Table 1. Effect of araC on dNTP pools

Length of treatment* (hr)	dNTP levels (% control) dATP	dTTP	dGTP	dCTP
3	144	113	108	135
6	143	127	185	157
12	276	204		

* HeLa Cells were treated with 10^{-4} M araC + 10^{-5} M THU and, at indicated times, extracts were prepared and dNTP determinations performed as described in Materials and Methods.

support of the "self-potentiation" concept whereby araA activity is increased by a decrease in dATP, which competes with the active form of the drug, araATP. This "self-potentiation" was not observed in HeLa cells that were infected with herpes simplex virus.

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Department of Biochemistry and Pharmacology
Tufts University Schools of Veterinary Medicine and Medicine
Boston, MA 02111, U.S.A.

THOMAS W. NORTH

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Interactions of combretastatin, a new plant-derived antimitotic agent, with tubulin

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A cytotoxic principle extracted from the South African tree *Combretum caffrum* and named combretastatin (NSC-348103) has been purified by Pettit *et al.* [1]. These workers determined the structure of the drug, shown in Fig. 1. Combretastatin has significant activity in reversing the differentiation of AC glioma cells into astrocytes, a model which has demonstrated good selectivity for antimitotic agents [2]. Moreover, the structure of the drug is reminiscent not only of colchicine, but especially of the active colchicine analog 2-methoxy-5-(2′,3′,4′-trimethoxyphenyl)tropone (MTPT, also shown in Fig. 1) synthesized by Fitzgerald [3]. Both its ability to reverse astrocyte differentiation and its structure prompted us to examine combretastatin for antimitotic and antitubulin activity.

The preparation of purified tubulin and heat-treated microtubule-associated proteins from calf brain and the sources of all other materials have been described elsewhere [4, 5]. The binding of [^{3}H]colchicine to tubulin and tubulin-dependent GTP hydrolysis were measured as described previously [6, 7]. Tubulin polymerization was followed turbidimetrically [8] in a Gilford model 250 recording spectrophotometer. Temperatures were maintained with a Gilford "Thermoset" electronic temperature controller. Baselines were established with the reaction mixtures at 0°. At zero time the instrument was set at 37°, and the point at which the instrument readout reached 37° is indicated in the appropriate figures by an arrow on the abscissa (the time axis). Drug cytotoxicity studies and slide

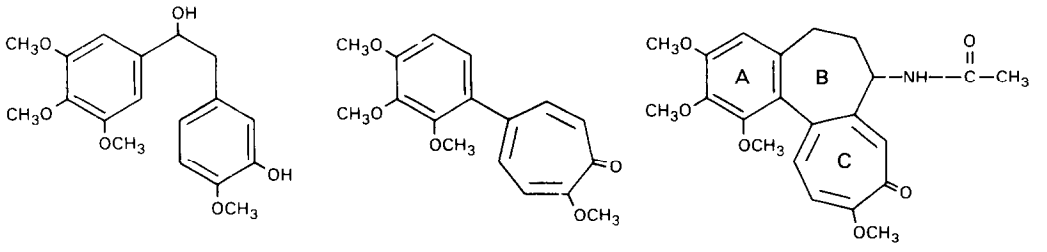

Fig. 1. Structures of combretastatin, MTPT and colchicine.

preparation for the determination of mitotic indices were performed as described by Wolpert-DeFilippes *et al.* [9].

Since all antimitotic agents which have thus far been examined exhibit significant stimulatory or inhibitory effects on tubulin-dependent GTP hydrolysis [6, 7, 10], we have proposed that this assay could be useful as a preliminary screen for antimitotic activity in drugs with an unknown mechanism of action [11]. Combretastatin was therefore examined for a potential effect on the GTPase activity of tubulin in 1 M glutamate [6, 7] and compared to colchicine and MTPT (Table 1). Like colchicine, combretastatin stimulated the reaction, as did MTPT. There are, however, differences between the three drugs. The maximum stimulation obtained with combretastatin was significantly lower than that obtained with either colchicine or MTPT. On the other hand, while the effect of colchicine at low drug concentrations is markedly enhanced by a drug–tubulin preincubation [6, 7], stimulation of GTP hydrolysis by combretastatin or MTPT was little affected by a preincubation. These preincubation effects probably reflect the rates of binding of the drugs to tubulin, since the binding of colchicine to tubulin is slow and temperature-dependent [12] while MTPT binds rapidly and in the cold [13]. It thus seems likely that combretastatin also binds rapidly to the protein.

Having obtained definite evidence from the GTPase assay that combretastatin interacted with tubulin, we wanted to establish that the drug was an antimitotic agent. L1210 cells (log phase) were grown in culture, and an initial 24 hr cytotoxicity determination was performed with combretastatin, as well as with colchicine and MTPT (Fig. 2). In this experiment, combretastatin was somewhat more cytotoxic than the other drugs, 50% inhibition occurring at about 0.06 μM combretastatin as opposed to about 0.2 μM colchicine and MTPT. Determination of mitotic indices in L1210 cells was performed at the same drug concentrations (Fig. 2) after 13 hr of drug exposure. A dramatic increase in the mitotic index was observed with all three drugs, and there was little difference between them. Moreover, there was a reasonable correlation between cytotoxic drug concentrations and the rise in the mitotic index. At the highest drug concentration examined (1 μM), 65% of the cells displayed mitotic figures with combretastatin, 69% with colchicine, and 51% with MTPT.

Both its structure and its stimulatory effect on tubulin-dependent GTP hydrolysis suggest that combretastatin should inhibit the binding of colchicine to tubulin. This was found to be the case, as demonstrated in Table 2. Combretastatin was more inhibitory than both nocodazole [14] and MTPT [13], but less inhibitory than podophyllotoxin [12]. As demonstrated by modified Dixon plots (Fig. 3), combretastatin is a competitive inhibitor of the binding of colchicine to tubulin, with an apparent K_i value of 1.1 μM. We were unable to demonstrate any inhibitory effect of combretastatin on the binding of vinblastine to tubulin

Table 2. Inhibition of colchicine binding to tubulin by combretastatin*

	Binding (% of control)	
Drug added	10 μM Drug	100 μM Drug
Combretastatin	38	7.9
MTPT	72	19
Nocodazole	45	16
Podophyllotoxin	8.9	1.4

* The binding of 5 μM [^{3}H]colchicine to tubulin was measured as described previously [7]. In the control reaction, 4.4 pmoles of colchicine was bound per μg of tubulin. Reaction mixtures contained 1% dimethyl sulfoxide.

Table 1. Stimulation of tubulin-dependent GTP hydrolysis by combretastatin, MTPT and colchicine*

	GTP hydrolyzed (% of control)		
	Not preincubated		Preincubated
Drug added	10 μM Drug	1 mM Drug	10 μM Drug
None	100	100	100
Combretastatin	225	215	238
MTPT	276	319	304
Colchicine	117	343	322

* Each 100 μl reaction mixture contained 1.0 mg/ml of purified tubulin, 1.0 M glutamate (pH 6.6), 0.1 mM [α-^{32}P]GTP and drugs as indicated. All drugs were dissolved in dimethyl sulfoxide, and reaction mixtures contained 10% (v/v) solvent. The preincubated reaction mixtures were incubated initially at 37° for 2 hr without GTP. They were then chilled on ice, and GTP was added. Incubation was at 37° for 20 min. In the nonpreincubated control, 13.9 nmoles GDP/ml of reaction was formed; in the preincubated control, 14.2 nmoles/ml.

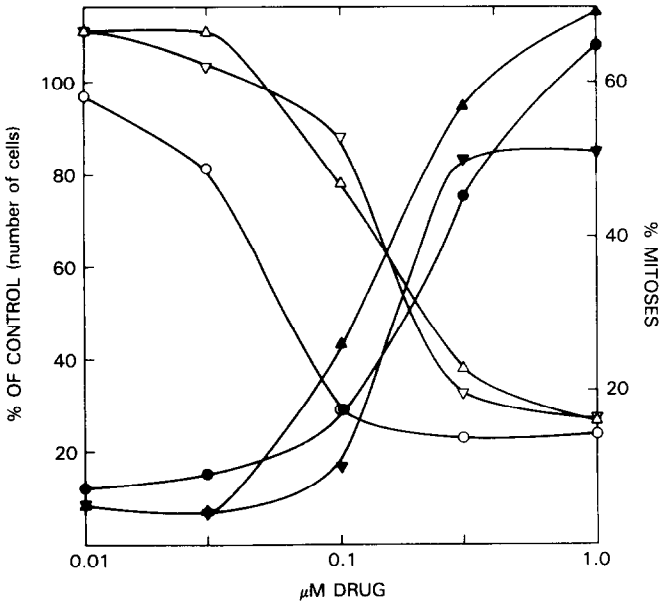

Fig. 2. Effects of combretastatin, MTPT and colchicine on the growth and mitotic index of L1210 cells. Duplicate cultures were inoculated with 10^5 cells/ml and drugs at the indicated concentrations (dimethyl sulfoxide concentration was 1%). In the cytotoxicity experiment (open symbols), the cells were counted after 24 hr of drug exposure (control, 5.28×10^5 cells/ml). In the mitotic index experiment (closed symbols), at least 400 cells were counted at each drug concentration (control, 4.4%). Key: combretastatin (○, ●); colchicine (△, ▲); and MTPT (▽, ▼).

(data not presented). This is not surprising, since vinblastine and colchicine are known to have distinct binding sites on tubulin [12].

Most antimitotic agents, including all those which inhibit the binding of colchicine to the protein [3, 7, 12–14], inhibit microtubule assembly *in vitro*. This is also the case with combretastatin. In a reconstituted system of purified tubulin [4] and heat-treated microtubule-associated proteins [5], progressive inhibition of microtubule assembly was observed at comretastatin concentrations from 1 to 10 μM

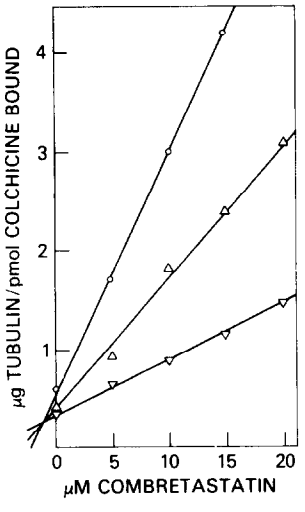

Fig. 3. Competitive inhibition of colchicine binding to tubulin by combretastatin. The concentration of [^{3}H]colchicine was as follows: (○) 1 μM; (△) 2 μM; and (▽) 4 μM. Reaction mixtures contained combretastatin at the indicated concentrations. The combretastatin concentration is plotted against the reciprocal of the pmoles of colchicine bound per μg of tubulin.

(Fig. 4A). To verify that tubulin, rather than a microtubule-associated protein, is the target for combretastatin, glutamate-induced polymerization of purified tubulin [4] was examined (Fig. 4B). This reaction was even more sensitive to combretastatin, complete inhibition being observed at 5 μM drug.

Combretastatin is thus an antimitotic agent which inhibits tubulin polymerization, stimulates tubulin-dependent GTP hydrolysis, and competitively inhibits the binding of colchicine to the protein. These properties of combretastatin are quite similar to those of MTPT, which also has antimitotic activity ([3], Fig. 2), stimulates the GTPase activity of tubulin (Table 1), inhibits tubulin polymerization [3], and is a competitive inhibitor of colchicine binding [13].

The two drugs have marked similarities, but also significant differences, in their structures. Both are bicyclic compounds, and, like colchicine, both contain a vicinal 1,2,3-trimethoxybenzene ring (the A ring of colchicine). In the case of MTPT, the second ring, a methoxytropone ring, is identical to the C ring of colchicine; but in combretastatin the second ring is a substituted benzene ring. Nonetheless, the vicinal methoxy and hydroxy groups in combretastatin's second ring seem analogous to the vicinal methoxy and carbonyl groups of the tropone ring of MTPT. In the case of MTPT, the tropone ring is directly attached at the 4-position of the 1,2,3-trimethoxybenzene ring, asymmetrically oriented relative to the methoxy groups. In combretastatin, the second ring is attached via a 2-carbon bridge at the 5-position of the trimethoxybenzene ring, symmetrically oriented relative to the methoxy groups. (This is similar to the attachment of the tetrahydronaphthol moiety of podophyllotoxin, but podophyllotoxin inhibits rather than stimulates net GTP hydrolysis by tubulin [6, 10].) We would, nevertheless, propose that the second substituted benzene ring of combretastatin is analogous to the C ring of colchicine and the methoxytropone ring of MTPT. If this is the case, then the seven-member tropone ring *per se* is not critical for the interaction of colchicine with tubulin. A similar conclusion has been reached by Fitzgerald [3], who noted that compounds with

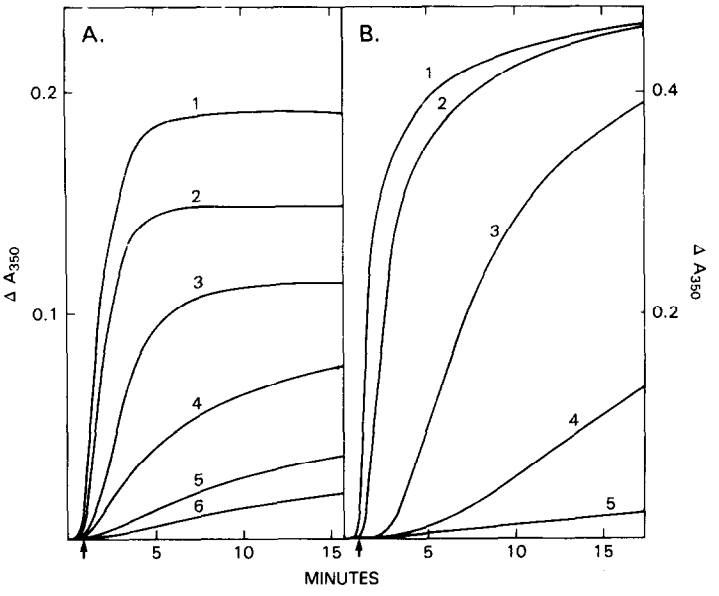

Fig. 4. Inhibition of tubulin polymerization by combretastatin. (A) Assembly dependent on microtubule-associated proteins. Each reaction mixture contained 1.0 mg/ml of tubulin, 0.4 mg/ml of microtubule-associated proteins, 0.1 M 2-(*N*-morpholino)ethanesulfonate (pH 6.9), 0.5 mM $MgCl_2$, 0.1 mM GTP, 1% dimethylsulfoxide and combretastatin as follows: curve 1, none; curve 2, 1 μM; curve 3, 4 μM; curve 4, 5 μM; curve 5, 8 μM; and curve 6, 10 μM. (B) Glutamate-induced polymerization of purified tubulin. Each reaction mixture contained 1.0 mg/ml of tubulin, 1.0 M glutamate (pH 6.6), 0.5 mM $MgCl_2$, 0.1 mM GTP, 1% dimethyl sulfoxide and combretastatin as follows: curve 1, none; curve 2, 1 μM; curve 3, 3 μM; curve 4, 4 μM; and curve 5, 5 μM.

a benzene ring replacing the tropone ring (*N*-acetylcolchinol, *N*-acetyliodocolchinol [15] and allocolchicine [3]) have substantial antitubulin activity.

In summary, the activity of combretastatin in reversing astrocyte differentiation suggested that this newly-isolated cytotoxic agent would have antimitotic activity; and its structure indicated that it is a colchicine analog. We have demonstrated here that combretastatin is an effective antimitotic agent, causing a marked rise in the mitotic index of L1210 cells. The drug, like colchicine, inhibited tubulin polymerization and stimulated tubulin-dependent GTP hydrolysis; and combretastatin competitively inhibited the binding of colchicine to tubulin.

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Laboratory of Medicinal Chemistry and Pharmacology
Developmental Therapeutics Program
Division of Cancer Treatment
National Cancer Institute
National Institutes of Health
Bethesda, MD 20205, U.S.A.

ERNEST HAMEL*
CHII M. LIN

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* Author to whom correspondence should be addressed.