Inhibition of Human Telomerase by Rubromycins: Implication of Spiroketal System of the Compounds as an Active Moiety

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ABSTRACT: We found that a group of rubromycins and their analogues, a class of quinone antibiotics that possesses benzofuran and benzodipyran rings to form a spiroketal system, strongly inhibited human telomerase as assessed with a modified telomeric repeat amplification protocol. β- and γ-Rubromycins and purpuromycin appeared to be the most potent telomerase inhibitors, with 50% inhibitory concentrations (IC_{50}) of about 3 μM, and griseorhodins A and C also showed comparable potencies for the inhibition (IC_{50} = 6−12 μM). In contrast, opening of the spiroketal system of β-rubromycin, giving rise to α-rubromycin, substantially decreased its inhibitory potency toward telomerase (IC_{50} > 200 μM), indicating the essential role of the spiroketal system in telomerase inhibition. A kinetic study of the inhibition by β-rubromycin revealed a competitive interaction with respect to the telomerase substrate primer, with a K_i of 0.74 μM, whereas a mixed type inhibition was observed with respect to the nucleotide substrate. β-Rubromycin was also potent in inhibiting retroviral reverse transcriptases but had virtually no effect on other DNA/RNA-modifying enzymes including DNA and RNA polymerases, deoxyribonuclease, and topoisomerase. Although β-rubromycin showed nonspecific cytotoxicities, reducing proliferation of cancer cells (IC_{50} ~ 20 μM), we conclude that β-rubromycin appears to be a lead structure for the development of more potent and selective inhibitors of human telomerase.

Since conventional DNA synthesis fails to copy the 3′-termini of dsDNA during chromosomal replication, successive cellular divisions lead to progressive loss of the telomeres, the large DNA−protein complexes that cap linear eukaryotic chromosomes. A reduction in the telomere length leads to genomic instability and aberrant chromosome fusion events, consequently resulting in a reduction in cellular longevity. Telomerase, a unique ribonucleoprotein enzyme, compensates for this underreplication by the de novo addition of simple guanine-rich sequence repeats to the end of the telomere. Telomerase activity is not detectable in most somatic cells but is upregulated in germ line cells and in most cancer cells in humans. If telomerase activity is a requirement for the continued growth of cancer cells, it would provide a potential target for cancer therapy (see reviews in refs *1*−*4*).

Development of increasingly specific inhibitors of human telomerase requires structural information about the enzyme. Recently, a catalytic subunit of telomerase was identified and cloned from protozoa, yeast, and humans (*5*−*9*). All seven previously defined reverse transcriptase (RT)[1] motifs have been identified in these telomerase RTs (TERTs), and the importance of some of these RT motifs for telomerase activity has been shown by mutagenesis, indicating that telomerase has a protein structure and function related to those of other RTs (see ref *10* for a review). To obtain lead structures for designing specific inhibitors of telomerase, the testing of the inhibition potency of compounds that have been shown to inhibit functionally related enzymes is an alternative strategy. For instance, a nucleoside analogue inhibitor of retroviral RTs was shown to cause progressive telomere shortening in immortalized human cell lines in vitro (*11*). A number of quinone antibiotics, such as streptonigrin, sakyomycin A, and rubromycins, also were demonstrated to inhibit retroviral RTs (*12*−*14*). In the present study, we examined the abilities of rubromycins and their analogues (Figure 1), a unique class of compounds containing benzofuran and benzodipyran rings to form a spiroketal system (*15*), to inhibit human telomerase. As a result, we found their spiroketal system to be a possible active moiety in telomerase inhibition.

EXPERIMENTAL PROCEDURES

Production, Isolation, and Purification of Rubromycins and Their Analogues. A strain of *Streptomyces* species (designated as 2202) isolated from a local field in Tateba-

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[1] Abbreviations: IC_{50}, 50% inhibitory concentration; RT, reverse transcriptase; TERT, telomerase RT; dNTP, deoxynucleoside triphosphate; HIV-1, human immunodeficiency virus type 1; M-MLV, moloney murine leukemia virus; Topo-1, topoisomerase I; TR, telomerase RNA.

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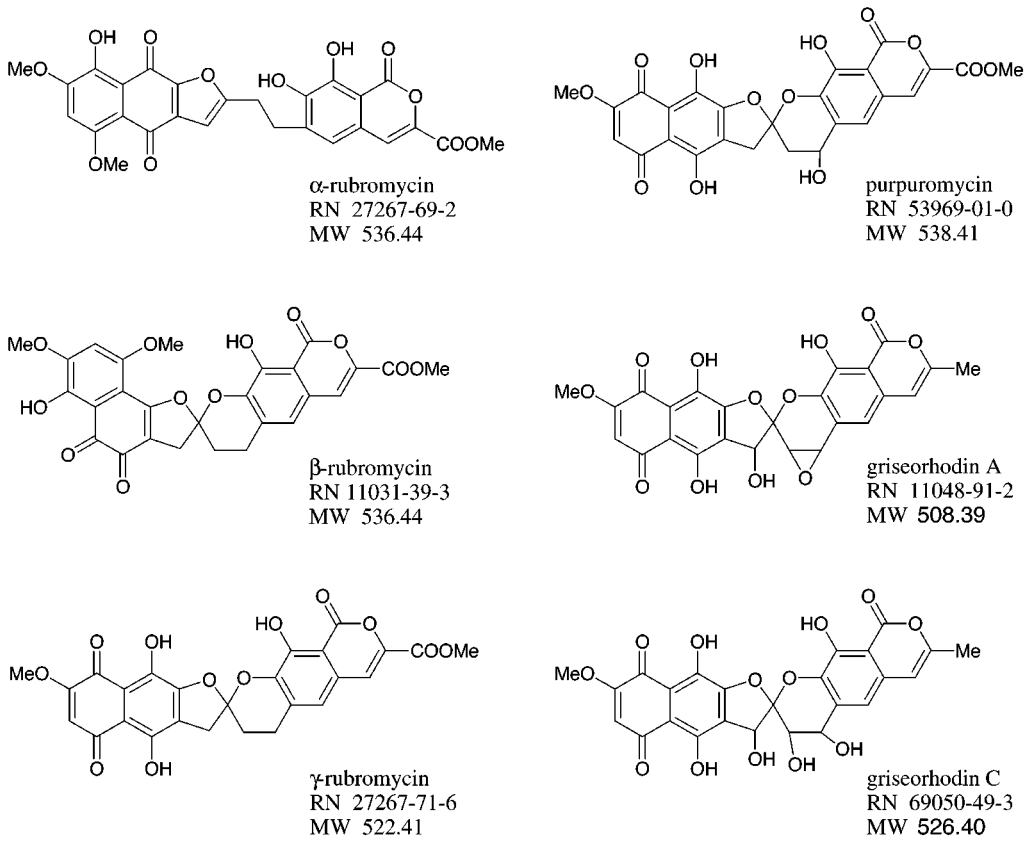

FIGURE 1: Structures of compounds used in this study. Registry number (RN) and molecular weight (MW) of each of the compounds are also shown.

yashi City, Gunma, Japan, was used to produce β- and γ-rubromycins (*16, 17*), whereas *Actinoplanes ianthinogenes* JCM3249 and *Streptomyces californicus* JCM6910 obtained from the Japan Collection of Microorganisms were used to produce purpuromycin (*18*) and griseorhodin A (*19*), respectively. A mature slant of either of these strains was inoculated into 5 mL of SG medium (2% soluble starch, 1% glucose, 1% Bacto Soytone, 0.1% NaCl, 0.1% K_2HPO_4, 0.05% $MgSO_4 \cdot 7H_2O$, and 0.2% $CaCO_3$, pH 7.3) for strain 2202 or into the same volume of AF/MS medium (2% glucose, 0.8% soybean meal, 0.2% yeast extract, 0.1% NaCl, and 0.4% $CaCO_3$, pH 7.3) (*20*) for JCM3249 and 6910 strains. After the culture had been shaken at 280 rpm and 30 °C for 2 days, a portion of the seed culture was transferred into each of several 500-mL baffled shake flasks, each containing 100 mL of the corresponding medium, and was cultured for 5 or 6 days at 30 °C. The mycelial cakes were then collected, washed with acetone, and extracted with ethyl acetate under acidic conditions. The resulting organic extract, red in color, was then applied to a silica gel column, which was developed with a 95:5:0.1 mixture of methylene chloride/methanol/acetic acid. Fractions containing each of the target compounds were pooled and concentrated to dryness on a rotary evaporator and then further purified by reversed-phase, high-performance liquid chromatography (HPLC) on an apparatus equipped with a preparative C_{18} column (Waters) operated with a mobile phase of acetonitrile/H_2O containing 0.1% acetic acid. Purities of β- and γ-rubromycins, purpuromycin, and griseorhodin A thus prepared were 85, 88, 98, and 98%, respectively, as assessed by analytical HPLC.

α-Rubromycin was obtained by heating β-rubromycin in refluxing pyridine for 15 min as previously described (*17*) followed by purification by preparative thin-layer chromatography and recrystallization. Griseorhodin C was obtained by incubating griseorhodin A in trifluoroacetic acid for 16 h at room temperature in the dark, as previously described (*21*), and was purified by silica gel column chromatography followed by HPLC as above.

Analytical data (UV, mass, and ^{1}H NMR spectra) obtained for all compounds prepared in this study were consistent with previously published information (*17, 21, 22*). Carbon NMR spectra of β- and γ-rubromycins were also analyzed and were identical to previously published values (*16, 17*).

Enzymatic Assay for Human Telomerase. K-562 cells grown in RPMI 1640 with 10% fetal calf serum (Irvine Scientific, Inc.) were collected, washed with phosphate-buffered saline (PBS), and suspended at a cell density of 10^7/mL in a lysis buffer containing 10 mM Tris-HCl (pH 7.5), 1 mM $MgCl_2$, 1 mM ethylene glycol bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid (EGTA), 10% glycerol, 5 mM 2-mercaptoethanol, and 0.1 mM 4-(2-aminoethyl)-benzenesulfonyl fluoride hydrochloride. Cell-free lysates were then prepared by freeze−thaw cycles, as previously described (*23*), and were used to measure telomerase activity.

Telomerase activity was determined by employing the telomeric repeat amplification protocol (TRAP) as described by Kim et al. (*24*) with modifications. The reaction mixture, containing 20 mM Tris-HCl (pH 8.3), 68 mM KCl, 1 mM EGTA, 0.05% Tween 20, 0.5 μM T4 gene 32 protein (Boehringer Mannheim), 8 μg/mL phage MS2 RNA (Boehringer Mannheim), 100 μg/mL bovine serum albumin (BSA), and K-562 lysate equivalent to 5000 cells, was preincubated for 10 min at room temperature in the presence or absence of various amounts of test compounds that had

been dissolved in dimethyl sulfoxide (DMSO). The telomerase reaction was then initiated by the addition of a 200 μM concentration of each of the four deoxynucleoside triphosphates (dNTPs) and a modified telomerase substrate primer, TS-A (5′-aaaaaaaaaaaaaatccgtcgagcagagtt-3′), in a total volume of 50 μL, and the mixture was subsequently incubated for 30 min at room temperature. After the reaction had been quenched by the addition of 20 μL of 0.1 M ethylenediaminetetraacetic acid (EDTA), pH 8.0, containing 20 μg/mL glycogen, TS-A oligonucleotides elongated by telomerase were extracted sequentially in phenol/chloroform/isoamyl alcohol and chloroform/isoamyl alcohol and then precipitated in ethanol. Precipitated materials were suspended in a mixture containing 20 mM Tris-HCl (pH 8.3), 68 mM KCl, 1 mM EGTA, 0.05% Tween 20, 100 μg/mL BSA, a 200 μM concentration of each dNTP, 0.5 unit of *Taq* polymerase, and 0.5 μM ACX primer (5′-gcgcggcttaccctacccttaccctaacc-3′) (*25*) with a fluorescent phosphoramidite, 6-FAM (PE Biosystems), at its 5′-end (ACX-FAM) and were then incubated at 94 °C for 30 s and at 60 °C for 45 s for 27 cycles to amplify the telomerase products. Resultant PCR-amplified telomerase products (1 μL) were mixed with a fluorescent size standard (1 μL of GS-350 ROX, PE Biosystems) and 20 μL of deionized *N*,*N*-dimethylformamide (DMF), denatured at 96 °C for 2 min, and then separated and visualized by capillary electrophoresis (Genetic Analyzer 310, PE Biosystems). The amounts of the products were integrated and quantified by a computer software, GeneScan Analysis 2.1 (PE Biosystems). Of note, the extraction step prior to PCR allowed us to amplify the telomerase product without any effect of the test compounds on PCR (see Results).

Enzymatic Assays for Other DNA/RNA-Modifying Enzymes. PCR buffers and supplements in the reaction mixture as well as the conditions for PCR were identical to those used in the modified TRAP assay as described above, with the exception that 100 copies of *Pvu*II-digested pBC KS+ plasmid (Stratagene) was used as a template DNA, and T7 (5′-taatacgactcactataggg-3′) and KS (5′-tcgaggtcgacggtatc-3′) were used as primers. A fluorescent phosphoramidite, HEX (PE Biosystems), was used to endlabel KS at its 5′-end (KS-HEX) to visualize the amplified products fluorescently.

Reverse Transcriptases. Activities of RTs of human immunodeficiency virus type 1 (HIV-1) and moloney murine leukemia virus (M-MLV) were determined by the DE-81 filter binding assay as previously described (*26*, *27*). The reaction mixture contained 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 3 mM $MgCl_2$, 100 μg/mL BSA, 1 μM poly(rA)·$(dT)_{12-18}$ (Amersham Pharmacia Biotech), 10 μM [^{3}H]-dTTP (Moravek), and HIV-1 (NEN) or M-MLV RT (Life Technologies). After a 20-min incubation at room temperature, the reactions were quenched by the addition of EDTA at a final concentration of 20 mM. Reaction products were collected on a DE-81 filter (Whatman), and the incorporated [^{3}H]-dTMP was quantified by a scintillation counter as described (*26*).

Type I Human Topoisomerase. Topoisomerase I (Topo-1) activity was determined with a Topoisomerase I assay kit using purified human Topo-1 (TopoGEN, Inc.) under conditions as suggested in the manufacturer's protocol. Briefly, the reaction mixture (25 μL) contained 10 mM Tris-HCl (pH 7.9), 1 mM EDTA, 150 mM NaCl, 0.1% BSA, 0.1 mM spermidine, 5% glycerol, 0.25 μg of supercoiled substrate DNA, and 1 unit of Topo-1 in the presence or absence of the test compounds dissolved in DMSO. After a 15-min incubation at room temperature, the reactions were quenched by the addition of 6 μL of a dye solution (5% Sarkosyl, 0.125% bromophenol blue, and 25% glycerol). The relaxed forms of circular DNA generated by Topo-1 were then separated in a agarose gel with ethidium bromide, and the band intensities were integrated by densitometric analysis.

Other Enzymes. Enzymatic activities of DNA polymerases of plant, *Escherichia coli*, and T4 phage, as well as those of T7 RNA polymerase and calf thymus DNase I, were determined as previously described (*28*, *29*). In brief, the template−primer and the nucleotide substrate used for DNA polymerases were 5 μM poly(dA)·$(dT)_{12-18}$ and 10 μM [^{3}H]-dTTP, respectively. For all assays, reaction products were collected on DE-81 filters as described above.

Cell Growth Inhibition Assay. Effects of the test compounds on proliferation of cancer cells were assessed by use of a [^{3}H]-thymidine incorporation assay, as described earlier (*29*). Briefly, K-562 or HeLa cells were maintained in an RPMI 1640 medium or in a minimum essential medium, respectively, supplemented with 10% fetal calf serum at 37 °C in 5% CO_2 atmosphere. Cells were collected and plated on a 96-well plate at a density of 5000 cells/well and then exposed to test compounds for 3 or 4 days for K-562 or HeLa cells, respectively. Afterward, [^{3}H]-thymidine (Amersham Pharmacia Biotech) was added to each well, and the plate was incubated for an additional 4 h. Cells were then harvested on glass fiber filters, and the filters were extensively washed with distilled water. Incorporated radioactivities that were retained on the glass filters were counted by a liquid scintillation counter.

RESULTS

Telomerase Assay. One of the most serious drawbacks of the telomerase assay employing the TRAP method is when a sample contains an agent that has the ability to block the PCR amplification step. Rubromycins and their analogues (see Figure 1 for structure) examined in this study appeared to have inhibitory activity toward PCR (see below; Figure 2). To evaluate the inhibitory potency of these compounds toward telomerase, we sought to remove the PCR-inhibiting compounds by applying chloroform extraction followed by precipitation of the telomerase products prior to amplification of the telomerase products by PCR. As shown in Figure 2, this extraction step completely eliminated the effect of β-rubromycin on the PCR amplification step and thereby enabled us to define its inhibition potency toward telomerase. This simple extraction was also effective for eliminating PCR interference by the other compounds examined in this study (data not shown).

Employing this modified TRAP assay along with the 5′-end fluorescence-labeled return primer (ACX-FAM) and capillary electrophoresis, we observed amplified DNA fragments at 6-base intervals (Figure 3). Pretreatment of the cell lysate with RNase A or protease K, or the addition of EDTA prior to initiation of the reaction by the addition of dNTPs, produced no elongated products (Figure 3), indicating that the products formed without any pretreatment were the result

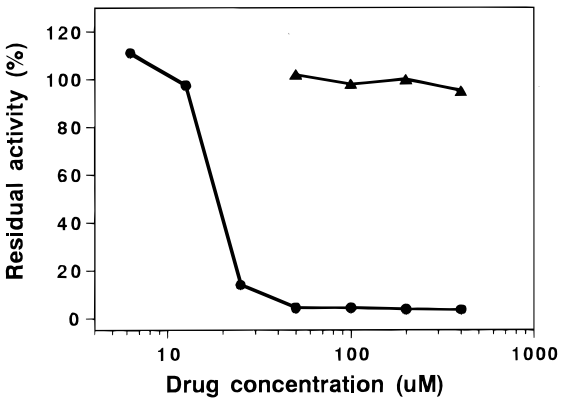

FIGURE 2: Effect of chloroform extraction on PCR. A mixture containing 20 mM Tris-HCl (pH 8.3), 68 mM KCl, 1 mM EGTA, 0.05% Tween 20, 100 μg/mL BSA, 200 μM concentration of each dNTP, 0.4 μg of glycogen, 0.5 unit of *Taq* polymerase, 100 copies of *Pvu*II-digested pBC KS+ plasmid, and 0.1 μM concentration of each of T7 (5′-taatacgactcactataggg-3′) and KS-HEX primers (5′-HEX-tcgaggtcgacggtatc-3′) in the presence or absence of the indicated concentrations of β-rubromycin was subjected to the thermal cycling reaction to assess the ability of β-rubromycin to inhibit PCR amplification (●). An identical mixture containing β-rubromycin was subjected to extraction with phenol/chloroform/isoamyl alcohol and chloroform/isoamyl alcohol, followed by precipitation of DNAs in ethanol to isolate DNAs from the compound. Resultant DNA was suspended in the identical mixture in the absence of the compound, and then the thermal cycling reaction was conducted (▲). Note that the IC_{50} value determined from the graph is given in Table 2.

of an enzymatic reaction requiring ribonucleoprotein and divalent cations. In addition, the amounts of integrated products were proportional to time (Figure 3), to the amount of cell lysate added to the reaction (Figure 4A), and to the concentrations of both substrates, TS-A (Figure 4B and 5A) and dNTPs (Figure 5B). When the data obtained for the telomerase activity as a function of the concentration of each of the substrates were applied to the conventional Michaelis−Menten equation, apparent K_m values of 0.22 ± 0.087 and 2.84 ± 1.2 μM for TS-A and dNTPs, respectively, were obtained (Figures 4B and 5). It should be noted that these K_m values of telomerase were in good agreement with those determined by Hayakawa et al. (K_m = 0.18 and 1.7 μM for the TS primer and the dNTPs, respectively) (*30*), confirming our modified TRAP assay to be suitable for this study.

Effect of Rubromycins and Their Analogues on Telomerase Activity. We then examined the abilities of rubromycins and their analogues to inhibit the telomerase-mediated addition of hexameric nucleotide repeats $(TTAGGG)_n$ onto the ends of TS-A. Among the compounds tested, β- and γ-rubromycins as well as purpuromycin appeared to be the most potent inhibitors of telomerase, with 50% inhibitory concentrations (IC_{50}) of about 3 μM; and griseorhodins A and C also appeared to be comparably potent inhibitors, with IC_{50} values of 12.2 and 5.87 μM, respectively (Table 1). In contrast, opening of the spiroketal system of β-rubromycin, giving rise to α-rubromycin (Figure 1), resulted in a marked reduction in the inhibitory potency toward telomerase (IC_{50} > 200 μM; Table 1), suggesting the essential role of this spiroketal system in telomerase inhibition. It should be noted that when the telomerase assay was conducted with

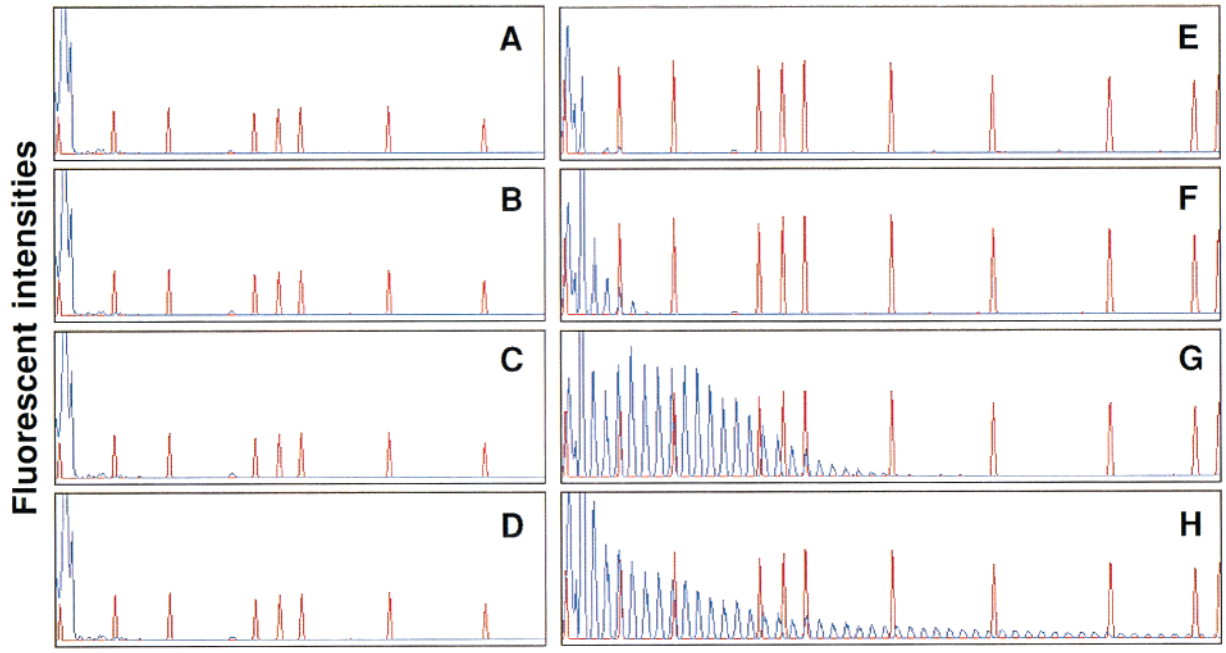

FIGURE 3: PCR-amplified telomerase products as visualized by capillary electrophoresis. Telomerase reactions were conducted in a mixture containing 20 mM Tris-HCl (pH 8.3), 68 mM KCl, 1 mM EGTA, 0.05% Tween 20, 0.5 μM T4 gene 32 protein, 8 μg/mL phage MS2 RNA, 100 μg/mL BSA, 200 μM concentration of each dNTP, 2.0 μM telomerase substrate primer (TS-A), and K-562 lysate equivalent to 5000 cells, and the mixtures were incubated at room temperature for an appropriate time. After the reaction had been quenched by the addition of EDTA, telomerase products were extracted in phenol/chloroform/isoamyl alcohol and chloroform/isoamyl alcohol and then precipitated in ethanol. Precipitated DNAs were amplified by PCR by use of the ACX-FAM primer. The amplified products (1 μL) were mixed with a fluorescent size standard (0.5 or 1 μL for panels A−D or E−H, respectively) and deionized DMF and then analyzed by capillary electrophoresis. Fluorescent signals derived from ACX-FAM are shown in blue, and those from the size standard are shown in red. Sizes of the markers were 50, 75, 100, 139, 150, 160, 200, 250, 300, and 340 bases from left to right in each panel. Panel A, no TS-A substrate added to the reaction; panel B, pretreatment of cell lysate with RNase A; panel C, pretreatment of cell lysate with protease K; panel D, addition of EDTA prior to initiation of the reaction; panel E, 1 min after the initiation of the reaction; panel F, 2 min thereafter; panel G, 10 min thereafter; panel H, 30 min thereafter.

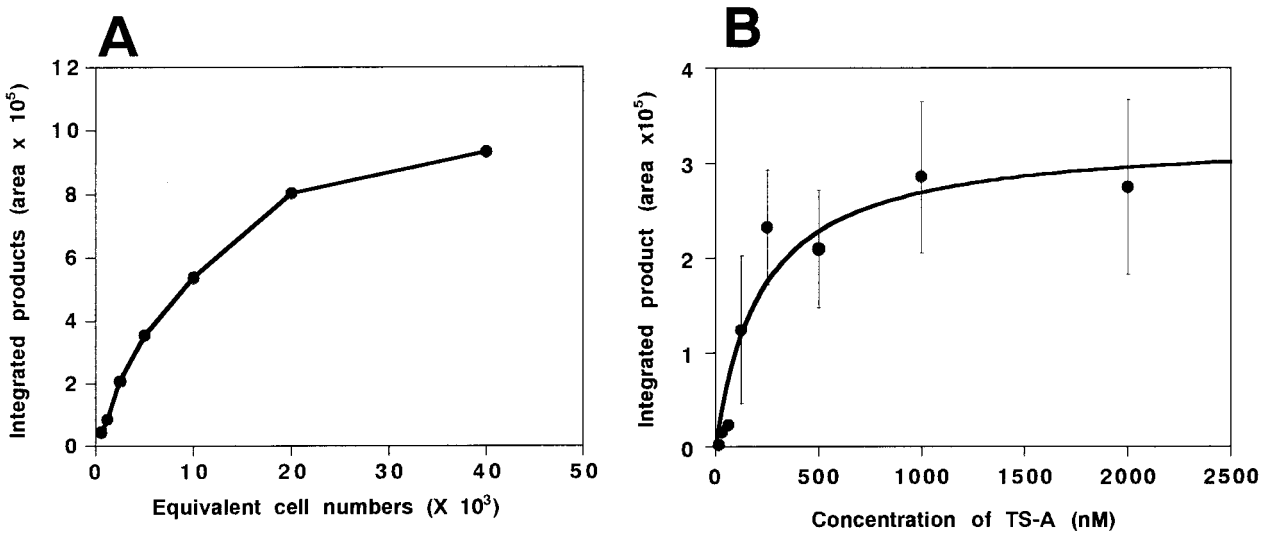

FIGURE 4: Telomerase assay as a function of concentrations of cell lysate and TS-A substrate. Telomerase reactions were conducted as described in Experimental Procedures with a 2.0 μM concentration of TS-A primer and the indicated numbers of K-562 lysate (panel A) or with use of 5000 cells equivalent numbers of K-562 lysate and indicated concentrations of TS-A primer (panel B). Data shown in panel B represent the mean ± SD of quadruplicate determinations, and the solid line represents the nonlinear square fit of the data to the conventional Michaelis−Menten equation, calculating an apparent K_m value of TS-A substrate of 0.22 ± 0.087 μM.

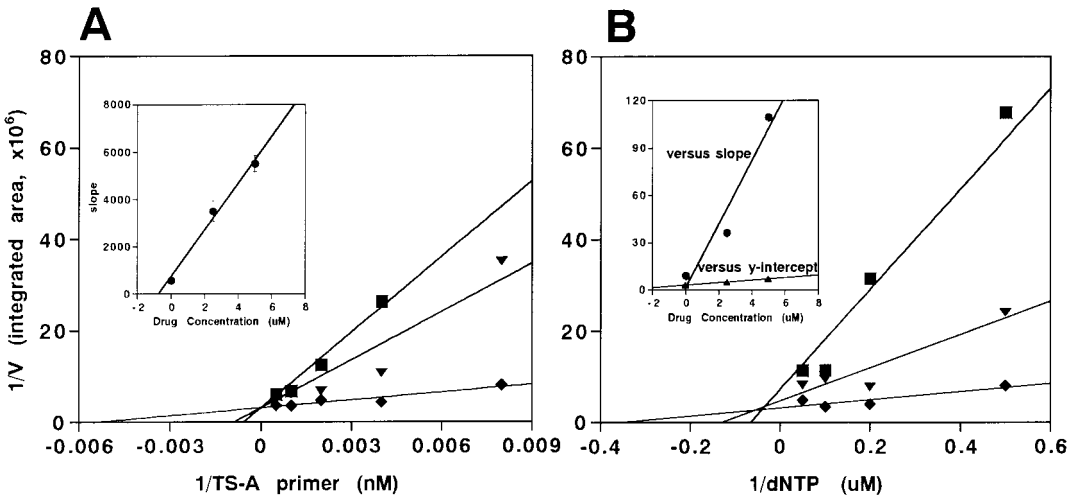

FIGURE 5: Lineweaver−Burk plot analysis of telomerase inhibition by β-rubromycin. Telomerase reactions were conducted as described in Experimental Procedures with K-562 lysate equivalent to 5000 cells and various concentrations of the TS-A substrate (panel A) or the dNTP substrate (panel B) in the absence (◆) or presence of 2.5 (▼) or 5.0 (■) μM β-rubromycin. Inset represents the replot of the slope (●) and the *y* intercept (▲) versus β-rubromycin concentration. Note that the inhibition constants of β-rubromycin determined from the *x* intercept of the replots are K_{is} = 0.74 μM with respect to TS-A (panel A) and K_{is} = 0.065 μM and K_{ii} = 3.68 μM with respect to dNTPs as variable substrates (panel B).

Table 1: Inhibition of Telomerase by Rubromycins and Their Analogues[a]

compounds	IC_{50} (μM) at TS-A concentration of 0.2 μM	2 μM
α-rubromycin	>200	>200
β-rubromycin	3.06 ± 0.85	8.64 ± 2.3
γ-rubromycin	2.64 ± 0.09	17.8 ± 2.0
purpuromycin	3.19 ± 0.45	15.9 ± 2.1
griseorhodin A	12.2 ± 3.1	41.6 ± 9.8
griseorhodin C	5.87 ± 0.44	25.8 ± 1.7

[a] Telomerase activities were determined by the modified TRAP method with a 0.2 or 2 μM concentration of a telomerase substrate primer, TS-A, as described in Experimental Procedures. Data are presented as the mean (± SD) of quadruplicate determinations.

[^{32}P]-dCTP and polyacrylamide gel electrophoresis as described by Kim et al. (*24*), virtually identical IC_{50} values (3.2 μM for β- and γ-rubromycins) were obtained (data not shown), further confirming our modified TRAP assay to be suitable for this study.

Analysis of Mode of Action of Rubromycins. To investigate the mode of action of rubromycins, we first examined the telomerase inhibition activity of β-rubromycin as a function of the concentrations of TS-A and dNTPs. From the Lineweaver−Burk plots shown in the Figure 5, β-rubromycin appeared to be a competitive inhibitor of telomerase with respect to the TS-A substrate, with K_{is} = 0.74 μM: whereas a mixed-type inhibition was observed with respect to the nucleotide substrates, with K_{is} = 0.065 μM and K_{ii} = 3.68 μM.

To partly confirm that the mode of action of the other rubromycins and their analogues with a spiroketal system were similar to that of β-rubromycin, we also tested the effect of the TS-A concentration on the telomerase-inhibiting activity of these other compounds. Increasing the concentra-

Table 2: Inhibition of Retroviral RTs and PCR by Rubromycins and Their Analogues[a]

compounds	IC_{50} (μM)		
	HIV-1 RT	M-MLV RT	PCR
α-rubromycin	>200	7.34 ± 0.73	1.06 ± 0.09
β-rubromycin	33.3 ± 2.4	3.42 ± 0.28	14.4 ± 0.93
γ-rubromycin	19.9 ± 1.6	4.37 ± 0.58	6.51 ± 0.65
purpuromycin	22.9 ± 0.45	3.69 ± 0.22	8.65 ± 1.4
griseorhodin A	10.5 ± 1.2	7.38 ± 0.58	2.07 ± 0.05
griseorhodin C	11.4 ± 1.8	9.37 ± 0.62	4.08 ± 0.34

[a] All enzymatic assays were conducted as described in Experimental Procedures. In brief, activities of RTs were analyzed with poly(rA)·$(dT)_{12-18}$ as a template−primer and [^{3}H]-dTTP as a nucleotide substrate. Incorporated [^{3}H]-dTMPs on the template−primer were determined by the DE-81 filter binding assay. PCR was conducted with 100 copies of *Pvu*II-digested pBC KS+ plasmid as a template and T7 and KS-HEX as primers. Amounts of amplified DNAs were visualized and quantified by capillary electrophoresis with a fluorescence detector. The dose−response curve used to determine the IC_{50} of β-rubromycin toward PCR is shown in Figure 2. Data are presented as the mean (± SD) of quadruplicate determinations.

tion of TS-A to a saturating level (2 μM) in the reaction resulted in decreased inhibition potencies of all of the compounds tested (Table 1), suggesting the competitive interaction between TS-A and rubromycins at the identical site on telomerase.

Effect of Spiroketal System on Other DNA/RNA-Modifying Enzymes. To assess the effect of the spiroketal system on other DNA/RNA-modifying enzymes, we first examined the inhibition potency of rubromycins on RTs of retroviruses (Table 2), since telomerase is a member of the RT family (*10*). β- and γ-Rubromycins appeared to be potent inhibitors of M-MLV RT, with IC_{50} values of 3.4 and 4.4 μM, respectively; whereas, they showed slightly weaker inhibition of HIV-1 RT, with IC_{50} values of 33 and 20 μM, respectively (Table 2). It is of interest that M-MLV RT appeared to be significantly sensitive to α-rubromycin (IC_{50} = 7.3 μM), whereas HIV-1 RT was quite insensitive to it (IC_{50} > 200 μM), suggesting the distinct structure and function between the two RTs, at least at the binding site of α-rubromycin.

Rubromycins were next tested for their ability to block PCR amplification. α-Rubromycin appeared to be the most potent inhibitor of PCR (IC_{50} = 1.06 μM), whereas β-rubromycin was about 14-fold less potent relative to α-rubromycin (Table 2), indicating that the spiroketal system in β-rubromycin was not very effective in inhibiting the PCR mediated by *Taq* polymerase. It should be noted, however, that the inhibitory activities of rubromycins and their analogues toward *Taq* polymerase were weak (IC_{50} > 50 μM) as assessed by the conventional primer extension assay similar to that used for other polymerases in this study (data not shown), most likely due to the multicycle aspect of PCR, which would tend to increase the apparent inhibitory activities of the compounds.

We further examined the effects of α- and β-rubromycins on a panel of DNA/RNA-modifying enzymes (Figure 6). Neither compound showed significant inhibition activity toward the enzymes examined, indicating a selective inhibitory activity of β-rubromycin toward RTs including telomerase.

Effect of Rubromycins on Proliferation of Human Cancer Cells. We then tested α- and β-rubromycins for their abilities to inhibit proliferation of cancer cells in vitro as assessed with [^{3}H]-thymidine incorporation assay. When K-562 and HeLa cells were exposed to each of the compounds for 3 and 4 days, respectively, α-rubromycin significantly inhibited the proliferation of both K-562 and HeLa cells with IC_{50} values of 2.46 and 2.35 μM, respectively, whereas β-rubromycin showed an approximately 10-fold decreased potency (IC_{50} = 19.5 and 22.7 μM for K-562 and HeLa cells, respectively) as compared with α-rubromycin, indicating that the spiroketal group in β-rubromycin was associated with decreased cytotoxic activity toward these human cancer cells. In addition, when the cytotoxicities of rubromycins were examined by counting the cells under a microscope and by measuring the metabolic activity of cellular enzymes using a tetrazolium dye, IC_{50} values obtained for K-562 and HeLa cells were all comparable to these determined by [^{3}H]-thymidine incorporation assay (data not shown), indicating that the effect of rubromycins on proliferation of these cancer cells was cytotoxic rather than cytostatic. It is, however, to be noted that since the induction of cell crisis and senescence caused by telomere shortening was shown to require long-term culture of cells (>20 population doublings) (*31, 32*), the cytotoxic activities of the compounds as observed toward the present short-term culture of cells are likely to be a side effect rather than a direct effect of telomerase inhibition. Neither cytotoxicity, cell crisis, nor senescence appeared to be induced when K-562 cells were cultured over 30 days (~30 population doublings) in the presence of 5 μM β-rubromycin (data not shown).

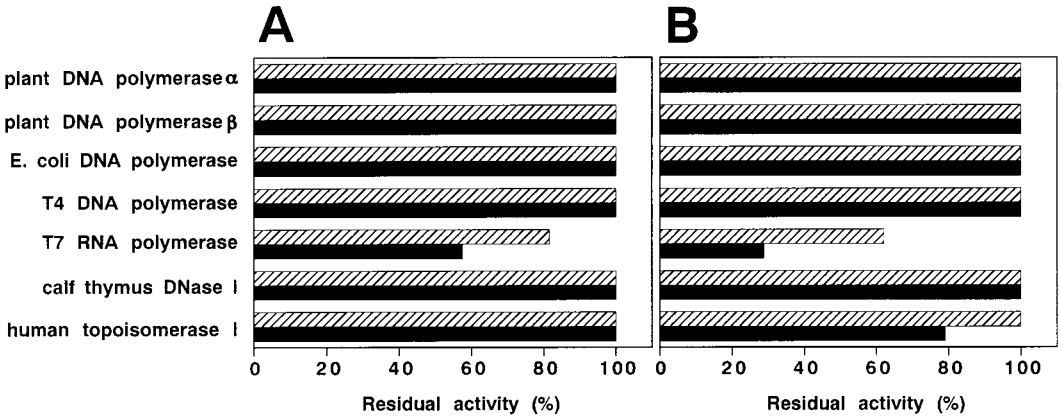

FIGURE 6: Inhibitory activities of α- and β-rubromycins toward various DNA/RNA-modifying enzymes. Residual activities of indicated enzymes in the presence of a 10 (hatched bars) or 100 (black bars) μM concentration of α- (panel A) or β-rubromycin (panel B) are shown.

DISCUSSION

The recently reported ectopic expression of the gene encoding human TERT (hTERT) in normal human cells resulted in the extension of the life-span of normal cells (*33*) and also the direct tumorigenic conversion of normal cells in combination with the simian virus 40 large-T oncoprotein and an oncogenic allele of H-ras (*34*). Moreover, a catalytically inactive mutant hTERT expressed ectopically in human cancer cells resulted in death of tumor cells and elimination of tumorigenicity in vivo (*32*). These studies corroborate telomerase as an attractive target for the development of potential anticancer agents, although the roles of telomerase in human tumors requires further testing, and some other concerns remain, including the other telomere maintenance systems. Accordingly, a number of groups have described different approaches to investigate telomerase inhibitors such as antisense oligonucleotides and peptide-nucleic acids (*35*), ribozymes (*36*), nucleoside analogue RT inhibitors (*11*), G-quadruplex-interacting compounds (*37, 38*), and screening of large numbers of chemical compounds (*30*). Natural products including tea catechins (*39*), alterperylenol (*40*), and diazaphilonic acid (*41*) have also been reported to be telomerase inhibitors. There is, however, no telomerase inhibitor shown to have anticancer activity in vivo, at least thus far, and thereby, several other classes of inhibitors with distinct structures are still needed for designing more potent and specific telomerase inhibitors.

In the present study, we focused on rubromycins and their analogues, a series of quinone antibiotics that possess benzofuran and benzodipyran rings that form a spiroketal system, and tested them for their abilities to inhibit human telomerase in a cell-free assay. As a result, rubromycin analogues that contained the spiroketal system showed comparable potency toward telomerase ($IC_{50} = 3-12$ μM), whereas α-rubromycin, a compound formed by the opening of the spiroketal system of β-rubromycin, showed substantially decreased inhibitory potency toward telomerase, indicating the essential role of the spiroketal system in the inhibition of telomerase. It should be noted that the roles of the quinone moieties of these compounds in telomerase inhibition have not been investigated yet. However, α-rubromycin or naphthoquinone itself did not inhibit telomerase at concentrations of >200 μM (T. Ueno, unpublished observations), suggesting that the quinone moiety in these compounds is not sufficient to afford inhibition of telomerase. Furthermore, steady-state kinetic analysis of inhibition of telomerase by β-rubromycin revealed the competitive interaction of the compounds with the telomerase substrate primer, TS-A, with a K_i of 0.74 μM. This result indicates that β-rubromycin could interact with the human telomerase RNA (hTR) and/or the catalytic subunit of telomerase (i.e., hTERT) since the binding site of TS-A should be located on both hTR and hTERT. Considering that purpuromycin, which is one of the derivatives of rubromycins and was shown to inhibit telomerase comparably as β-rubromycin, preferentially binds to bacterial tRNAs (see below) (*42*), it is possible that rubromycins and their analogues may have a capacity to bind to RNAs as well. In this regard, one might consider that rubromycins are di- or trivalent metal chelators, which tend to polymerize around a metal center and ultimately bind to nucleic acids, as observed for the DNA minor groove binders such as chromomycins and mithramycins (*43, 44*). It is, however, noted that the addition of excess amounts of plasmid DNAs, M13 single-stranded DNAs, or MS2 RNA in the reaction did not decrease the apparent inhibitory activity of β-rubromycin toward telomerase (data not shown), suggesting that nonspecific binding between β-rubromycin and nucleic acids is unlikely to be involved in the mode of action of the rubromycins, although whether rubromycins polymerize around the metal center has not been directly examined yet. Further analysis to elucidate the mechanism of the molecular interactions between telomerase and rubromycins with the spiroketal system are under investigation.

Rubromycins and their analogues were originally found as antibiotic pigments with a broad spectrum of activity against bacteria, fungi, and protozoa (*16, 17, 45*). Studies on the mechanism of action of purpuromycin, which also inhibits telomerase, revealed that purpuromycin binds to tRNAs with high affinity and inhibits their aminoacylation, leading to consequent inhibition of protein synthesis in these microorganisms (*42, 46*). β- and γ-Rubromycins were shown to inhibit HIV-1 RT by competitive interaction at the RNA/DNA template−primer site but did not show anti-HIV activity due to their cytotoxicities in the host cells in vitro (*14*). β- and γ-rubromycins were also shown to inhibit calf thymus DNA polymerase α with IC_{50} values of 18.6 and 5.5 μM, respectively (*14*). In our study, rubromycins and their analogues with the spiroketal system showed potent inhibitory activities toward both telomerase and retroviral RTs but had only subtle effects on the other DNA/RNA-modifying enzymes tested. However, proliferation of human cancer cells was shown to be inhibited by short-term exposure to rubromycins in vitro, suggesting the existence of other target molecules of rubromycins in human cancer cells. It is noteworthy that α-rubromycin appeared to inhibit rat DNA polymerase β activity ($IC_{50} \sim 1$ μM), whereas β-rubromycin inhibited it with 10-fold weaker potency as compared with α-rubromycin (Mizushina et al., submitted for publication). Such potencies of rubromycins likely provide a possible explanation for the cytotoxic activities of rubromycins as observed in this study, although further analysis of the effect of rubromycins on several classes of human DNA and RNA polymerases and on human cancer cells are needed.

It should be noted that IC_{50} values of β- and γ-rubromycins (0.98 and 0.62 μM, respectively) toward HIV-1 RT determined by Goldman et al. (*14*) are markedly lower than those determined in this study. The assay conditions used by Goldman et al. and us were not significantly different except for the viral strains used for RT preparations: Goldman et al. used NY-5, whereas we used HXB2. Genetic diversity of HIV-1 is well recognized, and there is, indeed, a 26-amino acid difference in p66/p51 heterodimer RTs between NY-5 and HXB2 (*47*), and such a difference is likely to cause a change in the binding between RTs and rubromycins. In fact, Beard et al. (*48*) reported a >250-fold difference in K_m values for template−primer between RTs from NY-5 and HXB2.

In summary, on the basis of structural and functional similarities of telomerase and retroviral RTs, we found rubromycins and their analogues having a spiroketal system to be potent and selective telomerase inhibitors but to show less activity toward other classes of DNA/RNA-modifying

enzymes. Since the inhibition potencies of these compounds toward telomerase in the cell-free assay are comparable to those reported thus far by others (*30*, *37*−*39*), it is thus intriguing to consider the development of more potent and selective telomerase inhibitors based on β-rubromycin as a novel lead structure, although potencies of β-rubromycin to induce cell crisis and senescence in human cancer cells have not yet been confirmed.

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