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ACCELERATED COMMUNICATION

Inhibition of Human Immunodeficiency Virus-1 Reverse Transcriptase Activity by Rubromycins: Competitive Interaction at the Template·Primer Site

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SUMMARY

Rubromycins, a class of quinone antibacterials, were discovered to selectively inhibit human immunodeficiency virus-1 (HIV-1) RNA-directed DNA polymerase (reverse transcriptase) (RT) activity more potently than cellular DNA polymerase α. β- and γ-rubromycin each inhibited equipotently HIV-1 RT and avian myeloblastosis virus RT, in a concentration-dependent manner, and were significantly weaker as inhibitors of calf thymus DNA polymerase α. These agents inhibited HIV-1 RT reversibly, were competitive with respect to template·primer, and were noncompetitive with respect to TTP. Dixon analyses yielded HIV RT K_i values of 0.27 ± 0.014 and 0.13 ± 0.012 μM for β- and γ-rubromycin, respectively. Similarly, using DNA polymerase α, the K_i values were 25.1 ± 4.3 and 3.9 ± 0.6 μM for β- and γ-rubromycin, respectively. Because these agents were toxic to noninfected human T lymphoid cells using concentrations at or above 6 μM, HIV-1 infectivity studies were carried out at 0.8–6 μM. At these concentrations, which are below the range expected to provide protection, no significant antiviral activity was observed. Although β- and γ-rubromycins did not possess sufficient HIV RT inhibitory potency or selectivity versus mammalian DNA polymerase to demonstrate antiviral activities, these studies support the hypothesis that specific molecules containing quinone functional groups can selectively inhibit viral polymerase activities over cellular polymerase activities. In addition, these studies suggest that rubromycins may be lead structures for the development of more potent and selective agents.

The identification of a retrovirus, HIV-1, as the etiologic agent of the acquired immune deficiency syndrome has renewed interest in the enzymology of RT and RT inhibitors (1–3). This enzyme, present only in retroviruses and retrovirus-infected cells, plays a central role in the incorporation of the viral genome into host cells. Reverse transcription of the viral RNA genome to double-stranded DNA involves a complex series of events including RNA-dependent DNA polymerase activity, DNA-dependent DNA polymerase activity, and RNase H activity, as well as interstrand and intrastrand molecular rearrangements (4, 5). Once synthesized, the double-stranded DNA is inserted into the host cell genome to form the integrated provirus.

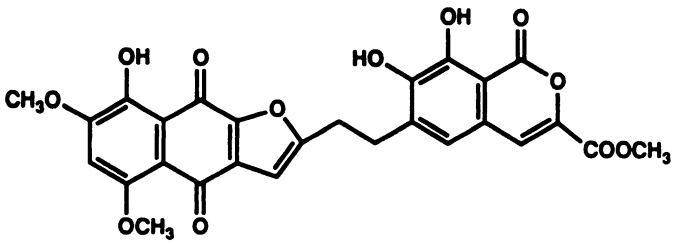
Because RT is a functionally novel DNA polymerase and is present in cells only after viral infection, this enzyme is a logical target for antiviral drug development. A selective RT inhibitor should prevent incorporation of the viral genome into the host and, therefore, block progression of the infection. Unlike inhibitors of other polymerases (RNA polymerase II, DNA polymerase α) (6, 7), selective RT inhibitors have not been identified as yet. The clinical effectiveness of nucleoside analogs (AZT, dideoxyinosine), which integrate into nascent viral DNA and cause chain termination (3, 8), provides evidence that classical enzyme inhibitors may also be clinically effective. As a result of the dose-limiting toxicities of nucleoside analogs, their requirement for intracellular phosphorylation, and the recent emergence of AZT-resistant strains of HIV-1 (8–10), novel RT inhibitors are needed.

Quinone antibiotics with antiviral activity, such as streptonigrin and sakyomycin A, have been shown to inhibit RT by interacting with a single unique site on the enzyme molecule, termed the quinone pocket (11–14). In the present study, the abilities of rubromycins (Fig. 1), a class of known quinone antibacterials (15), to inhibit viral and cellular polymerases were investigated.

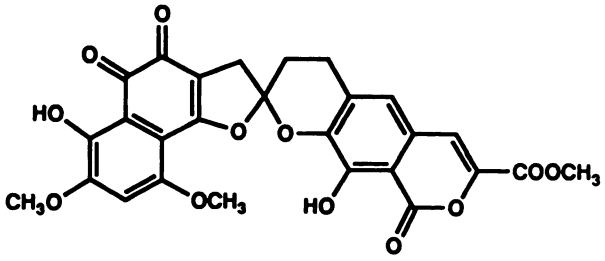
Experimental Procedures

Materials. Materials used in this study were purchased from the following sources: IPTG, aprotinin, phenylmethylsulfonyl fluoride, Tri-

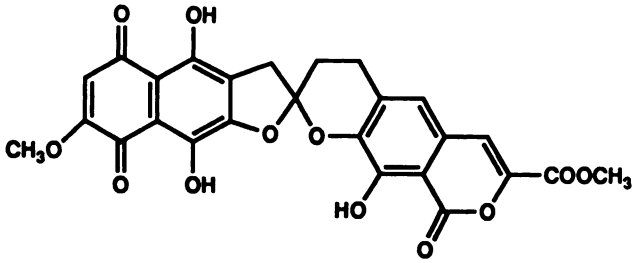
ABBREVIATIONS: HIV-1, human immunodeficiency virus type 1; RT, reverse transcriptase; DTT, dithiothreitol; AMV, avian myeloblastosis virus; BSA, bovine serum albumin; rA·dT, poly(rA)·oligo(dT_{12-18}); AZT, azidothymidine; IPTG, isopropyl-β-D-thiogalactopyranoside; EGTA, ethylene glycol bis (β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; dA·dT, poly(dA)·oligo(dT).



α-Rubromycin



β- Rubromycin



γ- Rubromycin

Fig. 1. Structural formulas of α-rubromycin, β-rubromycin, and γ-rubromycin.

ton X-100, DTT, lysozyme (from chicken egg white-type 1), and BSA (fraction V) from Sigma Chemical Co. (St. Louis MO); rA·dT and TTP from Pharmacia (Piscataway, NJ); DE-52 and P11 from Whatman (Hillsboro, OR); dA·dT from Supertechs (Rockville, MD); AMV RT from Molecular Genetic Resources (Tampa, FL); Aquassure cocktail from Dupont (Wilmington, DE); glycerol from Fisher (Pittsburgh, PA); and dialysis tubing (molecular weight 8000) from Spectrum Medical Industries (Los Angeles, CA). All other supplies were of the highest grade available.

Expression/purification of recombinant HIV-1 RT. The region of the HIV-1 genome (NY-5 isolate) encoding the *pol* gene was inserted into a plasmid derived from pKK223 (A63-16a) and transformed into lon-1 *Escherichia coli* (16). Based upon DNA sequence and Western blot analyses, this clone expresses the full length authentic RT sequence. Starter cultures (50 ml of Luria broth containing 100 μg/ml ampicillin) were grown overnight at 37° with 250 rpm agitation. Four cultures each 300 ml, were inoculated with 6 ml of the starter culture. After 4 hr, IPTG was added to a final concentration of 500 μM and the cultures were grown overnight at 37° with 250 rpm agitation. The cells were then centrifuged at 4000 × *g* for 10 min and the supernatant fluids were replaced with lysis buffer (30 ml/300 ml culture; 50 mM Tris·HCl, pH 7.8, 4 mM EDTA, 1 mM sodium chloride, 48 μg/ml aprotinin, 10 μg/ml, phenylmethylsulfonyl fluoride, and 1 g/100 ml lysozyme). After incubation at 0° for 15 min, Triton X-100 was added to a final concentration of 1% and incubated for another 15 min on ice. The preparation was then sonicated for two 1-min periods. DTT was added to a final concentration of 4 mM. The preparation was then centrifuged at 10,000 × *g* for 60 min. The supernatant fluid was removed and adjusted to 50% saturation with ammonium sulfate. The solution was stirred for 30 min in an ice bath and centrifuged at 30,000 rpm for 120 min (Ti60 rotor, Beckman L8-80 centrifuge). The resulting pellets were resuspended in column buffer [50 mM Tris·HCl, pH 7.7, 50 mM potassium chloride, 1 mM DTT, 0.2 mM EDTA, 0.1% Triton X-100 (v/v), 20% glycerol (v/v), and 0.2 mM PMSF] and dialyzed overnight against column buffer. The dialyzate was applied to a DE-52 column (1 × 20 cm) that was preequilibrated in column buffer. Using a flow rate of 0.5 ml/min, 2.5-ml fractions were collected. Peak fractions possessing HIV-1 RT activity (see below) were pooled and applied to a P11 phosphocellulose column (1 × 20 cm) that was preequilibrated in column buffer. After 40 min at 0.5 ml/min, a linear gradient of 0.05–0.5 M potassium chloride (200 ml) was run. Typically, HIV-1 RT activity eluted at 0.21–0.24 M potassium chloride. This material was divided into aliquots and stored at −20° in the presence of 10 mg/ml BSA.

HIV-1 RT assay. The final concentrations of the components of the reaction mixture (50 μl in Skatron tube strips) were 55 mM Tris·HCl, pH 8.2, 80 mM potassium chloride, 12 mM magnesium chloride, 1 mM DTT, 50 μM EGTA, 2.5 μg/ml rA·dT, 0.5 μCi of [^{3}H]TTP, 10 μM TTP, 1 mg/ml BSA, and 0.01% Triton X-100. Reactions were initiated upon addition of enzyme (2 units; 1 unit = 10 pmol of [^{3}H]TMP incorporated/60 min at 37°, using the above conditions) and placement of racks in a water bath maintained at 37°. At the completion of the 60-min incubation, the racks were placed in an ice-water bath and 200 μl of 13% trichloroacetic acid/10 mM sodium pyrophosphate were added to each tube. After 30 min, the acid-precipitable material was collected on filters (presoaked in 13% trichloroacetic acid/10 mM sodium pyrophosphate) using a Skatron semiautomatic cell harvester. The filters were washed with 1 M hydrochloric acid/10 mM sodium pyrophosphate (5.4 ml/filter). The filters were punched into Skatron scintillation vials, and 2 ml of Aquassure cocktail were added to each vial. Radioactivity was determined by liquid scintillation counting.

AMV RT assay. The conditions were the same as for the HIV-1 RT assay, except that 35 μM TTP, 2 μg/ml rA·dT, and 0.05 units of AMV RT were used (1 unit is the amount of enzyme activity that incorporates 1 nmol of [^{3}H]TMP into acid-insoluble products in 10 min at 37°).

DNA polymerase α assay. The DNA polymerase α assay was carried out as described for the HIV-1 RT assay, except that the final components of the reaction mixture were 55 mM Tris·HCl, pH 8.2, 0.5 mM manganese chloride, 1 mM DTT, 0.5 μCi of [^{3}H]TTP, 7 μM TTP, 15 μM deoxyadenosine, 1.5 μM deoxythymidine; 0.01% Triton X-100, and 1 mg/ml BSA. Immunopurified DNA polymerase α was prepared as described by Chang *et al.* (17).

Cell-based toxicity and infectivity assays. H9 human T lymphoid cells were maintained in RPMI 1640 medium containing 10% fetal bovine serum. The test compounds were dissolved in dimethyl sulfoxide and diluted to the appropriate concentrations in medium. Cells were treated with the compounds (0.78–6 μM) for 24 hr before infection. Infection was carried out at a multiplicity of infection (determined by dilution method) of 0.01, using the IIIb isolate of HIV-1. Fresh compound was added at the time of infection and every 2–3 days thereafter. Spread of virus through the culture was assessed by virus-specific immunofluorescence, using anti-HIV-1 antiserum. Toxicity controls on uninfected cell cultures were performed in parallel. Cell viability was monitored by trypan blue exclusion.

Fermentation conditions. Agar slants of pure cultures (HCA87), were used to prepare frozen vegetative mycelium in seed medium. Seed medium contained (in g/liter of distilled water): dextrose, 2.0; dextrin,

10.0; beef extract, 3.0; Ardamine pH, 5.0; NZ-Amine E, 5.0; $MgSO_4 \cdot 7 H_2O$, 0.050; K_2HPO_4, 0.37; and $CaCO_3$, 0.5. No pH adjustment was required. The medium was dispensed at 54 ml/250-ml three-baffle Erlenmeyer flask. Cotton closures were used. The medium was sterilized at 121° for 20 min.

Seed cultures of HCA87 were inoculated with the culture and grown on a gyratory shaker (220 rpm; 5.1-cm throw) for 48 hr at 27°. The seed cultures were used to inoculate production medium which contained (in g/liter of distilled water): corn gluten meal, 5.0; Primatone, 2.5; sucrose, 5.0; malt extract, 10.0; yeast extract, 1.0; $MgHPO_4 \cdot 3 H_2O$, 0.5; and $CaCO_3$, 5.0. Calcium carbonate was added after the presterile pH was adjusted to 7.0–7.2. The medium was dispensed at 54 ml/250-ml three-baffle Erlenmeyer flask. Cotton closures were used. The medium was sterilized for 20 min at 121°. A portion of the seed culture (1.5 ml) was used to inoculate each production flask. Production cultures were incubated on a gyratory shaker, as described above, for 4–5 days at 27°.

Isolation/purification of rubromycins. β- and γ-rubromycins were isolated from the mycelia of HCA87. Cultured broth (3.0 liters) was filtered with the aid of Supercel and the mycelial cake was extracted twice with 1.5-liter portions of acetone. The combined extracts were concentrated to an aqueous suspension, adjusted to pH 3.0 with 1 N HCl, and extracted three times with methylene chloride. The extracts were dried to a deep red residue (756 mg) and half of the material was applied to a silica gel column (Kieselgel 60, 230–400 mesh), which was developed with 96:4:0.1 methylene chloride/methanol/acetic acid. Fractions containing β- and γ-rubromycin were separately pooled and each was further purified by preparative thin layer chromatography on silica gel that was developed in 95:5:0.1 methylene chloride/methanol/acetic acid. Pure β-rubromycin (R_F = 0.55, 38 mg) and pure γ-rubromycin (R_F = 0.76, 8 mg) were thus obtained.

β-Rubromycin was subjected to mass spectral analysis (Finnegan MAT-212). The molecular ion was observed at m/z 536 and was found by high resolution mass spectrometry (peak-matching-method with perfluorokerosene as the internal standard) to correspond to an empirical formula of $C_{27}H_{20}O_{12}$ (found, 536.0949; calculated, 536.0955). Proton NMR (Varian XL-300) data for both rubromycins were identical to previously reported values (18, 19).

α-Rubromycin was obtained by heating β-rubromycin in refluxing pyridine for 15 min, as previously described (19). Analytical data (UV, mass spectral, and ^1H NMR) were consistent with published information (19).

Data analysis. Experiments were carried out in triplicate and results were expressed as the mean ± standard error of at least three experiments. Data were analyzed according to the standard procedures of Lineweaver and Burk (20); Ackermann and Potter (21), and Dixon (22).

Results

Inhibition of polymerases. The effects of each rubromycin on viral and cellular polymerases were characterized. β-Rubromycin inhibited recombinant HIV-1 RT activity in a concentration-dependent manner, with an IC_{50} value of 0.98 μM and a selectivity of 19-fold over calf thymus DNA polymerase α (Fig. 2A; Table 1). γ-Rubromycin was slightly more potent than β-rubromycin (IC_{50} = 0.62 μM) but only 9-fold selective versus DNA polymerase α (Fig. 2B; Table 1). The Hill coefficients for inhibition of HIV-1 RT by β- and γ-rubromycin in these experiments were 1.12 ± 0.06 and 1.22 ± 0.05, respectively, using rA·dT as the template·primer. When β-rubromycin titration studies were carried out in the presence of calf thymus DNA, which would act as a scavenger for intercalators, there was no significant change in potency (results not shown). Neither of the agents showed a significant selectivity for one viral polymerase over the other (Fig. 2; Table 1). One other

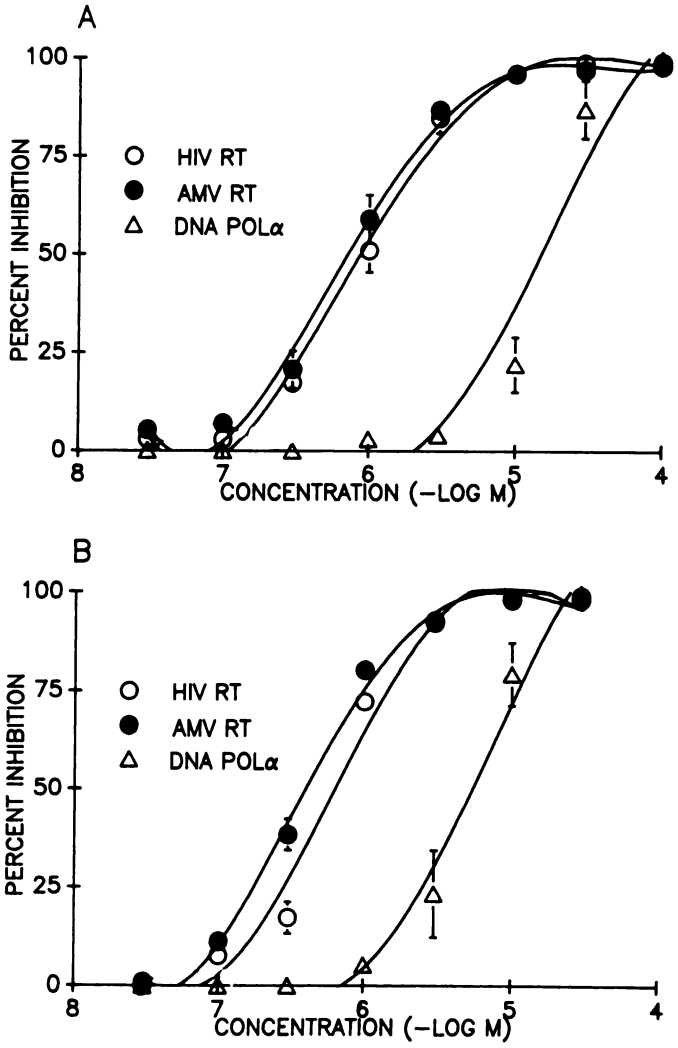


Fig. 2. Inhibition of polymerase activities by β-rubromycin (A) or γ-rubromycin (B). Polymerase assays were carried out in the presence of dimethyl sulfoxide, β-rubromycin, or γ-rubromycin at the indicated concentrations. At the completion of the incubation, trichloroacetic acid/sodium pyrophosphate was added to each tube. After 30 min, the acid-precipitated material was collected by filtration and radioactivity was determined. Values represent the mean ± standard error of three separate experiments, each carried out in triplicate. *POLα*, polymerase α.

natural rubromycin, α-rubromycin (Fig. 1), was a significantly weaker HIV-1 RT inhibitor and was not pursued further (Table 1).

Analysis of enzyme inhibition. When enzyme activity was measured as a function of the HIV-1 RT concentration in the presence of various concentrations of β- or γ-rubromycin, Ackermann-Potter plots resulted in curves intersecting at the origin, as would be expected for reversible inhibitors (Fig. 3). Further, double-reciprocal plot analysis demonstrated that both β- and γ-rubromycin were competitive inhibitors with respect to the rA·dT template·primer (Fig. 4). These agents inhibited HIV-1 RT noncompetitively at the TTP site (results not shown). In similar studies using DNA polymerase α instead of HIV-1 RT, β-rubromycin and γ-rubromycin were, likewise, competitive inhibitors with respect to the dA·dT template·primer and noncompetitive inhibitors with respect to TTP (results not shown).

Based upon the demonstration of competitive inhibition by

TABLE 1
Summary of polymerase inhibition data

Rubromycin	IC_{50}				K_i		
	HIV RT	AMV RT	DNA polymerase α	Selectivity (DNA/HIV)	HIV RT	DNA polymerase α	Selectivity (DNA/HIV)
	μM				μM		
β	0.98 ± 0.16	0.79 ± 0.14	18.6 ± 6.2	19	0.27 ± 0.04	25.1 ± 4.3	93
γ	0.62 ± 0.01	0.41 ± 0.04	5.5 ± 1.2	9	0.13 ± 0.01	3.9 ± 0.6	30
α	116 ± 16		149				

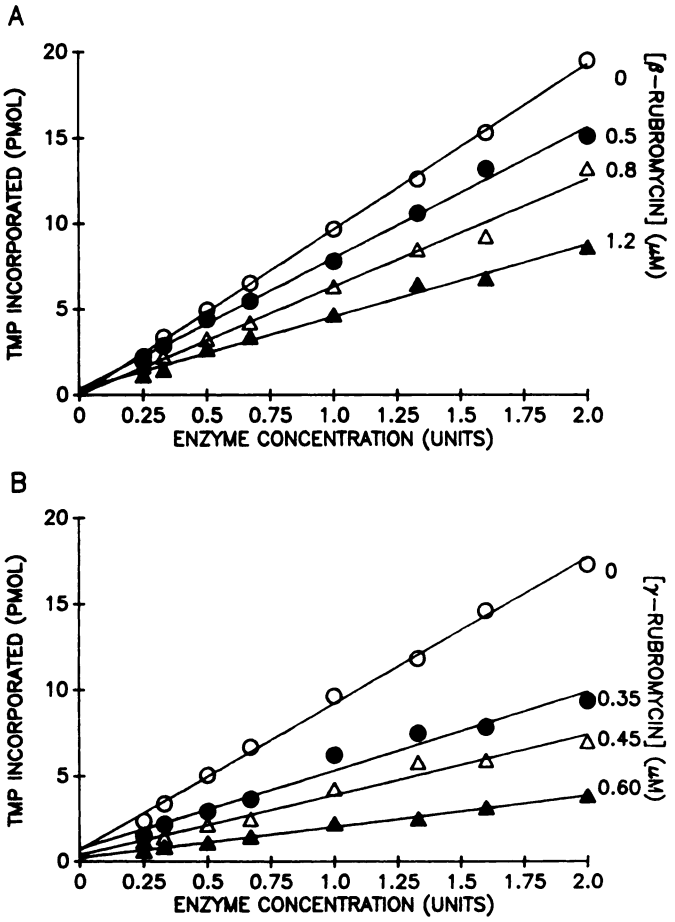


Fig. 3. Inhibition of HIV-1 RT by β-rubromycin (A) or γ-rubromycin (B) as a function of enzyme concentration: Ackermann-Potter plots. TMP incorporation into acid-precipitable material was measured as a function of the HIV-1 RT concentration at 0–1.2 μM β-rubromycin (A) or 0–0.6 μM γ-rubromycin (B). Results represent the mean of triplicate determinations of a representative experiment.

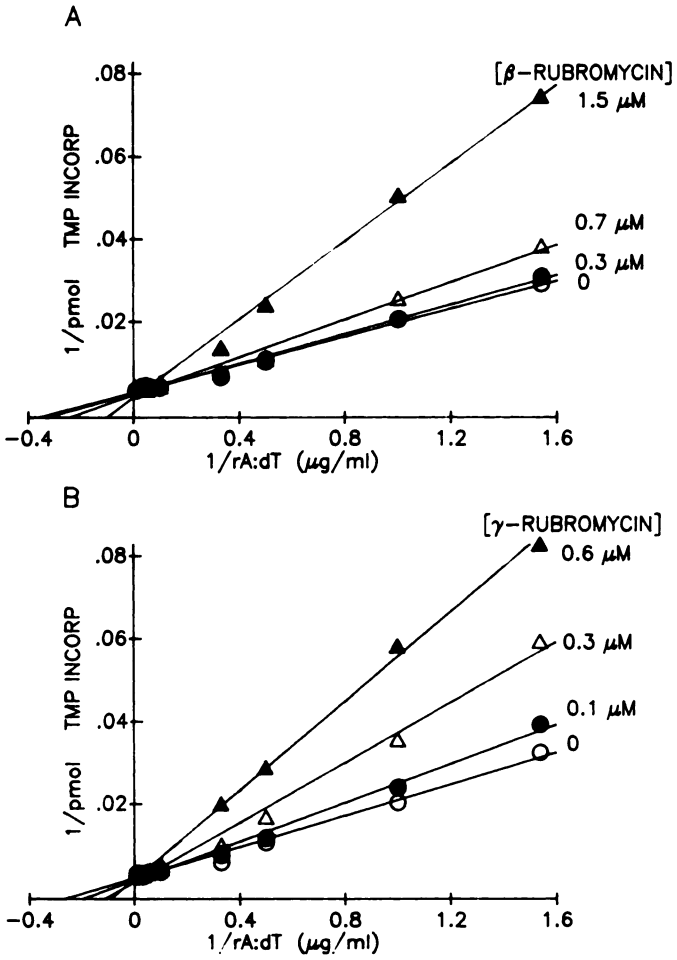


Fig. 4. Lineweaver-Burk analysis of the inhibition of HIV-1 RT by β-rubromycin (A) or γ-rubromycin (B) as a function of the template·primer concentration. Inhibition studies were carried out at 0–1.5 μM β-rubromycin (A) or 0–0.6 μM γ-rubromycin (B) at different rA·dT concentrations. Values represent the mean of triplicate determinations from a representative experiment.

each rubromycin with template·primer, dose-response studies were carried out at several template·primer concentrations (Dixon analyses). The K_i values for HIV-1 RT with rA·dT were 0.27 ± 0.041 and 0.13 ± 0.012 μM for β- and γ-rubromycin, respectively (Fig. 5; Table 1). Similarly, the β- and γ-rubromycin K_i values for DNA polymerase α with dA·dT were 25.1 ± 4.3 and 3.9 ± 0.6 μM, respectively. As found with the IC_{50} studies, the selectivity (DNA polymerase α/HIV-1 RT) was greater for β- than γ-rubromycin (Table 1).

Cell-based toxicity and HIV-1 infectivity studies. As a preface to determining the potential antiviral activity of each of the rubromycins, the compounds were tested for toxicity to noninfected H9 human T lymphoid cells in culture. In the range of 0.04–6 μM, the rubromycins did not affect cell viability (>94% of the cells excluded trypan blue). At concentrations above 6 μM, there was a significant decrease in cell viability over the 10-day culture period. Hence, in the virus inhibition assay using HIV-1, concentrations of 0.78–6 μM were used. The results demonstrated that, compared with dimethyl sulfoxide controls, neither β- or γ-rubromycin reduced viral infectivity at 3, 6, or 8 days after infection.

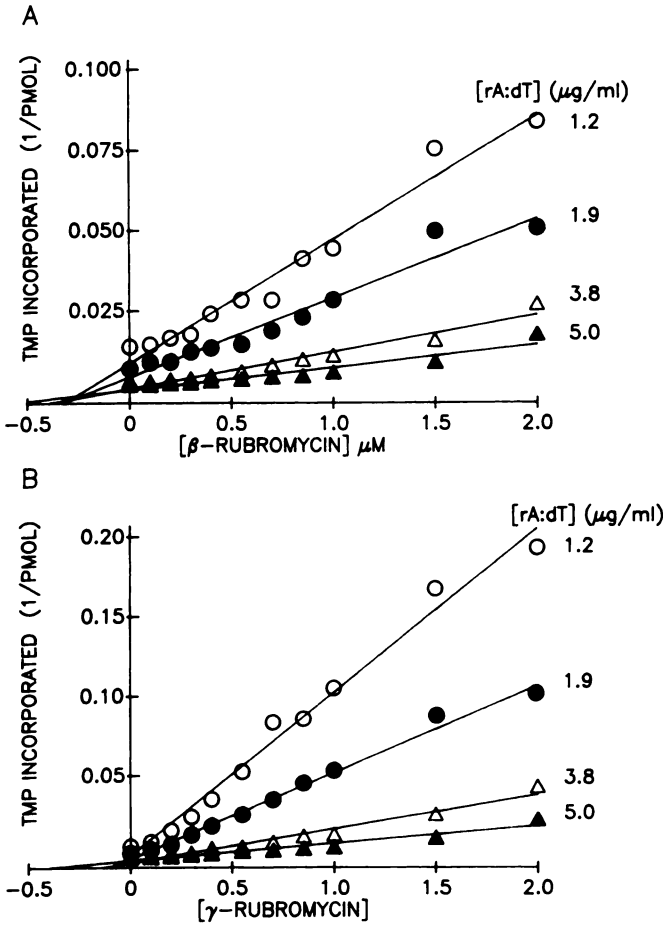


Fig. 5. Dixon analysis of the inhibition of HIV-1 RT by β-rubromycin (A) or γ-rubromycin (B). Dose-response curves were carried out at rA·dT concentrations of 1.2–5.0 μg/ml. Values represent the mean of triplicate determinations from a representative experiment.

Discussion

Ideal RT inhibitors should interact directly with the enzyme, preferably in a reversible manner, and show significant selectivity for retroviral versus cellular enzymes. Clinically effective nucleoside analogs, such as AZT and dideoxyinosine, do show more than 100-fold selectivity for RT-induced polymerization over cellular DNA polymerase α-induced polymerization (3, 9). These agents, however, inhibit the "process" of reverse transcription by causing chain termination rather than by directly inhibiting the enzyme. Although the chain terminators show sufficient selectivity versus DNA polymerase α, these agents significantly inhibit DNA polymerase β and DNA polymerase γ (23, 24). Recent studies suggest that the dose-limiting chain terminator-induced peripheral neuropathy may be mediated through the inhibition of DNA polymerase γ (23, 25). Because the clinical utilities of RT inhibitors, such as suramin and phosphonoformate, have not been clearly demonstrated (26, 27), further efforts to identify new RT inhibitors are warranted.

Based upon the demonstration that certain quinone antibiotics inhibit RNA-dependent DNA polymerases and possess antiviral activities (11, 12), a series of quinones was evaluated for selective RT-inhibitory properties. β- and γ-rubromycins were found to inhibit HIV-1 and AMV RTs in a concentration-dependent manner, with K_i values significantly less than 1 μM. In comparison with other natural quinone antibiotics, these agents appear to be the most potent inhibitors identified thus far. Direct comparisons, however, are difficult because studies from other laboratories used only AMV RT and/or were carried out with one or two inhibitor concentrations, so that accurate potency values cannot be determined (11–14). β- and γ-rubromycins were 92- and 30-fold weaker, respectively, as inhibitors of cellular DNA polymerase α.

The mechanism of RT inhibition by the rubromycins was different from that reported for streptonigrin. Whereas streptonigrin inhibited RT activity uncompetitively with respect to template·primer (13, 28), β- and γ-rubromycin were competitive. Streptonigrin (13) and the rubromycins inhibited enzyme activity noncompetitively with respect to TTP.

In our experience, agents typically inhibit the spread of HIV-1 in culture at concentrations 100- to 1000-fold above their potencies in the biochemical assays. Because β- and γ-rubromycin reduced the viability of noninfected H9 T lymphoid cells at concentrations of 6 μM and above, rubromycin concentrations of 0.8–6 μM were used. At these concentrations, however, there was no reduction of virus infectivity. Possible reasons for the lack of antiviral activity at 6 μM include 1) poor penetration of the compound into the cell, 2) breakdown of the compound in the culture medium, and 3) high concentrations of template·primer overcoming the inhibition of RT activity by the rubromycins.

In summary, the present studies demonstrate that β- and γ-rubromycins selectively inhibit the enzyme activities of retroviral polymerases over cellular DNA polymerase α through competitive interaction with the template·primer site. The rubromycins were cytotoxic to human T lymphoid cells in culture, so antiviral properties could not be demonstrated. These studies support the hypothesis that certain quinone antibiotics interact with a unique site on the enzyme molecule (13) and suggest that structural modifications of these agents may improve potency and selectivity.

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