In Vitro Inhibition of Viral DNA Polymerase Activity by Litmomycin

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Abstract □ Litmomycin, an antibiotic isolated from *Streptomyces litmogenes*, is highly active against Gram-positive bacteria and possesses antitumor activity. It inhibited viral DNA polymerase activity *in vitro*. The amount of litmomycin required to cause 50% inhibition of enzyme activity was 80–100 μg (180–225 nmoles)/ml of reaction mixture. The enzyme inhibition was observed when polyriboadenylate–oligodeoxythymidylate, polydeoxyadenylate–oligodeoxythymidylate, polyribocytidylate–oligodeoxyguanylate, activated DNA, and 70S RNA were used as templates. Reaction kinetics and the mechanism of enzyme inhibition are discussed. The results suggest that litmomycin interacts with the template primer and not with the enzyme protein to stop the polymerization process.

Keyphrases □ Litmomycin—effect on viral DNA polymerase activity *in vitro*, reaction kinetics and mechanism □ Viral DNA polymerase—effect of litmomycin on *in vitro* activity, reaction kinetics and mechanism □ DNA polymerase, viral—effect of litmomycin on *in vitro* activity, reaction kinetics and mechanism □ Enzymes—viral DNA polymerase, effect of litmomycin on *in vitro* activity, reaction kinetics and mechanism □ Antibiotics—litmomycin, effect on viral DNA polymerase activity *in vitro*, reaction kinetics and mechanism

RNA-directed DNA polymerase (reverse transcriptase) is the unique enzyme found (1–4) in nucleocapsids of RNA tumor viruses. This enzyme is required for productive infection and transformation of normal cells by RNA tumor viruses (5–7). Recent publications (8–10) indicate that RNA tumor viruses are involved in at least some forms of human cancer (leukemia). Current evidence indicates that reverse transcriptase is required only to initiate, but not to maintain, the transformed state and the continuous production of new virus particles by these transformed cells (10, 11).

The relationship between the biochemical characteristics of reverse transcriptase from human acute leukemia cells and several RNA tumor viruses is well documented (12). In the event that RNA tumor viruses are involved in human cancer, reverse transcriptase is the important enzyme in the onset of oncogenesis (13). Therefore, specific inhibitors of reverse transcriptase might find use in the development of drugs for leukemia and cancer chemotherapy. In an effort to develop *in vitro* test systems for antiviral activity, an inhibition of reverse transcriptase activity by fagaronine was previously studied (14). This paper reports the effect of litmomycin on the purified protein fraction of reverse transcriptase from RNA tumor viruses.

Litmomycin (granaticin A[1]), an antibiotic isolated from a new *Streptomyces* species, *S. litmogenes* (15), is highly active against Gram-positive bacteria but has little or no activity against Gram-negative bacteria, mycobacterium, molds, and yeast (16). It has significant antitumor activity against P-388 lymphocytic leukemia in mice and cytotoxicity against KB cells (17).

[1] Granaticin B showed a distinct inhibition of various transplanted tumors in rodents after repeated intraperitoneal application (H. Bickel and K. Scheibli, Ciba-Geigy Ltd., Basel, Switzerland, personal communication).

EXPERIMENTAL

Materials—Purified DNA polymerase from avian myeloblastosis virus[2] had a specific activity of 10,108 units/mg and a protein content of 0.35 mg/ml. The enzyme was purified according to the published method (18). DNA polymerase from simian sarcoma virus type 1[3] was derived from tissue culture fluids of the SSV-1-NC-37 cell line and purified by the method described previously (14). It possessed an activity of 6.00 nmoles of thymidine monophosphate incorporation/30 min/ml. Purified DNA polymerase from Rauscher murine leukemia virus[4] propagated in the JLS-V9 cell line had an activity of 4.00 nmoles of thymidine monophosphate incorporation/30 min/ml. Viral 70S RNA[5] was obtained from Rauscher murine leukemia virus, which was purified by the published method (19).

The templates[6], polyriboadenylate–oligodeoxythymidylate (I), polydeoxyadenylate–oligodeoxythymidylate (II), and polyribocytidylate–oligodeoxyguanylate (III), were dissolved in a buffer containing 0.01 *M* tromethamine (IV) (pH 7.0), 0.10 m*M* ethylenediaminetetraacetic acid tetrasodium salt (V), and 0.10 *M* NaCl. ^{3}H-Thymidine triphosphate and ^{3}H-guanosine triphosphate (specific activity of 14 Ci/mmole[7]), unlabeled thymidine triphosphate[6], and guanosine triphosphate[6] were obtained commercially. Activated calf thymus DNA was prepared by incubating 4.50 mg of bovine serum albumin in 10 ml of buffer containing IV (0.05 *M*, pH 7.0) and 5.00 m*M* $MgCl_2$ with 0.01 mg of crystalline pancreatic deoxyribonuclease[8] for 15 min at 37°. It was chilled at 4°, further incubated at 70° for 5 min, and stored at 4°.

All other chemical reagents[9] were of analytical grade. Appropriate concentrations of litmomycin (mol. wt. 444) were made in dimethyl sulfoxide.

Viral DNA Polymerase Assay and Enzyme Inhibition—One hundred microliters of enzyme assay mixture contained 5.00 μmoles of IV (pH 7.30), 8.00 μmoles of potassium chloride, 0.10 μmole of manganese chloride, 0.50 μmole of dithiothreitol, 2.00 μmoles of ^{3}H-thymidine triphosphate (170 cpm/pmole), 20.00 μg of bovine serum albumin, 2.00 μg of I, 10% glycerol, and purified enzyme preparation. The reaction mixture was incubated at 37° for 30 min. The enzyme reaction was stopped by chilling at 4° and by the addition of V (25 μl, 0.10 *M*).

Each reaction mixture (100 μl) was uniformly spotted onto 2.50-cm filter paper[10] disks and kept at room temperature for 15 min. The filter paper disks were washed batchwise six times with 5% $Na_2HPO_4 \cdot 7H_2O$ (10 ml/filter paper disk), followed by two washings each of water and ethanol (10 ml/filter paper disk). Then these disks were dried, and the radioactivity incorporated into an acid-insoluble material was determined[11] in a toluene base scintillation fluid.

For testing the enzyme inhibition, two reaction mixtures (A and B) were prepared. Mixture A contained enzyme, buffer, and bovine serum albumin. Mixture B contained template, substrate, dithiothreitol, and glycerol. The final concentration of ingredients in Mixtures A and B (100 μl) was the same as mentioned previously. Appropriate concentrations of litmomycin were mixed with Mixture A and allowed to react for 10 min at 4° before Mixture B was added. Control assays contained an equivalent volume of dimethyl sulfoxide. The results were expressed as percent of control.

[2] Life Science Research Labs., St. Petersburg, Fla., through the courtesy of Dr. J. Beard. The enzyme preparation contained 3538 units of reverse transcriptase/ml (1 unit of enzyme activity is expressed as the incorporation of 1 nmole of deoxythymidine monophosphate into an acid-insoluble product in 10 min at 37°).
[3] Pfizer, Inc., Maywood, N.J.
[4] Bionetics Laboratory Products, Kensington, Md.
[5] Gift from Mr. Z. Salahuddin, Litton Bionetics Inc., Bethesda, Md.
[6] P.L. Biochemicals Inc., Milwaukee, Wis.
[7] Schwarz-Mann.
[8] Worthington Biochemicals Corp.
[9] Sigma Chemical Co., St. Louis, Mo.
[10] DE-81, Whatman.
[11] Unilux III liquid scintillation unit.

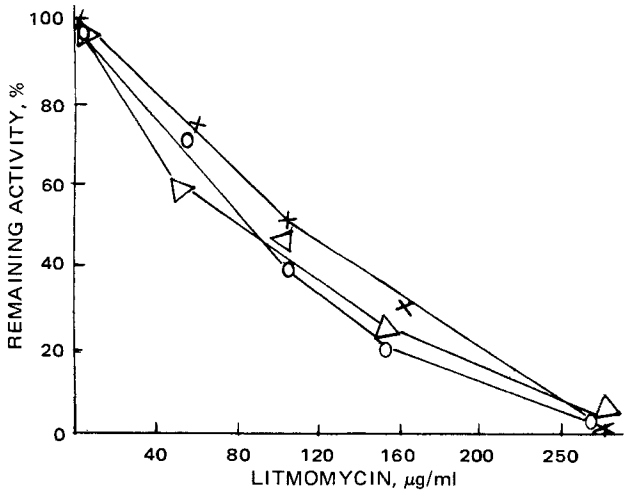

Figure 1—*Effect of litmomycin on the viral DNA polymerase activity. In a standard assay mixture (0.10 ml) containing 5 μl of avian myeloblastosis virus (○), 10 μl of simian sarcoma virus type 1 (△), or 10 μl of murine leukemia virus (×) DNA polymerase, different concentrations of litmomycin were added for enzyme inhibition.*

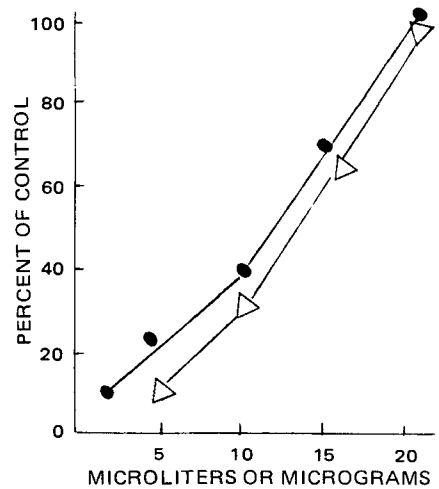

Figure 2—*Effect of increasing concentrations of avian myeloblastosis virus DNA polymerase and template primer on litmomycin-inhibited reaction mixture. The standard reaction mixture (0.10 ml) contained 5 μl of avian myeloblastosis virus DNA polymerase (△) or 2 μg of I (●). Twenty micrograms of litmomycin was used for each assay.*

RESULTS AND DISCUSSION

The inhibition of DNA polymerase activity from murine leukemia virus, simian sarcoma virus type 1, and avian myeloblastosis virus at increasing concentrations of litmomycin (0–240 μg/ml) was undertaken. The enzyme activity gradually declined when the litmomycin concentration in the reaction mixture was increased. The inhibition curves were very close and similar to each other (Fig. 1). Fifty percent inhibition of enzyme activity (ID_{50}) was obtained in the range of 80–100 μg (180–225 nmoles)/ml of the reaction mixture.

To explain the mode of enzyme inhibition, different template primers such as I, II, III, activated DNA, and 70S RNA were employed (Table I); different degrees of enzyme inhibition were obtained with different template primers. A high degree of enzyme inhibition in the presence of I or II, as compared to III, could be due to a strong binding affinity of litmomycin with adenine–thymine template primers. These results suggest that litmomycin did not interact with the enzyme protein. If litmomycin interacted with the enzyme, then the same degree of enzyme inhibition would have been observed regardless of the template primer used.

The interaction or competition of litmomycin with potassium chloride and ^{3}H-thymidine triphosphate or ^{3}H-guanosine triphosphate was not considered since they were present in the reaction mixture in eight- or 25-fold, respectively, in excess of the ID_{50} of the litmomycin concentration. The enzyme activity remained unchanged when the amount of manganese chloride in the assay mixture was increased (data not shown), indicating that the metal ions did not interact with the litmomycin. Therefore, litmomycin interacted with the template primers and not with the enzyme.

Effects of increasing concentrations of avian myeloblastosis virus DNA polymerase and template primer were observed on the litmomycin-inhibited reaction mixture to explain further the mechanism of enzyme inhibition. The enzyme activity of the litmomycin-inhibited reaction mixture was linearly restored when concentrations of I in the assay mixture were increased from 2 to 20 μg and the enzyme concentration was increased from 5 to 20 μl (Fig. 2). These results suggest that the enzyme–template–antibiotic complex in the reaction mixture was completely dissociated in the presence of excess template primers or the enzyme.

In the assay mentioned, litmomycin was added to the reaction mixture containing enzyme, template primer, metal ions, and substrate before incubation. The inhibitory effects of litmomycin were thus primarily directed to the free template primer and its complexes with the enzyme, substrate, and metal ions. In other assays where the enzyme molecule had already initiated the DNA synthesis, with subsequent elongation of polynucleotide chains, the effect of lit-

Table I—Effect of Different Template Primers on Avian Myeloblastosis Virus DNA Polymerase Inhibition by Litmomycin

Template Primer	Radioactive Substrate	Inhibition[a], %
I[b]	Thymidine triphosphate	80
II[b]	Thymidine triphosphate	86
III[c]	Deoxyguanosine triphosphate	15
Activated DNA[d]	Thymidine triphosphate	75
70S RNA[e]	Thymidine triphosphate	70

[a] Litmomycin, 20 μg/assay, was employed for each inhibition. [b] See standard assay conditions as specified in *Experimental* section. [c] Standard assay mixture contained 2 μg of this template primer and 2.0 nmoles of ^{3}H-deoxyguanosine triphosphate (370 cpm/mole). [d] Standard assay mixture contained 2.20 μg of activated DNA, 10 nmoles each of deoxyadenosine, deoxycytidine, and deoxyguanosine triphosphates, and 2.0 nmoles of ^{3}H-thymidine triphosphate. [e] In the standard assay conditions, 70S RNA (murine leukemia virus) with an optical density of 0.06 (260 nm) was used as the template.

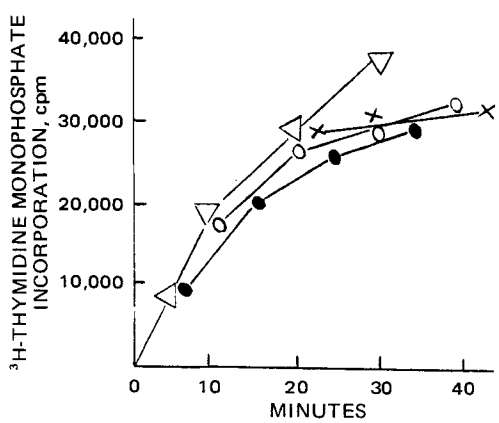

Figure 3—*Effect of addition of litmomycin at different time intervals during avian myeloblastosis virus DNA polymerase reaction. Two milliliters of standard assay mixture contained 100 μl of enzyme. The mixture was divided into four aliquots: A (0.60 ml), B (0.50 ml), C (0.50 ml), and D (0.40 ml). From A, a 0.10-ml sample was withdrawn at 0 min; A–D were incubated at 37°. One-tenth-milliliter samples from A (control, △) were withdrawn after 5, 10, 20, and 30 min of incubation. Litmomycin (250 μg/ml) was added after 5 (●), 10 (○), and 20 (×) min of incubation to B, C, and D, respectively. The enzyme activity was determined 1 min after the addition of litmomycin from the withdrawn samples (0.10 ml) at 10-min intervals.*

momycin was observed. Litmomycin was added to such a mixture at different time intervals during the reaction.

When litmomycin was added at 5 and 10 min after initiation of the polymerization reaction, the enzyme activity continued for about 20 min, comparable to the control. But when the litmomycin was added after 20 min, the enzyme activity stopped instantly, as indicated by the ^{3}H-thymidine monophosphate incorporation (Fig. 3). The results suggest that litmomycin did not inhibit the initial formation of an RNA–DNA hybrid but blocked the subsequent phase of the polymerization processes.

In conclusion, litmomycin inhibits DNA polymerase activity of RNA tumor viruses by interaction with (riboadenylate)$_n$–(deoxythymidylate)$_n$ or adenine–thymine bases and (ribocytidylate)$_n$–(deoxyguanylate)$_n$ or cytosine–guanine bases with a different degree of binding. It may be of interest to investigate the antiviral activity of litmomycin in cell culture.

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GLC Determination of Indoprofen in Plasma

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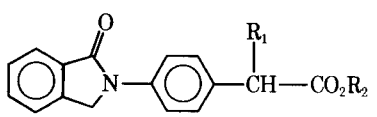
Abstract □ Implementation of human bioavailability studies with the analgesic indoprofen required a rapid, sensitive, and convenient assay in blood plasma. A procedure based on ether extraction of acidified plasma, derivatization with diazomethane, and GLC analysis using a 3% OV-1 column was sufficiently sensitive for measurement of plasma indoprofen concentrations in the 0.4–16-μg/ml range. An average recovery of 99.0 ± 7.6% (*SD*) was achieved when the pentanoic acid homolog was employed as an internal standard.

Keyphrases □ Indoprofen—GLC analysis, human plasma □ GLC—analysis, indoprofen in human plasma □ Analgesics—indoprofen, GLC analysis in human plasma

Indoprofen, *dl*-2-[4-(1-oxo-2-isoindolinyl)phenyl]propanoic acid (I), possesses promising analgesic and anti-inflammatory properties (1, 2). In humans, I reportedly appears unchanged in plasma (3). Furthermore, enantiomeric enrichment does not seem to be significant in humans (4). Implementation of human bioavailability studies in this laboratory required a rapid, sensitive, and convenient assay of I in blood plasma.

Two GLC methods were reported for the determination of I in plasma (4, 5). Both procedures employ a lengthy series of partition steps. In one (4), a trifluoroethyl ester



I: $R_1 = CH_3$, $R_2 = H$

II: $R_1 = CH_2CH_2CH_3$, $R_2 = H$

III: $R_1 = CH_3$, $R_2 = CH_3$

IV: $R_1 = CH_2CH_2CH_3$, $R_2 = CH_3$

is utilized after preparation with boron trifluoride–trifluoroethanol. The latter reagent is corrosive and difficult to eliminate prior to GLC development. The other method (5) involves preparation and resolution of a diastereomeric pair of amides obtained through reaction of I (as its acid chloride) with *l*-α-methylbenzylamine. While useful in evaluating *in vivo* enantiomeric interconversions, this procedure is not amenable to analyses of large numbers of samples because of its length.

A simplified assay based on a single ether extraction and GLC of I and the internal standard, *dl*-2-[4-(1-oxo-2-isoindolinyl)phenyl]pentanoic acid (II), as their methyl esters (III and IV) was developed and is the subject of this report.