INHIBITION OF REVERSE TRANSCRIPTASE ACTIVITY OF RNA-TUMOR VIRUSES BY FAGARONINE[+]

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SUMMARY: Fagaronine, a benzophenanthridine alkaloid from roots of Fagara zanthoxyloides (Rutaceae), has been reported to possess anti-leukemic activity. It inhibited RNA-directed DNA polymerase activity from avian myeloblastosis virus, Rauscher leukemia virus and simian sarcoma virus. With poly rA·oligo dT, the alkaloid concentration for 50% inhibition of the enzyme activity from these viruses was in the range of 6-12 μg (15 - 31 nmoles) per ml of reaction mixture. The enzyme reaction was also inhibited with activated DNA and 70S RNA as templates; however, with poly rC·oligo dG no inhibition of enzyme activity was obtained. These results suggest that fagaronine inhibits enzyme activity by interaction with the A:T template-primer.

Nucleocapsids of RNA tumor viruses contain RNA-directed DNA polymerase (reverse transcriptase) which catalyzes the formation of DNA from RNA template (1,2). This enzyme is involved in viral DNA (provirus) formation which on integration with the host genome causes cell transformation (1-5). If RNA tumor viruses are involved in human neoplasia (6-9), reverse transcriptase is a key enzyme for oncogenesis. Specific inhibitors of this enzyme might therefore be useful prophylactic agents in leukemia and cancer chemotherapy. With a view to set up *in vitro* test systems for antitumor activity, we are testing drugs and natural products for inhibition of virus transformation, viral and cellular replication and transcription enzymes (10,11). Fagaronine, a benzophenanthridine alkaloid, has been isolated from roots of Fagara zanthoxyloides (Rutaceae), and its structure and anti-leukemic activity have been recently reported (12,13). This paper deals with

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the effect of fagaronine on purified preparations of reverse transcriptase from RNA tumor viruses.

MATERIALS AND METHODS

Reagents, templates, viruses and alkaloid. Radioactive nucleoside triphosphates were purchased from Schwarz-Mann and unlabelled nucleotides were obtained from P. L. Biochemicals. All other reagents were of analytical grade. The templates poly rA·oligo dT, poly dA·oligo dT, poly rC·oligo dG and oligo dT were purchased from P. L. Biochemicals and were dissolved in a buffer containing 0.01 M Tris-HCl, pH 7.0, 0.1 mM EDTA and 0.1 M NaCl. Activated calf thymus DNA was prepared by incubating 4.5 mg of DNA, 5 mg of bovine serum albumin in 10 ml buffer containing 0.05 M Tris-HCl, pH 7.0 and 5 mM $MgCl_2$ with 0.01 mg of crystalline pancreatic DNase (Worthington) for 15 minutes at 37^0, chilled, further incubated at 70^0 for 5 minutes and stored at 4^0. Viral 70S RNA was prepared by pelleting 500 mg of avian myeloblastosis virus (AMV) onto a glycerol cushion, followed by 2x deproteination in 0.5% SDS-phenol, addition of tRNA carrier, ethanol precipitation and separation by velocity centrifugation on a 10-30% glycerol gradient. Simian sarcoma virus, type 1 (SSV-1) derived from the tissue culture fluids of the SSV-1-NC-37 cell line was obtained from Pfizer, Inc., Maywood, New Jersey. Chicken plasma containing AMV was obtained from Life Sciences Research Labs, St. Petersburg, Florida through the courtesy of Dr. J. Beard. Purified reverse transcriptase from Rauscher murine leukemia virus (MuLV), propagated in JLS-V9 cell line, was obtained from Bionetics Laboratory Products, Kensington, Maryland. Appropriate concentrations of fagaronine chloride (M.W=385) were made in dimethyl sulfoxide (DMSO).

Viral DNA polymerase assay and testing of enzyme inhibition. The enzyme assay mixture of 100 µl contained 5 µmoles Tris-HCl, pH 7.3, 8 µmoles KCl, 0.1 µmole $MnCl_2$, 0.5 µmoles dithiothreitol (DTT), 2 nmoles (^{3}H)TTP (120 cpm/pmole), 20 µg bovine serum albumin (BSA), 2 µg poly rA·oligo dT, 10% glycerol and purified enzyme fraction. The reaction mixture was incubated at 37^0 for 30 minutes. The enzyme reaction was terminated by chilling in ice and by the addition of 25 µl of 0.1 M EDTA. One hundred µl of each reaction mixture was spotted uniformly onto a 2.5 cm circular DE-81 (Whatman) filter, kept at room temperature for 15 minutes and washed batch-wise by swirling in 10 ml per filter of 5% $Na_2HPO_4.7H_2O$ for six times followed by two washings each with water and ethanol. Filters were dried and radioactivity counted in toluene-based scintillation fluid.

For enzyme inhibition studies, two reaction mixtures were made, one containing enzyme, buffer and BSA, and the other having template, substrate, salts, DTT and glycerol. The final concentration of the ingredients was as described above. Suitable concentrations of fagaronine were mixed with the first mixture and placed in ice for 10 minutes before adding the second mixture. Control experiments contained an equivalent amount of DMSO. Results were expressed as percent of control.

Purification of viral DNA polymerases. DNA polymerase from AMV and SSV-1 was purified according to a modified procedure of Abrell and Gallo (14). The virus solution was pelleted by centrifugation and the virus extracted with buffer containing 0.05 M Tris-HCl, pH 7.9, 0.05 M each NaCl and KCl, 0.02 M DTT, 1 mM EDTA and 0.25% Triton X-100. The viral extract was applied to a DEAE-cellulose column and eluted with a buffer containing 0.05 M Tris-HCl, pH 7.9, 1 mM EDTA, 1 mM DTT, 20% glycerol, 0.025% Triton X-100 and 0.30 M KCl. The eluate was extensively dialyzed and applied to a phosphocellulose column which was previously saturated with bovine serum albumin. DNA polymerase was eluted from the column by a linear KCl gradient, the peak activity eluting at

0.25 - 0.3 M KCl. The enzyme fractions were pooled, concentrated by dialysis against Tris-buffer containing 30% polyethyleneglycol 6000, and stored at -80^0. The amount of protein in the enzyme preparations, due to its very low content, was not determined. The activities of MuLV, SSV-1 and AMV DNA polymerases were 4, 6 and 20.3 nmoles, respectively, of TMP incorporation/30 minutes/ml.

RESULTS AND DISCUSSION

The inhibition of reverse transcriptase activity from MuLV, SSV-1 and AMV at the increasing concentrations of fagaronine (0-50 µg/ml) was examined. There is a sharp decline in enzyme activity at increasing concentrations of the alkaloid, and inhibition curves were very close and similar to each other (figure 1). The amount of alkaloid required to cause 50% enzyme inhibition

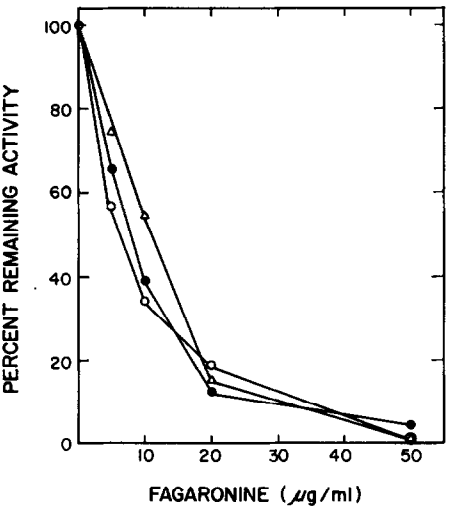

Figure 1. Effect of fagaronine on the viral DNA polymerase activity. In a standard assay mixture of 0.10 ml containing 10 µl of MuLV (o—o), 5 µl of SSV-1 (●—●) or 5 µl of AMV DNA polymerase (Δ—Δ), different concentrations of the alkaloid were used for enzyme inhibition.

(ID_{50}) was in the range of 6-12 µg (15 - 31 nmoles) per ml of reaction mixture.

In order to elucidate the mechanism of enzyme inhibition, different template-primers (poly rA•oligo dT, poly dA•oligo dT, poly rC•oligo dG, activated DNA and 70S viral RNA) were employed for inhibition studies. The enzyme activity with poly rA•oligo dT, poly dA•oligo dT, activated DNA and

Table 1. Inhibition of AMV DNA polymerase activity by fagaronine with different template-primers.

Template-primer	Radioactive substrate	Fagaronine (5 μg/assay)	Enzyme activity (pmoles/assay)	Percent Control
poly rA·oligo dT[1]	TTP	-	134	100
poly rA·oligo dT[1]	TTP	+	0.5	0.3
poly dA·oligo dT[1]	TTP	-	43	100
poly dA·oligo dT[1]	TTP	+	0.25	0.6
poly rC·oligo dG[2]	dGTP	-	35.6	100
poly rC·oligo dG[2]	dGTP	+	39.2	110
activated DNA[3]	TTP	-	2.1	100
activated DNA[3]	TTP	+	0.39	18
70S RNA[4]	TTP	-	1.2	100
70S RNA[4]	TTP	+	0	0
70S RNA + oligo dT[5]	TTP	-	5.2	100
70S RNA + oligo dT[5]	TTP	+	0	0

[1]Standard assay conditions as described in Materials and Methods were used.

[2]Standard assay mixture contained 2 μg of the template-primer and 2.2 nmoles of (^{3}H)GTP (340 cpm/mole).

[3]In the standard assay conditions, synthetic template was replaced by 2.25 μg of activated DNA; 10 nmoles each of dATP, dCTP and dGTP and 2 nmoles of (^{3}H)TTP were used.

[4]The assay conditions were the same as in 3, but 0.04 OD units (260 nm) of 70S AMV RNA were used as template.

[5]The assay conditions were the same as above, but in addition contained 2 μg of oligo dT as primer.

70S viral RNA was strongly inhibited by the alkaloid, but with poly rC·oligo dG there was no enzyme inhibition (Table 1). These results suggest that the alkaloid inhibition is not due to its interaction with the enzyme protein. Moreover, it is also not due to its interaction or competition with KCl or substrate, (^{3}H)TTP or (^{3}H)GTP, since they are present in the reaction mixture in 70 and 250 fold, respectively, in excess to that of ID_{50} of the alkaloid concentration. It was also observed that enzyme activity remained unchanged by increasing $MnCl_2$ in the assay mixture (data not shown) indicating that the metal ions did not interact with fagaronine. The data in Table 1 further suggest that the alkaloid has less affinity for (rC)n,(dG)n or (C)n·(G)n pairs. The strong inhibition of enzyme activity with poly rA·oligo dT and poly dA·

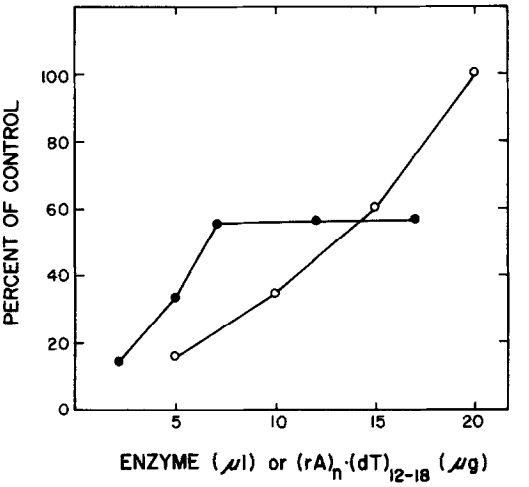

Figure 2. Influence of increasing concentration of AMV DNA polymerase and template-primer on fagaronine-inhibited reaction mixture. The standard reaction mixture of 0.10 ml contained 5 μl of enzyme and 2 μg of poly rA·oligo dT. Two μg of the alkaloid per assay were used. AMV DNA polymerase o–o, poly rA·oligo dT ●–●.

oligo dT suggests that the inhibition is due to interation of the alkaloid with (rA)n,(dT)n or A:T pairs. This interaction may further explain the inhibition of the enzyme activity with activated DNA or 70S viral RNA.

When poly rA·oligo dT concentration in the assay mixture was increased from 2 to 17 μg, the enzyme activity was restored to about 60%, whereas a 4 fold increase of enzyme concentration completely reversed the inhibition (figure 2). These results can be explained by assuming the formation of an enzyme-template-alkaloid ternary complex in the reaction mixture which is fully dissociated by addition of excess of enzyme, but is partially dissociated to the extent of 60% in the presence of excess of template-primer. The reasons for the partial dissociation of the ternary complex are unknown.

In the above-described experiments, fagaronine was added to the reaction mixture before incubation at 37^0 and inhibitor effects were directed to free template-primer and its complexes with enzyme substrate and metal ions. In another situation where the enzyme molecule was elongating the polynucleotide chains, the effect of fagaronine was studied. Addition of the

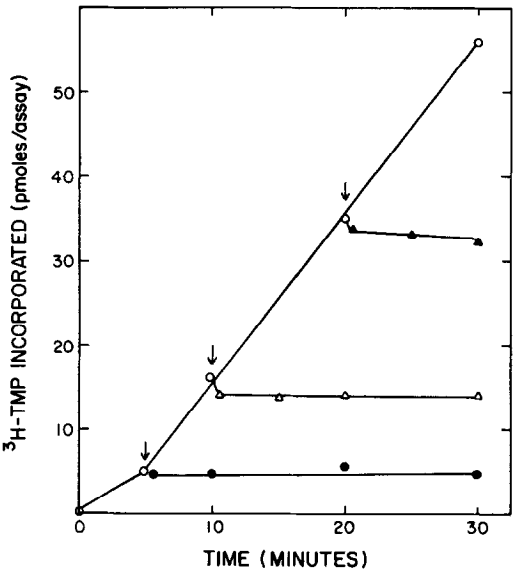

Figure 3. Addition of fagaronine during AMV DNA polymerase reaction kinetic. Two ml of standard assay mixture containing 50 µl of enzyme/ml were made and distributed into 0.6 ml, A, 0.5 ml, B, 0.5 ml, C, and 0.4 ml, D, aliquots. From A, 0.10 ml sample was withdrawn at 0 minutes. A-D were incubated at 37^0. One tenth ml samples from A were withdrawn at 5, 10, 20 and 30 minutes post incubation. Fagaronine (50 µg/ml) was added to B, C and D at 5, 10 and 20 minutes, respectively. One minute after addition of the alkaloid 0.1 ml samples were withdrawn from B, C and D at 5 minute intervals. The enzyme activity of each sample was determined. Control o–o, addition of drug after 5 minutes (●–●), 10 minutes (△–△) and 20 minutes (▲–▲).

alkaloid during reaction at 5, 10 and 20 minutes stopped the (^{3}H)TMP incorporation instantly, and there was no decrease of the synthesized product on further incubation (figure 3). The data clearly show that fagaronine stops the elongation reaction, probably by interacting with the template-primer. Moreover, these results further suggest that degradation of the enzyme product does not take place with the alkaloid.

In conclusion, fagaronine inhibits oncornavirus DNA polymerase by interacting with (rA)n,(dT)n or A:T pairs. However, it is yet to be determined whether fagaronine's anti-leukemic activity can be attributed to this property.

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