template-primer and free enzyme combining to form the first complex in the reaction sequence (39). The classical interpretation of the mechanism of an uncompetitive inhibitor is one in which two or more enzyme ligands bind in an obligate order. It was of interest to know if dNTP binding was necessary for the binding of MP. The fact that MP is able to exert its effect after the start of the polymerization reaction which has been shown to be processive in nature (39) would seem to support this idea. Preliminary studies using preincubation mixtures suggest that the enzyme-template-primer complex was unaffected by the inhibitor. The differential inhibition observed with different template-primer pairs can therefore be explained from the viewpoint of an uncompetitive inhibitor is one in which two or more enzyme-ligands are bound to the enzyme-template-primer complex was unaffected by the inhibitor. The differential inhibition observed with different template-primer pairs can therefore be explained from the viewpoint of an uncompetitive inhibitor is one in which two or more enzyme-ligands are bound to the enzyme in a manner that prevents further binding.

The relative resistance of the activated DNA template to the inhibitory effects of MP could be caused by the presence of the difference in potency of MP in inhibiting these two activities. In the (-)-emetine molecule, the polymerase active sites.

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REFERENCES

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