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BIOCHEMICAL EFFECTS OF *d*-TETRANDRINE AND THALICARPINE

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Abstract—The benzylisoquinoline alkaloids *d*-tetrandrine and thalicarpine inhibit the biosynthesis of DNA, RNA and proteins, when incubated with S180 cells *in vitro*. Oxidation of glucose[^{14}C] to $^{14}CO_2$ was not affected by either alkaloid at levels up to 100 μg/ml *in vitro*. Incorporation of labeled acetate into lipids was inhibited only by thalicarpine at 100 μg/ml. Inhibition of the incorporation of thymidine into DNA was also observed *in vivo* after treatment with these drugs at 30–120 mg/kg; under these conditions, the synthesis of RNA and protein was not inhibited. In an attempt to elucidate the mechanism for inhibition of nucleic acid synthesis, the interaction of DNA, RNA and polynucleotides with the alkaloids was studied by gel filtration and dialysis. The two drugs associated with both DNA and RNA, but exhibited different affinities for the five polynucleotides examined. Both alkaloids were bound by polyguanylic and polyadenylic acids, but whereas *d*-tetrandrine associated only poorly with polythymidylic acid and not at all with polyuridylic acid, it was polycytidylic acid that showed no affinity for thalicarpine.

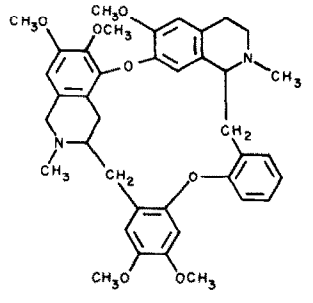
The benzylisoquinoline alkaloids *d*-tetrandrine and thalicarpine (Fig. 1) are pharmacologically active agents derived from members of the plant families *Menispermaceae* and *Ranunculaceae* (*Thalictrum* species) respectively. *d*-Tetrandrine has been described as having a *d*-tubocurarine-like effect [1] and anti-inflammatory activity [2], while thalicarpine shows a cardiotoxic action [3]. However, it was the reported activity of these compounds against Walker 256 carcinosarcoma in the rat [4, 5] and KB cells in monolayer culture [6] that led to their selection for clinical evaluation in the treatment of human cancers. Although the monomeric benzylisoquinolines and aporphines were devoid of antitumor activity, suggesting that a dimeric type of structure was needed, there seemed to be no stereospecificity requirements for biological activity among a series of bis (benzylisoquinolines) or aporphine-benzylisoquinoline derivatives studied [7]. In experiments with L1210 mouse leukemia cells in culture, thalicarpine was generally inhibitory for macromolecular biosynthesis, with DNA synthesis as the most sensitive parameter; no evidence was obtained for binding of the drug by DNA [8]. However, the same authors (Allen and Creaven [9]) subsequently found evidence of such binding by calf thymus DNA. Preliminary clinical pharmacological data indicate that *d*-tetrandrine is also an inhibitor of the incorporation of thymidine into DNA, in this case by human leukemic leukocytes *in vitro* [10].

The present study was carried out in order to obtain further information on mechanisms of action and to compare the biochemical effects of these two alkaloids. Inhibition of macromolecular synthesis seen in these experiments may result, at least in part, from association of the drugs with nucleic acids.

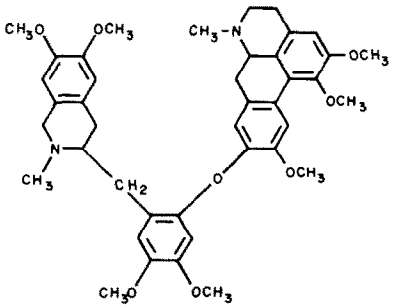
MATERIALS AND METHODS

Thalicarpine and *d*-tetrandrine were supplied by the National Cancer Institute, Bethesda, Md. Calf thymus DNA type I, polycytidylic, polythymidylic, polyguanylic, polyadenylic and polyuridylic acids were purchased from the Sigma Chemical Co. *d*-Tetrandrine[^{14}C] was synthesized by Monsanto Corp. and supplied by the National Cancer Institute. Thymidine-methyl[^{3}H], uridine[5-^{3}H], glycine[1-^{14}C] and sodium acetate[2-^{14}C] were purchased from the New England Nuclear Corp., and glucose[U-^{14}C] was purchased from CalBiochem.

RNA (combined ribosomal and transfer) was isolated from S180 cells, carried in CD-1 mice (Charles



D-TETRANDRINE



THALICARPINE

Fig. 1. Structural formulas of *d*-tetrandrine and thalicarpine.

River Breeding Laboratories). After freeing them from erythrocytes by exposure to hypotonic saline, the tumor cells were suspended in 0.01 M NaCl containing 0.01 M sodium phosphate buffer, pH 6.7, and sonified for 1 min with a Biosonic II. An equal volume of 90% phenol was then added, the mixture stirred for 1 hr at room temperature and centrifuged for 30 min at 7000 rev/min. The aqueous layer was removed, the lower phase re-extracted with an equal volume of buffered saline, and after centrifuging, the combined aqueous fraction was made to 2 per cent (w/v) with potassium acetate and RNA precipitated by adding 2 vol. ethanol. This crude material was reprecipitated twice more under the same conditions and dialyzed overnight against 0.03 M NaCl.

For dialysis experiments, DNA, RNA, synthetic polynucleotides and serum albumin (0.5 to 5 mg) were dissolved in 0.03 M NaCl solution together with varying amounts of *d*-tetrandrine or thalicarpine in a total volume of 10 ml. This solution (pH 4) was then placed inside dialysis tubing and dialyzed for periods of up to 26 hr against 100 ml of 0.03 M NaCl solution at room temperature in the dark. Equilibrium was achieved normally in 18–20 hr. The absorbance of the dialysate was read at frequent intervals with a Zeiss PMQ II spectrophotometer, using wavelengths of 282 nm for *d*-tetrandrine and 285 nm for thalicarpine. All samples were returned to the vessel after reading. In some experiments with *d*-tetrandrine, ^{14}C-labeled material was used, both as a control for the u.v. absorbance assay, and to determine directly the amount of drug bound by polynucleotide.

Gel filtration was carried out on 30 × 1 cm columns of Sephadex G-25 with 0.005 M NaCl at pH 4 as the eluent. Solutions of nucleic acid, protein or polynucleotide (0.3 mg) in 1 ml of 0.005 M NaCl were allowed to stand 30 min at room temperature and then applied to the columns. Fractions (2 ml) were collected, the absorbancies were read at 260 nm, and samples were counted in a Packard Tri-Carb liquid scintillation spectrometer.

The synthesis by S 180 cells *in vitro* of nucleic acids, residual proteins and lipids was measured by incorporation of thymidine-methyl[^3H] (1 μCi, 6.7 Ci/m-mole), uridine[5-^3H] (1 μCi; 3 Ci/m-mole), glycine-[1-^{14}C] (1 μCi; 44.9 mCi/m-mole) and sodium acetate[2-^{14}C] (2.5 μCi; 50 mCi/m-mole), respectively, using procedures that have been described previously [11, 12]. Incubation volumes were 3 ml (with 12% dialyzed calf serum and phosphate buffer [13]), and approximately 2.5 × 10^7 cells were used per flask. Time courses of up to 60 min were followed after an initial preincubation with drug but no tracer for 15 min. Oxidation of glucose was measured by incubating S180 cells with glucose[U-^{14}C] (0.05 μCi; 55 mCi/m-mole) in 3 ml of serum buffer in Warburg flasks. The reaction was stopped by tipping in perchloric acid (0.3 ml; 1 M) from the side arm; the $^{14}CO_2$ released during the incubation was collected on filter paper moistened with 0.1 ml of 1 N NaOH in the center well.

Uptake of nucleosides was measured in similar incubation systems to those used for studying incorporation, but at the end of the uptake period, samples were removed, layered over 0.25 M sucrose solution in Shevky–Stafford and McNaught tubes, and centrifuged for 3 min at 1600 *g*. The supernatant fluids were aspirated carefully and the radioactivity in the cell pellets was determined after suspending them in 0.2 ml water.

For experiments with S180 cells *in situ* in mice, the tumor-bearing animals received i.p. injections of thalicarpine or *d*-tetrandrine (30, 60 or 120 mg/kg) followed 0.5, 5.5 or 23.5 hr later by radioactive precursors (2 μCi; same specific activities as for studies *in vitro*). A period of 0.5 hr (1 hr for lipids) was allowed for metabolic utilization. The mice were killed, ascitic fluid was removed, red cells were lysed by exposure to hypotonic saline, and the tumor cells were extracted as for the studies carried out *in vitro*. Cell counts (Coulter counter model B) or DNA contents measured by the diphenylamine reaction [14] served for standardization.

RESULTS

Inhibition of biosynthetic pathways. The effects of *d*-tetrandrine and thalicarpine on the incorporation of radioactive precursors into DNA, RNA and residual protein by S180 cells *in vitro* are shown in Figs. 2 and 3. It is evident that DNA synthesis is the parameter most sensitive to thalicarpine, whereas the incorporation of precursors into DNA and RNA is about equally affected by *d*-tetrandine. The concentrations (in μg/ml) of thalicarpine and *d*-tetrandine, respectively, that were required to produce 50 per cent inhibition were: DNA, 14 and 46; RNA, 45 and 50; and protein, 76 and 80. Uptake of nucleosides was not inhibited. The oxidation of glucose[U-^{14}C] to $^{14}CO_2$ was unaffected by either drug at levels up to 100 μg/ml. Reproducible inhibition of acetate incorporation into lipids by S180 cells *in vitro* was only achieved with thalicarpine at 100 μg/ml. At this level, incorporation into neutral and phospholipid, respectively, was 26 and 61 per cent of control values.

When mice bearing the S180 tumor were treated with thalicarpine, the incorporation of thymidine into DNA was inhibited after dosages of 30 or 60 mg/kg,

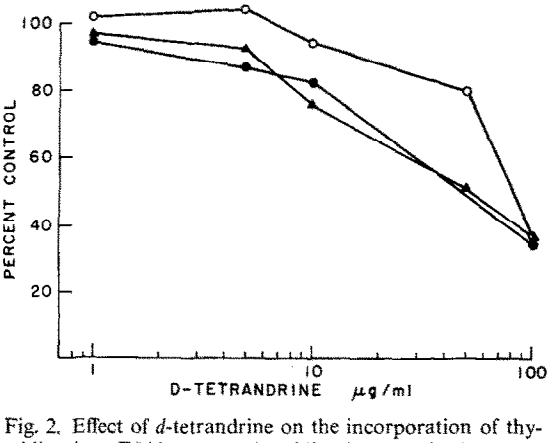


Fig. 2. Effect of *d*-tetrandrine on the incorporation of thymidine into DNA (●——●), uridine into RNA (▲——▲), and glycine into protein (○——○) of S180 cells incubated *in vitro*. Data are expressed as percentage of control values which were: DNA, 1618; RNA, 1900; protein 2410 cpm/10^6 cells.

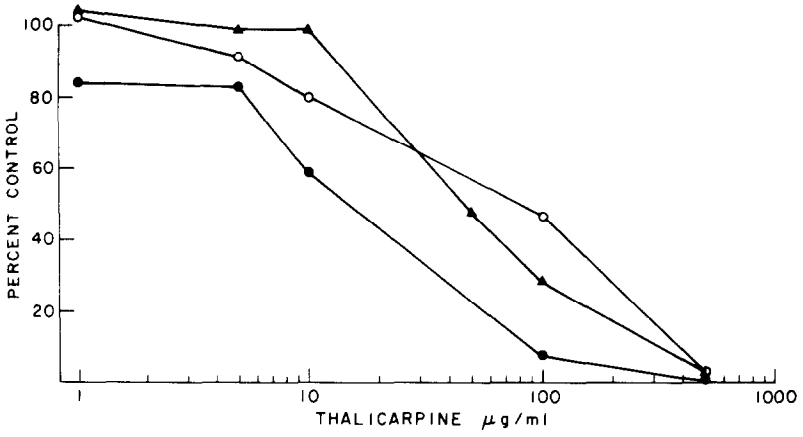


Fig. 3. Effect of thalicarpine on the incorporation of thymidine into DNA (●——●), uridine into RNA (▲——▲), and glycine into protein (○——○) of S180 cells incubated *in vitro*. Data are expressed as percentage of control values as listed in Fig. 2.

whereas a level of 120 mg/kg of *d*-tetrandrine was required to give minimal inhibition (Table 1). The incorporation of uridine into RNA, glycine into protein, and acetate into lipids was unaffected at these drug dosages.

Interaction with nucleic acids and polynucleotides. When solutions of DNA, RNA or several synthetic polynucleotides to which thalicarpine had been added were dialyzed, passage of drug across the membrane was retarded, and a lower external equilibrium concentration of free drug attained than for controls without polynucleotides. This effect was not seen with bovine serum albumin fraction V or polycytidylic acid. Similar effects were also observed in analogous experiments with *d*-tetrandrine, but in this case polyuridylic acid did not interact with drug at all and polythymidylic acid only minimally (Table 2). The same findings were made at other concentrations of drug. Using *d*-tetrandrine[^{14}C] in gel filtration experiments, this lack of affinity for polyuridylic acid was confirmed (Fig. 4). Only in the case of polyguanylic acid were significant ranges of drug and polymer concentrations examined by equilibrium dialysis to enable Scatchard plots [15] to be made (Fig. 5). The intercepts were such as to suggest that 51 and 104 moles thalicarpine and *d*-tetrandrine, respectively, were bound/mole (150,000 daltons) of polynucleotide, corresponding to 0.12 and 0.24 binding sites/nucleotide. The dissociation constants were calculated as 1.43×10^{-5} moles/liter for thalicarpine and 1.08×10^{-5} moles/liter for *d*-tetrandrine.

DISCUSSION

It is evident that similarities in the chemical structures of these two alkaloids are reflected in their biochemical actions. Incorporation of precursors into nucleic acids and proteins is inhibited at drug levels that are attainable *in vivo* [see Ref. 10, for example]. For thalicarpine, DNA synthesis is the most sensitive process in these as in earlier [8] studies. The other parameters examined—oxidation of glucose, incorporation of acetate into lipids, and total uptake of nucleosides by cells—were either unaffected or inhibited only at very high, unphysiological, levels of drug. The similarity in the dose response curves for incorporation of precursors into RNA and protein *in vitro* suggests an interdependence, although it cannot be ruled out that the protein effect is a separate drug action. The overall relevance of effects on macromolecular synthesis to cytotoxicity has not, of course, been established by these studies.

Table 1. Effect of treatment with alkaloids *in vivo* on the incorporation of thymidine into the DNA of S180 cells

Drug	Dose (mg/kg)	Time (hr)	Per cent inhibition*
Thalicarpine	30	1	11.8
		6	27.0
		24	48.4
Thalicarpine	60	1	35.4
		6	51.7
		24	83.1
d-Tetrandrine	120	1	20.7
		6	28.0
		24	10.9

* Mean incorporation of thymidine in untreated mice was $1.80 \pm 0.36 \times 10^5$ dis./min/10^6 cells. Animals received drugs i.p. at time zero and thymidine[^{3}H] (2 μCi; 6.7 Ci/m-mole) 0.5 hr before sacrifice.

Table 2. Effects of biopolymers on the rates and equilibria of alkaloid dialysis

Drug	Polymer*	Initial dialysis rate† (moles × 10^{-7} hr^{-1})	Drug bound at equilibrium (moles × 10^{-7})
Thalicarpine‡	None	9.08	
	pA	3.44	5.10
	pC	6.73	
	pG	3.02	10.10
	pU	3.75	9.18
	DNA	6.26	7.46
	RNA	8.91	4.28
	Protein	11.10	
d-Tetrandrine‡	None	10.17	
	pA	2.58	19.01
	pC	6.66	6.83
	pG	3.62	20.02
	pU	8.85	
	DNA	2.83	1.31
	RNA	4.42	2.19
	Protein	9.83	
Thalicarpine§	None	13.80	
	pT	9.80	4.36
d-Tetrandrine§	None	15.02	
	pT	13.81	0.61

* Abbreviations: pA, polyadenylic acid; pC, polycytidylic acid; pG, polyguanylic acid; pU, polyuridylic acid; pT, polydeoxythymidylic acid.
† Measured during the first 0.75 hr of dialysis.
‡ The system contained 5 mg polymer and 2 mg drug.
§ The system contained 0.5 mg polymer and 2 mg drug.

Association of drug with nucleic acids could serve as the underlying mechanism for inhibiting both nucleic acid and protein synthesis. In agreement with findings made elsewhere [8, 9], we were able to detect association of thalicarpine with DNA by equilibrium dialysis, but not by spectrophotometric assay. This applies also to the interactions with RNA and synthetic polynucleotides. It is interesting that the two alkaloids differ significantly in their base specificities. Both drugs associate effectively with purine bases, showing some preference for guanine. However, whereas thalicarpine does not associate with cytosine moieties, *d*-tetrandrine interacts only poorly with thymine and not at all with uracil residues. There is some

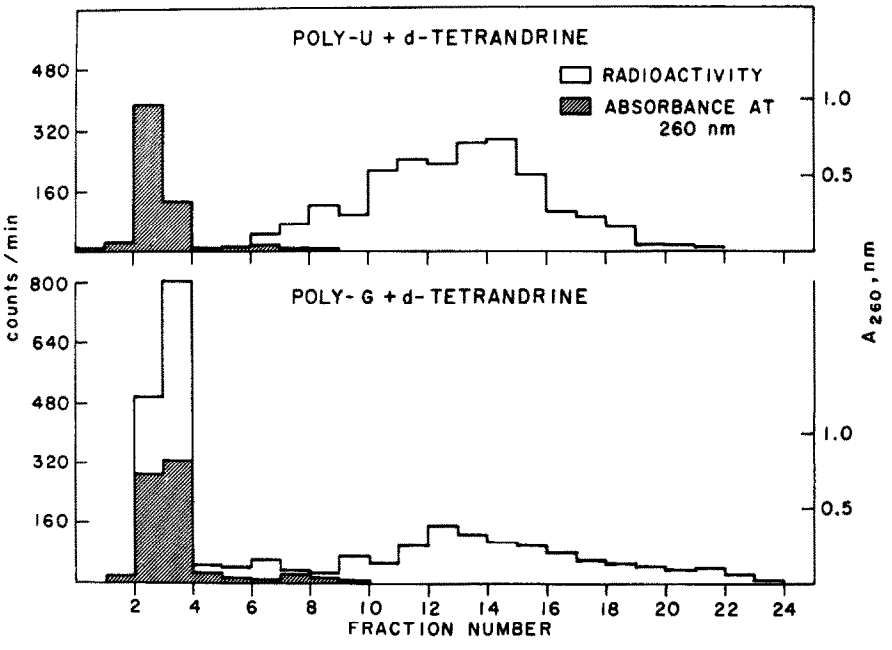


Fig. 4. Gel filtration of mixtures of *d*-tetrandrine[^{14}C] (0.2 mg, 2400 cpm) with 0.3 mg of polyguanylic or polyuridylic acids on 30 × 1 cm columns of Sephadex G-25. Fractions (2 ml) were collected during elution with 0.005 M NaCl.

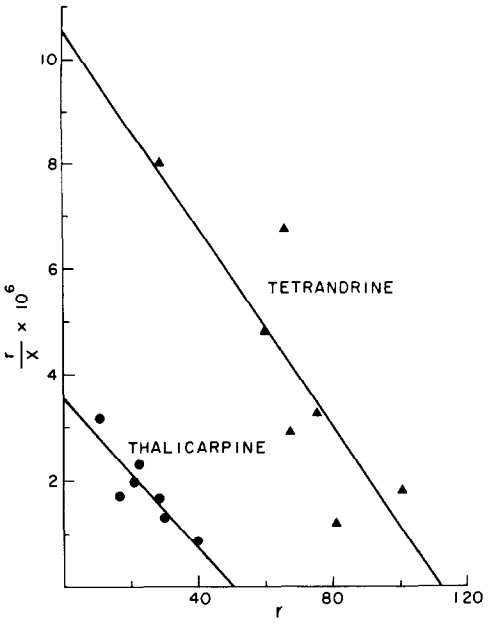


Fig. 5. Scatchard plots of the ratio of moles thalicarpine (●——●) and *d*-tetrandrine (▲——▲) bound/mole of polyguanylic acid to equilibrium concentration of free drug (r/x) vs moles of drug bound/mole of polymer (r). The intercept on the abscissa gives the maximum number of molecules of guanylic acid (n), while the intercept on the ordinate gives n divided by the equilibrium dissociation constant of the complex.

inconsistency between the relative binding of the two alkaloids by native nucleic acids and by synthetic polynucleotides (Table 2). The amount of *d*-tetrandrine bound by the two nucleic acids is routinely less than thalicarpine, whereas this is not true for polyadenylic, polyguanylic and polycytidylic acids. It is possible that the conformation of the native nucleic acids is such as to reduce the ability of the more rigid *d*-tetrandrine molecule, with its completed 16-membered inner ring (Fig. 1), to associate with the component bases. This might also account for the generally weaker inhibitory effects of *d*-tetrandrine on nucleic acid synthesis.

Direct comparison of the binding of these benzylisoquinoline alkaloids by polynucleotides with data obtained for other DNA binding agents is difficult, both because of different experimental conditions, and because our quantitative results apply only to polyguanylic acid. However, DNA has been shown to have 0.12 binding sites for daunorubicin/nucleotide [16], close to our figures for *d*-tetrandrine and thalicarpine of 0.24 and 0.12 respectively. The equilibrium binding constants for DNA with a number of actinomycins are close to 3×10^6 [17], giving equilibrium dissociation constants of about 0.3×10^{-6} moles/liter. Thus, the complexes of *d*-tetrandrine and thalicarpine with polyguanylic acid would be less stable than the actinomycin–DNA complexes, whose equilibrium dissociation constants are lower by more than an order of magnitude.

Quite apart from its significance in terms of the mechanism of action of these agents, binding to nucleic acids may be important in determining their distribution and excretion patterns. In patients treated with *d*-tetrandrine, there is prolonged retention of the drug coupled with slow urinary excretion [10], and the peripheral blood leukocytes are able to concentrate the drug to levels many times higher than those in the coincident plasma samples. Binding to nucleic acids may be a factor that influences this, as it is for other drugs such as chloroquine [18]. An additional feature involved in this distribution pattern is binding by plasma protein, which in the case of *d*-tetrandrine involves 58–72 per cent of total drug in the plasma [10]. Since in the present studies purified serum albumin fraction V did not bind *d*-tetrandrine, another plasma protein component must be responsible for the clinical findings.

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