BINDING OF MAYTANSINE TO RAT BRAIN TUBULIN

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Summary: Maytansine and vincristine binding to crude rat brain tubulin is a temperature- and ionic strength-dependent process. The K_A values for binding of vincristine and maytansine are 0.5×10^6 M^{-1} and 1.5×10^6 M^{-1}, respectively. Maytansine competitively inhibits vincristine binding with a K_i for maytansine of 0.4×10^{-6} M. The binding of both drugs is easily reversible. Unlabeled maytansine rapidly displaces the labeled drug whereas only about 75% of the bound maytansine was displaced by vincristine. Bound labeled vincristine is completely displaced by either vincristine or maytansine. Both drugs appear to share a common binding site, although an additional site specific for maytansine seems to be present. Colchicine did not affect either vincristine or maytansine binding.

Maytansine is an ansa macrolide of plant origin first isolated by Kupchan et al. (1). It possesses a significant antitumor activity, inhibiting the growth of the experimental tumors P388 lymphocytic leukemia and B16 melanocarcinoma in mice (1,2). L1210 cells when exposed to a low concentration of maytansine (10^{-8} M) show an increase in mitotic index, with the cells arrested in metaphase (3,4). The drug interferes with the formation of microtubules and also causes microtubule depolymerization (5,6). Its inhibitory action on dividing cells resembles that of colchicine and the vinca alkaloids (7,8). In sea urchin eggs maytansine was ca. 100-fold more effective than vincristine in inhibiting mitosis, whereas in vitro the polymerization of tubulin was inhibited by both drugs to almost the same extent (5). A possible similarity in the mode of action of the two drugs was also indicated by a cross-resistance to maytansine of a P388 cell line resistant to vincristine (3).

The similarity in biological activities of maytansine and vincristine

prompted us to study the binding of maytansine to microtubular protein, and to ascertain whether it shares a common binding site with vincristine.

MATERIALS AND METHODS

Adult male Sprague-Dawley rats (150-200 g) were decapitated, the brains removed and washed with 10 mM sodium phosphate buffer (pH 6.5) supplemented with 100 mM sodium glutamate. The brains were then homogenized at 4°C in a teflon-glass homogenizer with 3 volumes of the same buffer. The homogenate was centrifuged at 100,000 x g for 45 min at 4°C. The supernatant was used as a source of tubulin (9). Upon storage at -40°C for about 10 days no significant loss of vincristine-binding activity could be observed. Refrigeration of the protein (at 4°C) for 24 hr resulted in an 80% decrease in binding activity. Protein concentration was determined by the method of Lowry (10) with bovine serum albumin as standard.

[^{3}H]-Vincristine, specific activity 42.5 mCi/mmole, was prepared by Dr. James P. Kutney, University of British Columbia and repurified by high pressure liquid chromatography by Dr. Manford Castle, National Cancer Institute, NIH. Maytansine (NSC-153858) was provided by Dr. Harry Wood, Drug Research and Development, Division of Cancer Treatment, National Cancer Institute. Colchicine [ring C, methoxy-^{3}H], specific activity 18.5 Ci/mmole, and [^{3}H] maytansine, were from New England Nuclear (Boston, Mass.). The latter was purified using TLC plates, silica gel 60, thickness 0.25 mm (EM Reagents), with 10% ethanol in chloroform as solvent, and the specific activity found to be 0.3 Ci/mmole. For convenience of assay, the labeled maytansine and colchicine were diluted in the respective unlabeled drugs.

The binding assay mixture contained brain protein, 1 mg in 10 mM sodium phosphate buffer (pH 6.5) supplemented with 100 mM sodium glutamate or sodium chloride, in a total volume of 1 ml. After temperature equilibration in a water bath at 37°C, the drug (maytansine or vincristine) was added in a volume of 0.05 ml. The drug-tubulin complex was adsorbed on buffer-moistened DEAE-cellulose filters 2.5 cm diameter, Whatman DE81, as previously described by Borisy (11). To remove unbound drug, the filters were washed twice with 3 ml portions of the same buffer at 4°C, under mild suction. The filters were dried and counted in scintillation vials containing 10 ml Spectrafluor PPO-POPOP liquid scintillator (Amersham) diluted in toluene.

RESULTS

Effect of temperature and ionic strength on vincristine and maytansine binding.

Fig. 1 demonstrates that maytansine as well as vincristine binding is temperature-dependent, exhibiting an optimum at 36-38°C and a complete inactivation of the binder at 60°C. The effect of ionic strength on binding is somewhat different for the two. Whereas the binding of vincristine in the absence of sodium chloride is only about 30% of that seen at the optimal sodium chloride concentration, the binding of maytansine in the absence of sodium chloride is about 75% of that at the optimal sodium chloride concentration and

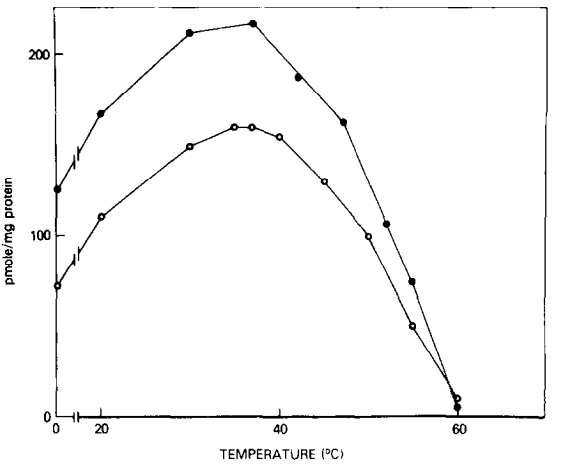

Figure 1. Effect of temperature on binding of vincristine and maytansine. Each reaction mixture consisting of 1 mg brain protein in 10 mM sodium phosphate buffer (pH 6.5) supplemented with 100 mM sodium glutamate was equilibrated in a thermostated water bath prior to initiation of the reaction by addition of [^{3}H] vincristine, 0.5 μM (○) or [^{3}H] maytansine, 0.5 μM (●). After 5 min incubation the binding was terminated following the procedure described in Materials and Methods.

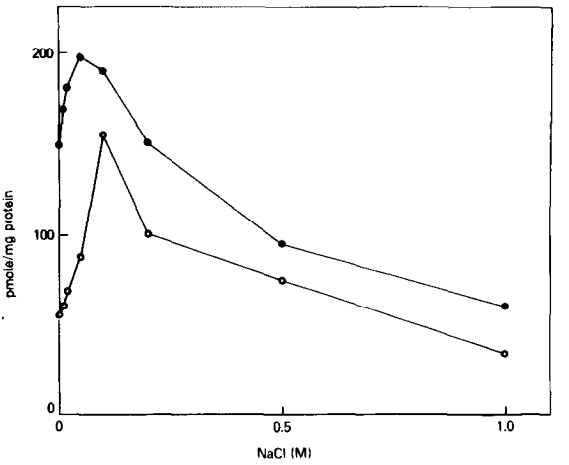

Figure 2. Effect of ionic strength on binding of vincristine and maytansine. The reaction mixtures containing various concentrations of sodium chloride were incubated at 37° for 5 min. Other experimental conditions and symbols were as in Figure 1.

decreases at salt concentration above 0.05 M. Vincristine binding exhibits an optimum at 0.1 M sodium chloride.

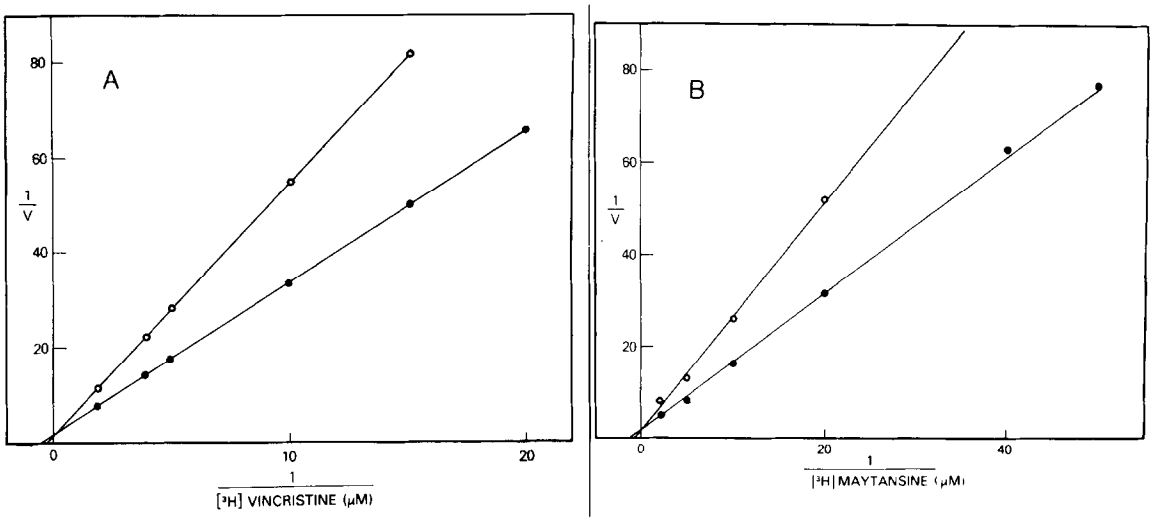

Figure 3. Reciprocal plot of vincristine and maytansine binding vs. concentration. 1 mg protein was incubated for 2 min with various A., vincristine (●) or B, maytansine (●), concentrations. A, (○) binding of vincristine in the presence of maytansine (0.2 μM); B (○), binding of maytansine in the presence of vincristine (10 μM). V = nmole of drug bound per mg protein.

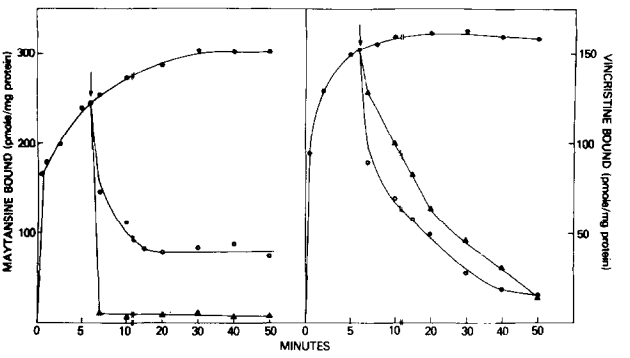

Figure 4. Exchange of bound vincristine or bound maytansine. The protein (1 mg/ml) was incubated in a reaction mixture containing [^{3}H]-maytansine, 0.5 μM, or [^{3}H] vincristine, 0.5 μM, in a total volume of 4 ml. Unlabeled vincristine (○) or maytansine (▲) were added, as indicated by arrows, to a final concentration of 50 μM. At various time intervals, 0.4 ml portions were removed and filtered. Controls (●), showing the effect of time on binding were run in each experiment.

Binding of vincristine and maytansine as a function of concentration. The binding of both drugs increases with increasing concentration. The association constants (K_A) for vincristine and maytansine as calculated from reciprocal

plots (Fig. 3A and 3B) were 0.5 x 10^6 M^{-1} and 1.5 x 10^6 M^{-1} respectively. Maytansine competitively inhibited vincristine binding with a K_i value for maytansine of 0.4 x 10^{-6} M (Fig. 3A). Fig. 3B demonstrates that binding of labeled maytansine was also competitively inhibited by vincristine with a K_i for the latter of 10 x 10^{-6} M.

Competitive displacement of bound vincristine and bound maytansine. Fig. 4 shows that binding of both drugs is easily reversible. Bound [^{3}H]-vincristine is displaced both by unlabeled vincristine and maytansine at about the same rate. The displacement of labeled maytansine exhibits a different pattern. Unlabeled maytansine causes a very rapid displacement of the bound drug, whereas the displacement by unlabeled vincristine is significantly slower and not complete. About 25% of bound maytansine could not be exchanged, indicating presence of an additional binder for maytansine not shared by vincristine.

Specificity of binding. Vincristine at a concentration 200 times higher than that of maytansine resulted in only ca. 80% inhibition in the binding of the

Table 1. Specificity of [^{3}H]-maytansine binding

Additions (nmole)	Maytansine bound per mg protein (pmole)	Inhibition %
None	310	-
Maytansine 50.0	5	98
Vincristine 100.0	60	81
Colchicine 100.0	300	3

Tubulin was incubated for 10 min in 1 ml reaction mixtures containing [^{3}H]-maytansine (0.5 nmole) in the presence of the added compounds indicated. The reaction mixtures with colchicine were incubated for 45 min. Controls were run to determine the possible decay of tubulin binding activity due to prolonged incubation.

latter (Table 1), in agreement with the results obtained when vincristine was added after labeled maytansine (Fig. 4). Colchicine, an antimitotic drug known to possess a different binding site on tubulin than that for vinca alkaloids (12-14), did not affect maytansine binding. Similar

Table 2. Specificity of [^{3}H] vincristine binding

Additions (nmole)	Vincristine bound per mg protein (pmole)	Inhibition %
None	160	-
Vincristine 50.0	0	100
Maytansine 50.0	0	100
Colchicine 100	158	<1

All experimental conditions were as in Table 1. The concentration of [^{3}H]-vincristine was 0.5 nmole/1 ml reaction mixture.

Table 3. Specificity of [^{3}H] colchicine binding

Additions (nmole)	Colchicine bound per mg protein (pmole)	Inhibition %
None	105	-
Colchicine 50.0	2	98
Vincristine 100.0	106	none
Maytansine 100.0	109	none

The reaction mixtures were incubated for 45 min. The concentration of colchicine was 1 nmole/1 ml reaction mixture.

results were obtained for the specificity of vincristine binding: maytansine inhibited vincristine binding, whereas colchicine was completely ineffective (Table 2). To further confirm these results we have shown that maytansine or vincristine did not inhibit colchicine binding (Table 3).

DISCUSSION

Data on labeled vincristine and vinblastine binding to crude and purified rat, pig and chick embryo brain tubulin have been presented previously by other workers (9,12-14). In the present study, the results obtained for vincristine binding and its specificity were in good quantitative agreement to those of Owellen et al. (9).

We have shown that the new antitumor agent maytansine binds to tubulin and shares a common binding site with vincristine (Tables 1,2 and Fig. 3). It exhibits a higher affinity for the binder than that of vincristine (Fig. 3A, 3B), an observation which could be of aid in explaining its higher biological activity in vivo (3,5). Furthermore, the binding of both drugs at the same site may also explain the cross-resistance phenomenon observed in vincristine-resistant P388 cells (3). Although maytansine and vincristine share the same binding site as shown by competitive inhibition and by displacement (Fig. 4), the mechanism of binding for the two drugs appears to be somewhat different as shown by a different effect of ionic strength (Fig. 2), by more rapid maytansine exhange, and by its higher binding rate (Fig. 4). Since a high concentration of vincristine could not completely displace the bound maytansine and since also, in the case of the simultaneous addition of both drugs, about 20% of maytansine remained bound (Table 1), an additional binder, specific for maytansine may be present, although not evident from the linear, monophasic reciprocal plot obtained with the latter agent (Fig. 3B). A possibility that another cellular component, not tubulin, possesses maytansine binding capacity not shared by vincristine cannot be excluded. Purification of the binding protein and elucidation of the role of the α and β components of tubulin (15) will undoubtedly contribute to

further understanding of the biological interaction between maytansine and vincristine.

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