Enzyme Inhibition VI: Inhibition of Reverse Transcriptase Activity by Protoberberine Alkaloids and Structure–Activity Relationships

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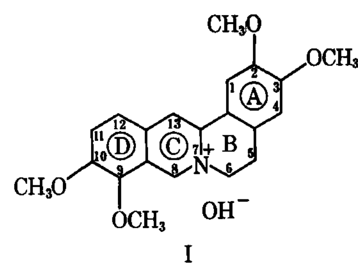
Abstract ☐ Protoberberine alkaloids such as palmatine (I), 13-methylpalmatine iodide (II), 2,3-methylenedioxy-10,11-dimethoxy-13-methylprotoberberine iodide (III), 2,3-methylenedioxy-9,10-dimethoxy-13-methylprotoberberine chloride (IV), and berberine (V) showed inhibition of reverse transcriptase activity of RNA tumor viruses in the presence of polyriboadenylic acid·oligodeoxythymidylic acid (VI), polydeoxyadenylic acid·oligodeoxythymidylic acid (VII), activated calf thymus deoxyribonucleic acid (IX), and 70S ribonucleic acid (X), but not in the presence of polyribocytidylic acid·oligodeoxyguanylic acid (VIII). These results indicated that the alkaloids caused inhibition of the enzyme activity by interacting with the template primer, particularly of the adenine–thymine base pair. Furthermore, the alkaloids competed with the template primer-binding site of the enzyme. The time course inhibition indicated that the alkaloids stopped the DNA synthesis instantly when added after the initiation of polymerization processes. Inhibition of reverse transcriptase activity was correlated with the structure and antileukemic activity of the protoberberine alkaloids.

Keyphrases ☐ Protoberberine alkaloids—inhibition of reverse transcriptase activity, reaction kinetics, structure–activity relationships ☐ Reverse transcriptase—effect of protoberberine alkaloids, reaction kinetics, structure–activity relationships ☐ Enzyme inhibition—effect of protoberberine alkaloids on viral DNA polymerase, reaction kinetics, structure–activity relationships ☐ Structure–activity relationships—protoberberine alkaloids, *in vitro* inhibition of reverse transcriptase activity

Protoberberine alkaloids are a class of isoquinoline alkaloids that possess a wide variety of biological properties (1) [*i.e.*, antimicrobial (2–4), uterine contracting or stimulating (5, 6), and anticancer (7–9)]. Berberine chloride, one of the protoberberine alkaloids has been extensively studied, and exhibits antibacterial, antifungal, and antiprotozoal properties (10, 11). It also antagonizes cholera toxin (12, 13) and possesses intercalating (14–16) and mutagenic properties (17). Recently, berberine (V), 13-methylpalmatine (II), and 2,3-methylenedioxy-10,11-dimethoxy-13-methylprotoberberine (III) were reported to possess antitumor activity against experimental tumors (18), but lacked antileukemic activity against P-388 murine lymphocyte leukemia (19).

In view of the observation that some benzophenanthridine alkaloids are potential inhibitors of reverse transcriptase activity (20–22), the structurally related protoberberine alkaloids were examined for their effect on reverse transcriptase activity of RNA tumor viruses. It was considered worthwhile to screen these alkaloids for their antireverse transcriptase activity and correlate this activity with their structures and their antileukemic activity.

Reverse transcriptase (RNA-directed DNA polymerase) enzyme was discovered in RNA tumor viruses (retroviruses) by Baltimore (23) and Temin and Mizutani (24) in 1970. Since this discovery and association of the retroviruses with leukemia, lymphoma, and sarcoma of several vertebrates including primates (25), massive literature on the isolation, characterization, cell transformation, inhibitors, *etc.*, has accumulated over the past decade (26, 27). Recent findings of retroviral information in human leukemia (28, 29) and human cutaneous T-cell lymphoma cell line (30, 31) suggests a link between the RNA tumor viruses and human cancer. Consequently, the inhibitors of viral reverse transcriptase may contribute to approaches made toward the prevention of cancer.



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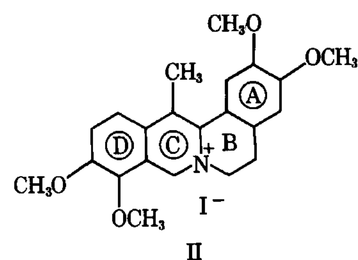

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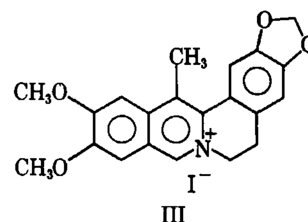

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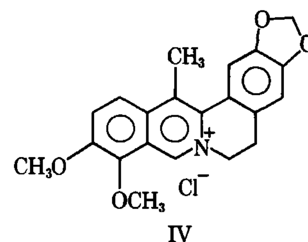

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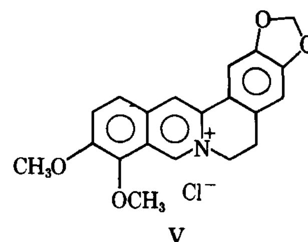

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Table I—Effect of Different Template Primers on Inhibition of AMV Reverse Transcriptase Activity by Protoberberine Alkaloid [a]

Alkaloid	Template Primer [b]	Inhibition, %
I	VI	100
	VII	99
	VIII	1
	IX	98
	X	98
II	VI	98
	VII	97
	VIII	2
	IX	97
	X	96
III	VI	95
	VII	95
	VIII	3
	IX	94
	X	94
IV	VI	97
	VII	96
	VIII	4
	IX	96
	X	95
V	VI	90
	VII	85
	VIII	5
	IX	84
	X	80

[a] Alkaloid used for each inhibition was 12 μg/assay and the standard assay conditions as published previously (21) were followed. DNA and RNA polymerases of *E. Coli* under the standard assay conditions were less sensitive than viral polymerases to alkaloid inhibition. [b] Standard assay conditions were followed using thymidine triphosphate radioactive substrate except where otherwise specified under *Experimental*. The names of template primers and templates used are given in *Materials and Methods*.

EXPERIMENTAL

Materials and Methods—Purified reverse transcriptase from avian myeloblastosis virus[1] (AMV) contained specific activity of 92,472 U/mg of protein. The enzyme preparation contained 10,072 U/ml of reverse transcriptase activity and a protein content of 0.11 mg/ml. One unit of enzyme activity was expressed as the incorporation of 1 nmole of deoxythymidine monophosphate into an acid-insoluble product in 10 min at 37°. The purity of the enzyme preparation was determined by an established procedure (32). Reverse transcriptase from Rauscher murine virus[2] (MuLV), propagated from JLS-V9 cell line had an activity of 4.00 nmoles of thymidine monophosphate incorporation/30 min/ml. DNA polymerase from simian sarcoma virus type 1[3] (SSV) was derived from tissue culture fluids of the SSV-I-NC-37 cell line and purified by a previous method (20). It had an activity of 6.00 nmoles of thymidine monophosphate incorporation/30 min/ml. The 70S RNA[4] from MuLV was purified by a published method (33). Reverse transcriptase assay and enzyme inhibition were carried out by a method previously reported (21). Appropriate concentrations of the protoberberine alkaloids[5,6] were dissolved in dimethyl sulfoxide. Control assays were performed without the alkaloids but contained an equivalent volume of dimethyl sulfoxide. The results were expressed as the percent of control activity.

The protoberberine alkaloids tested were palmatine[5] (I,NSC 209407), 13-methylpalmatine iodide[6] (II), 2,3-methylenedioxy-10,11-dimethoxy-13-methylprotoberberine iodide[5] (III,NSC 276348), 2,3-methylenedioxy-9,10-dimethoxy-13-methylprotoberberine chloride[6] (IV), and berberine chloride[5] (V,NSC 163088).

The template primers or templates used were polyriboadenylic acid·oligodeoxythymidylic acid (VI), polydeoxyadenylic acid·oligodeoxythymidylic acid (VII); polyribocytidylic acid·oligodeoxyguanylic acid (VIII) (2.00 nmoles of [^{3}H]deoxyguanosine triphosphate, 380 cpm/mole substrate), activated calf thymus DNA (IX) (2.20 μg activated deoxyribonucleic acid, 10 nmoles each of deoxyadenosine, deoxycytidine, and deoxyguanosine triphosphates and 2.50 nmoles of [^{3}H]thymidine triphosphate substrates); 70S ribonucleic acid (X) (MuLV) of 0.05 optical density units (260 nm).

[1] Life Science Research Labs., St. Petersburg, Fla., through the courtesy of Dr. J. Beard.
[2] Bionetics Laboratory Products, Kensington, Md.
[3] Pfizer, Inc., Maywood, N.J.
[4] Gift from Dr. M. Reitz, Litton Bionetics, Bethesda, Md.
[5] Drug Synthesis and Chemistry, Division of Cancer Treatment, NCI, Bethesda, Md.
[6] Dr. Mark Cushman, Purdue University, West Lafayette, IN 47907.

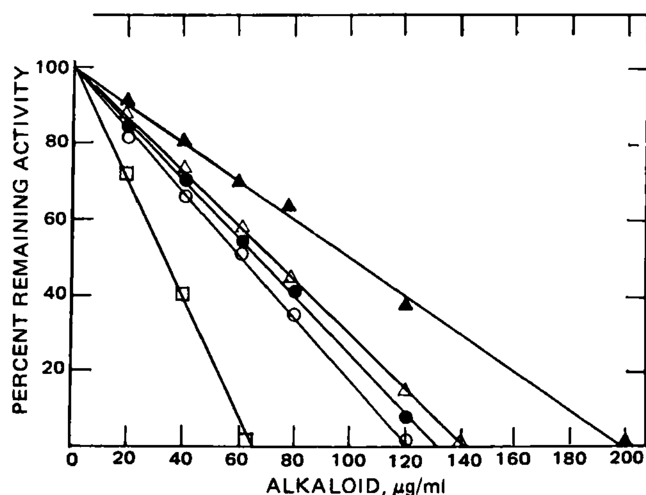

Figure 1—*Effect of protoberberine alkaloids on AMV reverse transcriptase activity. In a standard assay mixture (0.10 ml) containing 5 μl of enzyme, different concentrations of alkaloids I (□), II (○), III (●), IV (△), and V (▲) were used for enzyme inhibition.*

RESULTS AND DISCUSSION

The *in vitro* inhibition of AMV reverse transcriptase activity by protoberberine alkaloids I–V in the presence of template primer (VI) is shown in Fig. 1. Fifty percent enzyme inhibition by alkaloids I–V was in the range of 30–35 μg/ml (I), 60–65 μg/ml (II), 65–70 μg/ml (III), 70–75 μg/ml (IV), and 100–105 μg/ml (V). Similar results were obtained on MuLV and SSV reverse transcriptase activity (data not shown). Based on the nature of inhibitory curves and 50% inhibition of enzyme activity, alkaloids I, II–IV, and V were classified as potent, moderate, and very weak inhibitors of reverse transcriptase activity, respectively. Purified AMV, MuLV, and SSV reverse transcriptase activities were also inhibited by these protoberberine alkaloids in the presence of VII, IX, and X template primers, but a very low degree of inhibition of enzyme activity was observed when VIII template primer was used (Table I). The different percentage of inhibition of enzyme activity observed in the presence of these template primers indicated that the inhibition of the enzyme activity was due to the interaction of alkaloids with template primers and not with the enzyme protein; otherwise the same percentage of inhibition of enzyme activity would have been observed regardless of the different template primers used. A very low degree of inhibition of enzyme activity in the presence of VIII as compared with VI, VII, IX, and X was indicative of the strong binding affinity of alkaloids with the adenine–thymine base

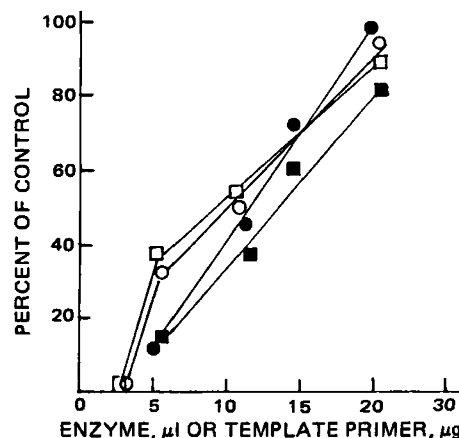

Figure 2—*Effect of increasing concentrations of AMV reverse transcriptase or template primer on protoberberine alkaloid-inhibited reaction mixture. The standard assay mixture (0.10 ml) contained 5 μl of AMV reverse transcriptase and 6 μg of I (●) or 12 μg of III (■) or 2.5 μg of VI template primer and 6 μg of I (○) or 12 μg of III (□).*

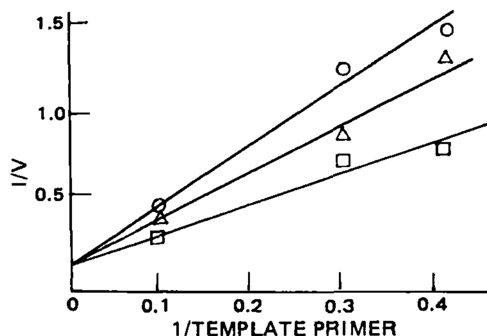

Figure 3—*Double-reciprocal plot (Lineweaver-Burk plot) of 1/velocity versus 1/template primer shown by alkaloid I. Units of velocity (V) are expressed as counts per min × 10^{-4} of methyl-[^{3}H]thymidine monophosphate incorporated into DNA synthesis. Concentrations of template primer (VI) are expressed in μg/ml. Alkaloid I concentrations were 0 (□); 0.3 μg/ml (△); 0.9 μg/(○) in the standard assay mixture.*

pair template primers. The interaction or competition of alkaloids with manganese chloride was ruled out, because the increasing concentration of this metal ion in the assay mixture did not change the enzyme activity (*data not shown*). The possibility of interaction of alkaloids with potassium chloride or substrate ([^{3}H]thymidine or guanosine triphosphate) was not considered, since the latter was present in the reaction mixture in 8- or 25-fold, respectively, in excess of the ID_{50} of the alkaloid concentration.

To explain further the mode of action of alkaloids I and III, the effect of increasing the concentrations of AMV reverse transcriptase enzyme or template primer (VI) on the alkaloid-inhibited reaction mixture was observed, as shown in Fig. 2. The increasing concentration of the enzyme from 5 to 20 μl or template primer from 3 to 20 μg in the assay mixture resulted in the full restoration of enzyme activity of the alkaloid-inhibited reaction mixture. Similar results were shown by alkaloids II, IV, and V (data not shown). These results could be due to the dissociation of the enzyme–template–alkaloid ternary complex in the reaction mixture in the presence of excess template primer or enzyme. The data support the previous observation that the alkaloids interacted with the template primer. The Lineweaver–Burk plot (Fig. 3) obtained by alkaloid I was of a competitive type indicating interaction of the alkaloid with the template primer binding site of the enzyme. Similar plots were obtained in the case of alkaloids II–IV (data not shown). The time course of inhibition of reverse transcriptase activity by alkaloids I and III is shown in

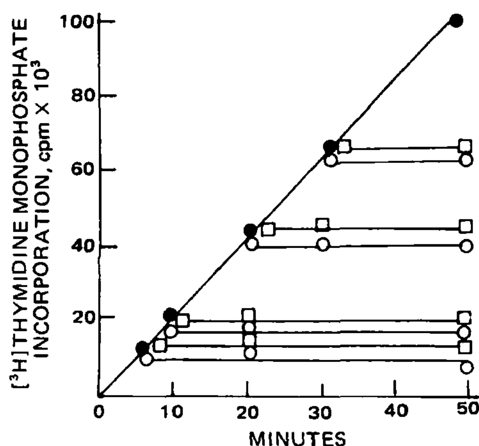

Figure 4—*Effect of addition of protoberberine alkaloids during AMV reverse transcriptase kinetic reaction. For each assay, 2 ml of standard assay mixture containing 100 μl of enzyme was divided into four parts: A(0.60 ml), B(0.50 ml), C(0.50 ml), D(0.40 ml). From A, a 0.10-sample was withdrawn at zero min, and the remaining quantity was incubated at 37°. One-tenth-milliliter samples from A(control, ●) were withdrawn at 5, 10, 20, 30, and 50 min after incubation. Parts B, C, and D were also incubated at 37°, and 60 μg/ml or 120 μg/ml solutions of alkaloid I (○) or III (□), respectively, were added at 5, 10, 20, and 30 min after incubation. One minute after addition of the alkaloid, 0.10-ml samples were withdrawn from B, C, and D at 5-min intervals. Radioactivity of each sample was determined.*

Table II—Effect of Functional Groups of Protoberberine Alkaloids on Inhibition of Reverse Transcriptase Activity

Alkaloid	50% Inhibition, μg/ml	Functional Group[a]
I	32	Maximum inhibition due to methoxyl groups at positions 2, 3, 9, and 10
II	62	Decrease of inhibition due to methyl group at position 13
III	67[b]	Increase of inhibition due to methoxyl groups at positions 10 and 11 as compared with positions 9 and 10
IV	72	Decrease of inhibition due to methyl group at position 13
V	102	Decrease of inhibition due to methylenedioxy group at positions 2 and 3

[a] For functional groups refer to the individual structure of the alkaloids. [b] This increase in 50% inhibition is relative to the inhibition exhibited by alkaloid IV. Other 50% inhibitions are compared with the maximum inhibition displayed by alkaloid I.

Fig. 4. The controlled activity of the reaction mixture was determined without the alkaloids. During the time course of the reaction, the alkaloids were added at 10, 20, 30, and 50 min after initiation of DNA synthesis. As soon as the alkaloids were added, the enzyme activity was abruptly changed, as indicated by the [^{3}H]thymidine monophosphate incorporation. Further incubation of the reaction mixture did not change the kinetics of DNA synthesis or degrade the product. The immediate cessation of the polymerization reaction by the alkaloids may be due to the interaction of the alkaloids with the template primer. Similar results were obtained with alkaloids II, IV, and V (data not shown).

The individual members of the protoberberine alkaloids showed different degrees of inhibition of reverse transcriptase activity, although the mode of action was similar in each case. Therefore, variation in 50% inhibition of enzyme activity by the protoberberine alkaloids could be attributed to the structure of the alkaloids. A maximum of 50% inhibition of enzyme activity (32 μg/ml) was observed in alkaloid I (base or iodide), which has two methoxyl groups at positions 2 and 3 of ring A and two methoxyl groups at positions 9 and 10 of ring D (Table II). In the structure of alkaloid I, when an additional methyl group was present at position 13 of ring C, 50% enzyme inhibition by alkaloid II was decreased considerably (62 μg/ml) indicating the influence of a methyl group. On the other hand, with substitution of a methylenedioxy group at positions 2 and 3 of ring A in place of methoxyl groups at these positions of alkaloid I, 50% inhibition of enzyme activity by alkaloid V was almost lost (102 μg/ml). However, the presence of a methyl group at position 13 of ring C resulted in an increase of 50% enzyme inhibition (72 μg/ml) by alkaloid IV (Table II). Alkaloid III showed slightly more enzyme inhibition (67 μg/ml) than alkaloid IV, although two methoxyl groups in alkaloid III were present at positions 10 and 11 of ring D. Whether the presence of methoxyl groups at positions 9 and 10 or positions 10 and 11 of ring D play an important role in the inhibition of enzyme activity is not yet conclusively determined. However, previous results in structurally related benzophenanthridine alkaloids (22) indicated that the presence of substituent groups at positions analogous to 10 and 11 of ring D were important to exhibit increased inhibition of reverse transcriptase activity. In fact, alkaloid III was a better inhibitor of reverse transcriptase activity than alkaloid IV, which indicated that the presence of methoxyl groups at positions 10 and 11 of ring D (alkaloid III) increased the inhibition of enzyme activity. In any case, the present investigation reveals that the placing of methoxyl groups at positions 2 and 3 of ring A, regardless of such groups at positions 9 and 10 or 10 and 11 of ring D, was an essential requirement in possessing the inhibitory effect of the alkaloids.

The cytotoxic alkaloids I–V displayed an effective antitumor activity against experimental tumors (18) but did not show antileukemic activity against P-388 lymphocytic leukemia in mice (19). Earlier studies have indicated that the inhibition of reverse transcriptase activity by benzophenanthridine alkaloids corresponded well with their antileukemic activity (22). Surprisingly, except alkaloid V, protoberberine alkaloids failed to exhibit such a correlation. The lack of antileukemic activity of alkaloid V was, thus, in agreement with the observation that this alkaloid exhibited very weak inhibition of reverse transcriptase activity. However, lack of antileukemic activity of alkaloids I–IV, but possession of inhibition of reverse transcriptase activity by these alkaloids, could not be explained at the present time. Further work is in progress to establish the relationship of enzyme inhibition and antileukemic or antitumor activity.

Alkaloids I–V showed less inhibition of reverse transcriptase activity compared with the analogous benzophenanthridine alkaloids. Such a difference in reverse transcriptase inhibition by the protoberberines and benzophenanthridines could be due to the stereochemistry, presence of *N*-methyl (—N—CH_3) group in ring C and/or influence of counterions of different salts of benzophenanthridine alkaloids. A previous report related an antileukemic activity of certain cytotoxic protoberberine alkaloids with the conformations and DNA binding properties of the alkaloids (19). Other studies reported that the biological activity of alkaloid V (34) (and a few protoberberines) was due to their binding to double-helical DNA by intercalation (35, 36). Furthermore, protoberberine alkaloids were reported to inhibit a number of enzymes such as NADH oxidase (37), horse liver alcohol dehydrogenase (38, 39), and xanthine oxidase (40) by different mechanisms. The present study reports inhibitory effect of protoberberine alkaloids on another enzyme (reverse transcriptase) for the first time. It would be worthwhile to investigate the exact mechanism of action of protoberberine alkaloids in order to justify the cytotoxicity and diverse biological activities of the alkaloids.

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