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Interactions of Tubulin with Potent Natural and Synthetic Analogs of the Antimitotic Agent Combretastatin: a Structure-Activity Study

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SUMMARY

Combretastatin, an antineoplastic and antimitotic agent, was isolated from the bark of *Combretum caffrum* [*Can. J. Chem.* **60:** 1374–1376 (1982); *Biochem. Pharmacol.* **32:**3864–3867 (1983)]. Structurally, combretastatin consists of two substituted benzene rings linked by a saturated, hydroxy-substituted two-carbon bridge. A large number of combretastatin analogs have now been synthesized or obtained from *C. caffrum*. These vary in substituents on the phenyl rings or bridge carbons, bridge length, unsaturation of the bridge (i.e., stilbene derivatives, with the two phenyl rings oriented either *cis* or *trans*), and in precise ring structure (two major variants, with the bridge incorporated into a third six-member ring to form a phenanthrene structure or a methyl group eliminated from vicinal methoxy substituents to form a benzodioxole ring). Available analogs (17 natural products and 22 synthetic agents) were examined for antimitotic and cytotoxic activity and for effects on tubulin polymerization and colchicine binding. Nineteen compounds inhibited cell growth by 50% or more at concentrations of 1 μM or less, and 14 inhibited tubulin polymerization by at least 50% at stoichiometric drug concentrations. The most potent cytotoxic agents generally strongly inhibited both tubulin polymerization and the binding of colchicine to tubulin. The most promising compound is the (*cis*)-stilbene derivative (*cis*)-1-(3,4,5-trimethoxyphenyl)-2-(3′-hydroxy-4′-methoxyphenyl)ethene, which has been named combretastatin A-4. This compound inhibited cell growth by 50% at 7 nM, inhibited tubulin polymerization by 50% at 2.5 μM (¼ molar equivalent), and competitively inhibited colchicine binding with an apparent K_i of 0.14 μM.

A major function of microtubules in eucaryotic cells is formation of the mitotic spindle required for cell division. Consequently, many antimitotic drugs interfere with normal formation of microtubules by interaction with their major component, the protein tubulin. A large number of these agents are plant derived and include the *Colchicum* alkaloids colchicine (1) and cornigerine (1–3), the *Catharanthus* (*Vinca*) alkaloids vinblastine and vincristine (4), *Podophyllum*-derived agents represented by podophyllotoxin (5), steganacin and congeners obtained from *Steganotaenia araliacea* (6, 7), maytansine and congeners from *Maytenus serrata* (8, 9), and taxol from *Taxus brevifolia* (10, 11).

The South African tree *Combretum caffrum* is a newly discovered source for multiple antimitotic compounds and these are perhaps the simplest natural products yet described that bind to tubulin. The first antineoplastic compound isolated from extracts of the bark of the tree was termed combretastatin and consisted of two phenyl rings linked by a two-carbon bridge (12, 13) (see below for structure). This agent caused mitotic arrest in L1210 murine leukemia cells, inhibited microtubule assembly, competitively inhibited the binding of [^{3}H]colchicine to tubulin (apparent K_i = 1.1 μM), and stimulated tubulin-dependent GTP hydrolysis (14).

A number of still more potent compounds, with comparably simple structures, have been isolated from the *C. caffrum* bark extract (15–17), and additional analogs have been chemically synthesized. These compounds vary in substituents on the phenyl rings, substituents on the bridge carbons, length of the bridge, saturation of the bridge, and precise ring structure. In this report we present studies of structure-function relationships among the *C. caffrum*-derived and -inspired compounds. Because the most potent *C. caffrum* compounds are the strongest inhibitors of the binding of colchicine to tubulin yet described, such studies should provide insights into key features of the colchicine/podophyllotoxin binding site on tubulin and may assist in the design of optimally active antineoplastic compounds that bind in this site.

Experimental Procedures

Materials. Electrophoretically homogeneous calf brain tubulin and heat-treated microtubule-associated proteins were prepared as described previously (18). Nonradioactive colchicine, GTP (repurified by triethylammonium bicarbonate gradient chromatography on DEAE-Sephadex A-25), and monosodium glutamate (adjusted to pH 6.6 with HCl) were obtained from Sigma Chemical Co. (St. Louis, MO) and [*ring A*-4-^{3}H]colchicine from Amersham (Arlington Heights, IL). All

drugs were dissolved in dimethylsulfoxide, and control reaction mixtures contained equivalent amounts of the solvent, which had no significant effect on the reactions studied.

Methods. L1210 murine leukemia cells were used to examine the effects of drugs on cell growth and the mitotic index as described by Wolpert-DeFilippes *et al.* (19). *In vitro* polymerization of tubulin was followed turbidimetrically (20) in Gilford model 250 and 2400S recording spectrophotometers equipped with electronic temperature controllers. Reaction mixtures (0.25 ml) contained 1.0 mg/ml (10 μM) tubulin, 1.0 M monosodium glutamate, 0.4 mM GTP, 1.0 mM $MgCl_2$, and drugs as indicated. Reaction mixtures were preincubated for 15 min at 37° before the addition of GTP. IC_{50} values were determined as described in detail elsewhere (21). For comparison with the *C. caffrum* compounds, the IC_{50} values of colchicine and podophyllotoxin in this system were, respectively, 4–5 μM and 2–3 μM. Colchicine binding was measured by the DEAE-cellulose filter assay (22) as described previously (14, 21).

Isolation of natural products and synthetic procedures for many of the new agents used here have been described elsewhere (12, 13, 15–17),[1] but synthesis of three compounds is described briefly here. Wittig reaction of 3-benzyloxy-4-methoxybenzyltriphenylphosphonium bromide with 3,4,5-trimethoxybenzaldehyde or with 2-(3,4,5-trimethoxyphenyl)acetaldehyde produced the corresponding olefins. Catalytic hydrogenation of the olefins yielded compounds **3** and **21**. Condensation of 3-benzyloxy-4-methoxybenzaldehyde (23) with 1-(3,4,5-trimethoxyphenyl)propenyl triphenylphosphonium bromide furnished the appropriate olefin, which on catalytic hydrogenation yielded compound **22**. Spectroscopic data and elemental analyses were consistent with the proposed structures (data not presented).

Results

Figs. 1–6 summarize our major findings with 17 pure natural products derived from extracts of the stem bark of *C. caffrum* as well as with 22 structurally similar synthetic compounds.[2] In earlier reports describing the isolation of many of the natural products (15, 16) we had noted their inhibitory effects on microtubule assembly in reaction mixtures containing tubulin and microtubule-associated proteins. Figs. 1–6 present more extensive data comparing inhibitory effects on the polymerization of purified tubulin induced by glutamate (24), on the binding of radiolabeled colchicine to purified tubulin, and on the growth of L1210 murine leukemia cells in culture. There was good correlation between the inhibitory effects on tubulin polymerization and on colchicine binding, but the correlation of these antitubulin effects with the cytotoxicity of the agents was imperfect. We therefore verified that all agents, except those with minimal cytotoxicity, actually exerted their effects on cells at the microtubule level by demonstrating that approximately equivalent concentrations (in most cases 2–5 times the cytotoxic IC_{50} level) of active compounds caused a marked rise in the mitotic index observed in cultured cells.

Fig. 1 presents our findings with substituted biphenyl compounds in which the two rings are connected by an unsaturated 2-carbon bridge, as well as with analogs in which the double bond of the bridge is reduced. The four unsaturated compounds derived from *C. caffrum* (compounds **1**, **6**, **9**, and **13**) all have the two phenyl rings in the *cis*-configuration and are thus derivatives of (*cis*)-stilbene. Although all four were toxic to cells, there was a 300-fold difference between the least (compound **13**) and most (compound **1**) active agents. Compound **13**, in addition, had almost negligible effects on *in vitro* tubulin-dependent reactions. Despite these functional differences, there were only seemingly minor structural differences between the two compounds—the position of one methyl group differed in the two agents, changing from the hydroxyl at position 5 in compound **1** to the hydroxyl at position 3′ in compound **13**. The intermediate activities of compounds **9** (which only differs from compound **1** by demethylation at the hydroxyl at position 5) and **11** (a synthetic derivative, which only differs from compound **1** by methylation at the hydroxyl group at position 3′) as both cytotoxic agents and as inhibitors of tubulin polymerization indicate that both demethylation at the hydroxyl at position 5 and methylation at the hydroxyl at position 3′ contribute to the reduced activity of compound **13**.

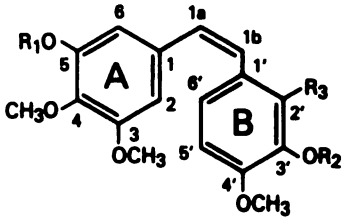
Comparison of compound **6** with compound **1** is of particular interest, as the former agent has an additional hydroxyl group at position 2′. Although the extra hydroxyl results in a marked reduction in the cytotoxicity of compound **6** as compared with compound **1** (but see below), the former agent is equally potent as an inhibitor of tubulin polymerization. The colchicine binding assay, however, yielded somewhat contradictory results. With a colchicine to inhibitor ratio of 1:1, compound **6** was reproducibly more inhibitory than compound **1**, but at lower inhibitor concentrations compound **1** was always more potent. (For comparison, under the reaction conditions used in these experiments, podophyllotoxin in a 1:1 ratio to colchicine inhibited binding of the latter by 85–90% and is thus less potent than both compounds **1** and **6** in binding at the colchicine site of tubulin.)

The patterns of inhibition of colchicine binding obtained with both compounds **1** and **6** were examined in detail (data not presented). With compound **1**, straightforward results were obtained. The agent is a competitive inhibitor of the binding of colchicine to tubulin, with an apparent K_i of 0.14 μM (with podophyllotoxin the apparent K_i is 0.4–0.6 μM). With compound **6**, the data obtained were more complex. Lineweaver-Burk plots were consistent with competitive inhibition, but Dixon analysis yielded parabolic curves, precluding determination of an apparent K_i value. This resulted from unexpectedly weak inhibition with low concentrations of compound **6**. This behavior of compound **6**, as well as its relatively weak cytotoxicity, appears to result from chemical instability (probably oxidation to an *o*-quinone), which has been observed in three compounds in this series with vicinal hydroxyls at the 2′ and 3′ positions (i.e., compounds **7** and **37**, as well as compound **6**).

Supporting this conclusion was the activity obtained with acetate esters of compounds **1** and **6** (i.e., compounds **4** and **8**, respectively; see Fig. 1). Although acetylation significantly reduced *in vitro* inhibition of both tubulin polymerization and colchicine binding, acetylation at the 3′-hydroxyl of compound **1** (to yield compound **4**) did not alter cytotoxicity whereas diacetylation at the 2′- and 3′-hydroxyls of compound **6** (to yield compound **8**) enhanced cytotoxicity 10-fold. These data suggest that the acetate groups did not interfere with penetra-

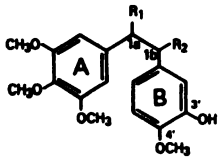
[1] G. R. Pettit and S. B. Singh, manuscript in preparation.

[2] Several of the compounds described here have been given names (based on the genus of origin) in previous papers, as follows: 1) combretastatin (compound **15**) (see Refs. 12–14); 2) combretastatin A-1 (compound **6**) and combretastatin B-1 (compound **7**) (see Ref. 15); 3) combretastatin B-2 (compound **10**), combretastatin B-3 (compound **25**), and combretastatin B-4 (compound **24**) (see Ref. 16); 4) combretastatin A-2 (compound **35**) and combretastatin A-3 (compound **9**) (see Ref. 17); and 5) combretastatin A-4 (compound **1**), combretastatin A-5 (compound **13**), and combretastatin A-6 (compound **14**) (manuscript in preparation).



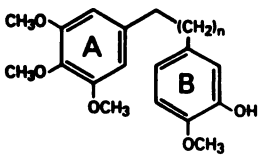
COMPOUND (NP or S)	STRUCTURAL DETAILS	IC_{50} (μM) L1210 GROWTH	% MITOSES (μM Drug)	IC_{50} (μM) TUBULIN POLYMERIZATION	% OF CONTROL-COLCHICINE BINDING Colchicine : Drug 1:0.2	1:1	1:10
1 (NP)	R_1= CH_3, R_2= R_3 = H	0.007	38 (0.01)	2-3	21	3.6	—
2 (S)	Trans Form of #1	0.02	58 (0.1)	7.5-10	—	25	5.9
3 (S)	#1, but Bridge Saturated	0.2	52 (0.8)	4-5	—	26	2.6
4 (S)	R_1 = CH_3, R_2 = $COCH_3$ R_3 = H	0.007	51 (0.03)	7.5-10	—	37	6.7
5 (S)	#4, but Bridge Saturated	0.1	44 (0.6)	>100	—	—	46
6 (NP)	R_1= CH_3, R_2= H, R_3= OH	0.6	44 (7)	2-3	27	1.3	—
7 (NP)	#6, but Bridge Saturated	2	25 (10)	4-5	—	18	—
8 (S)	R_1 = CH_3, R_2 = $COCH_3$ R_3 = $OCOCH_3$	0.06	47 (0.3)	20	—	62	19
9 (NP)	R_1= R_2= R_3 =H	0.04	41 (0.2)	4-5	—	22	8.4
10 (NP)	#9, but Bridge Saturated	3	30 (10)	40	—	73	19
11 (S)	R_1 = R_2 = CH_3, R_3 = H	0.3	51 (1)	4-5	—	44	6.2
12 (S)	#11, but Bridge Saturated	20	27 (30)	>100	—	88	63
13 (NP)	R_1= R_3= H, R_2= CH_3	2	55 (8)	75-100	—	90	43
14 (S)	Trans Form of #13	30	50 (100)	>100	—	101	83

Fig. 1. Effects of (*cis*)- and (*trans*)-stilbene derivatives and saturated analogs on the growth of murine L1210 leukemia cells in culture, on the inhibition of tubulin polymerization, and on the binding of radiolabeled colchicine to tubulin. In the cytotoxicity experiments, duplicate cultures were inoculated with 1–2 × 10^5 cells/ml and appropriate drug concentrations (dimethylsulfoxide concentration was 1%). The cells were counted after 24 hr of drug exposure. Cell cultures incubated with the indicated drug concentrations were evaluated for mitotic arrest as described in the text. The polymerization experiments were performed as described in the text. In the colchicine binding experiments, reaction mixtures (0.1 ml) contained 0.1 mg/ml (1.0 μM) tubulin, 5.0 μM [*ring A*-4-^{3}H]colchicine, and the indicated drug concentrations. Incubation was for 10 min at 37°. Some of the data presented here (or similar experiments) with compounds **6**, **7**, **10**, and **12** have been summarized elsewhere (Refs. 15 and 16). *NP*, natural product; *S*, synthetic.



COMPOUND (NP or S)	STRUCTURAL DETAILS	IC_{50} (μM) L1210 GROWTH	% MITOSES (μM Drug)	IC_{50} (μM) TUBULIN POLYMERIZATION	% OF CONTROL-COLCHICINE BINDING Colchicine : Drug 1:1	1:10	1:100
1 (NP)	$R_1 = R_2 = H$ 1a-1b Bond is Double Bond	0.007	38 (0.01)	2-3	3.6	–	–
2 (S)	Trans Form of #1	0.02	58 (0.1)	7.5-10	25	5.9	–
3 (S)	$R_1 = R_2 = H$	0.2	52 (0.8)	4-5	26	2.6	–
15 (NP)	$R_1 = OH$, $R_2 = H$ (R Configuration at 1a)	0.05	58 (0.2)	5-7.5	34	5.9	–
16 (S)	$R_1 = OH$, $R_2 = H$ (Racemic Mixture)	0.09	54 (0.4)	7.5-10	43	7.1	–
17 (S)	$R_1 = OCOCH_3$, $R_2 = H$ (Racemic Mixture)	0.2	44 (0.8)	7.5-10	51	11	–
18 (S)	$R_1 = H$, $R_2 = OH$ (Racemic Mixture)	0.9	36 (3)	75-100	–	52	11
19 (S)	$R_1 = H$, $R_2 = =O$	3	50 (10)	75-100	–	52	18
20 (S)	$R_1 = OH$, $R_2 = H$(Racemic) B Ring Substituents at Positions 3' and 4' Reversed	>100	–	>100	–	–	84

Fig. 2. Effects of modifications at the bridge carbons, in compounds with phenyl ring substituents identical to compound **1**, on cell growth, tubulin polymerization, and colchicine binding. Experimental conditions as described for Fig. 1. A detailed study with compound **15** has been presented previously (Ref. 15). *NP*, natural product; *S*, synthetic.



COMPOUND (NP or S)	STRUCTURAL DETAILS	IC_{50} (μM) L1210 GROWTH	% MITOSES (μM Drug)	IC_{50} (μM) TUBULIN POLYMERIZATION	% OF CONTROL-COLCHICINE BINDING Colchicine : Drug 1:1	1:10
3 (S)	n = 1	0.2	52 (0.8)	4-5	26	2.6
21 (S)	n = 2	1	63 (3)	10-15	58	13
22 (S)	n = 3	3	50 (10)	40-50	69	28

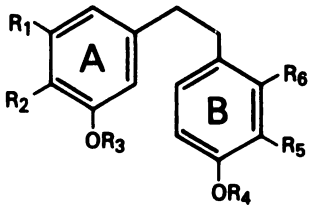
Fig. 3. Effects of bridge length between the two phenyl rings on cell growth, tubulin polymerization, and colchicine binding. Experimental conditions were as described for Fig. 1. *NP*, natural product; *S*, synthetic.

tion of the agents into cells but did reduce or prevent degradation of compound **8** as compared with compound **6**.

Of the seven (*cis*)-stilbenes presented in Fig. 1, saturated analogs of five compounds were available. Two of these were natural products (compounds **7** and **10**). Most notably, compound **3** has not yet been obtained from the *C. caffrum* extracts. In all cases, the saturated compound was less cytotoxic and less inhibitory with tubulin *in vitro* than the corresponding (*cis*)-stilbene.

Two analogs with the two phenyl rings in *trans*-configuration (derivatives of (*trans*)-stilbene) were available (Fig. 1). Both compound **2** and compound **14** were significantly less active than the corresponding (*cis*)-stilbenes, with the latter agent almost inert (corresponding to the relatively feeble activity of compound **13**). Nevertheless, compound **2** merits further comment, for, while less active than its *cis*-isomer, compound **1**, it has surprising cytotoxicity and activity against tubulin *in vitro*. Compound **2** is about one-third as cytotoxic as compound **1** and one fourth as effective as an inhibitor of tubulin polymerization. Although its effect on colchicine binding has not been studied in detail, compound **2** appears to be about one fifth to one tenth as effective as compound **1** in inhibiting the binding of radiolabeled colchicine to tubulin. Relative to the saturated analog compound **3**, compound **2** is 10 times as cytotoxic, half as effective as an inhibitor of polymerization, and nearly identical as an inhibitor of colchicine binding. (Thus far the only complete set of *cis*, *trans*, and saturated analogs available is represented by compounds **1**, **2**, and **3**.)

The data in Fig. 1 also indicate that interactions of this class of agents with tubulin are significantly reduced by bulkier substituents at the 3′ hydroxyl and that this effect is much more pronounced with a saturated bridge than with the two phenyl rings in the *cis*-configuration. In the (*cis*)-stilbene compounds, agents with either a methyl or acetyl group at the 3′ hydroxyl have reduced, but still substantial, activity as inhibitors of tubulin polymerization (cf. compounds **4** and **11** with compound **1**). With the comparable saturated series, agents with methyl and acetyl 3′-hydroxyl substituents (compounds **5**

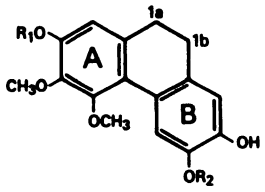


COMPOUND (NP or S)	STRUCTURAL DETAILS	IC_{50} (μM) L1210 GROWTH	% MITOSES (μM Drug)	IC_{50} (μM) TUBULIN POLYMERIZATION	% OF CONTROL-COLCHICINE BINDING Colchicine : Drug 1:1	1:10	1:100
3 (S)	$R_1=R_2=OCH_3, R_3=R_4=CH_3$ $R_5=OH$, $R_6=H$	0.2	52 (0.8)	4-5	26	2.6	—
5 (S)	$R_1=R_2=OCH_3, R_3=R_4=CH_3$ $R_5=OCOCH_3$, $R_6=H$	0.1	44 (0.6)	>100	—	46	30
7 (NP)	$R_1=R_2=OCH_3, R_3=R_4=CH_3$ $R_5=R_6=OH$	2	25 (10)	4-5	18	—	—
10 (NP)	$R_1=R_5=OH$, $R_2=OCH_3$ $R_3=R_4=CH_3$, $R_6=H$	3	30 (10)	40	73	19	7.2
12 (S)	$R_1=R_2=R_5=OCH_3$ $R_3=R_4=CH_3$, $R_6=H$	20	27 (30)	>100	88	63	37
23 (NP)	$R_1=OCH_3$, $R_2=R_6=H$ $R_3=R_4=CH_3$, $R_5=OH$	1	54 (6)	10-15	38	8.4	—
24 (NP)	$R_1=OCH_3$, $R_2=R_4=R_6=H$ $R_3=CH_3$, $R_5=OH$	1	41 (10)	25-30	75	31	19
25 (NP)	$R_1=R_2=OCH_3$, $R_3=CH_3$ $R_4=R_6=H$, $R_5=OH$	3	41 (10)	>100	92	82	48
26 (NP)	$R_1=OCH_3, R_2=R_4=R_5=R_6=H$ $R_3=CH_3$	4	50 (10)	>100	87	45	13
27 (S)	$R_1=R_5=OCH_3, R_2=R_6=H$ $R_3=R_4=CH_3$	30	56 (100)	>100	90	80	34
28 (NP)	$R_1=R_2=OCH_3$, $R_3=CH_3$ $R_4=R_5=R_6=H$	50	34 (100)	>100	89	75	43
29 (S)	$R_1=R_2=R_5=R_6=OCH_3$ $R_3=R_4=CH_3$	>100	—	>100	—	—	94
30 (S)	$R_1=R_3=R_4=R_6=H$ $R_2=R_5=OCH_3$	>100	—	>100	—	—	80

Fig. 4. Effects of modifications in the substituent pattern in the two phenyl rings on cell growth, tubulin polymerization, and colchicine binding. Experimental conditions were as described for Fig. 1. Some of the data presented here (or similar experiments) with compounds **23, 24, 25, 26, 27,** and **28** have been summarized elsewhere (Refs. 15, 16). *NP*, natural product; *S*, synthetic.

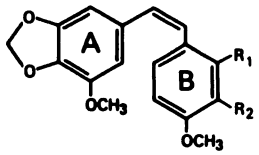
and **12**) have negligible activity, whereas compound **3** is highly inhibitory.

In Fig. 2 we have summarized data obtained with all available agents having identical substituent patterns on the two phenyl rings but differing in the structures of the two-carbon bridge. Besides compound **1**, the only natural product in this group is compound **15**, the original combretastatin (12, 14), now known to have the *R*-configuration at position 1a (13). Much less active in all assays than compound **1**, compound **15** was more comparable in its activity to compound **3**, the analog with the saturated bridge, differing only from the latter compound by a hydroxyl substituent at position 1a. Although compound **3** had slightly greater inhibitory effects in the *in vitro* tubulin assays, compound **15** was somewhat more cytotoxic. The racemic compound **16** was less active than compound **15** in all assays, suggesting that the *R*-configuration at position 1a is of functional importance. Acetylation of the bridge hydroxyl (compound **17**) caused a minor reduction of activity in all assays. In particular, the loss of activity in the polymerization and colchicine assays was negligible in comparison with the greater effects noted above for acetylation of the 3′-hydroxyl, suggest-



COMPOUND (NP or S)	STRUCTURAL DETAILS	IC_{50} (μM) L1210 GROWTH	% MITOSES (μM Drug)	IC_{50} (μM) TUBULIN POLYMERIZATION	% OF CONTROL-COLCHICINE BINDING Colchicine : Drug 1:10	1:100
31 (NP)	$R_1 = R_2 = CH_3$ 1a-1b Bond is Double Bond	1	50 (4)	100	20	6.0
32 (NP)	$R_1 = R_2 = CH_3$	3	31 (10)	>100	44	12
33 (NP)	$R_1 = CH_3$, $R_2 = H$	20	–	>100	94	45
34 (NP)	$R_1 = H$, $R_2 = CH_3$	20	–	>100	84	32

Fig. 5. Reduced inhibitory activity of natural products with phenanthrene-like structures on cell growth, tubulin polymerization, and colchicine binding. Experimental conditions were as described for Fig. 1. *NP*, natural product; *S*, synthetic.



COMPOUND (NP or S)	STRUCTURAL DETAILS	IC_{50} (μM) L1210 GROWTH	% MITOSES (μM Drug)	IC_{50} (μM) TUBULIN POLYMERIZATION	% OF CONTROL-COLCHICINE BINDING Colchicine : Drug 1:0.2	1:1	1:10
35 (NP)	$R_1 = H$, $R_2 = OH$	0.1	71 (0.7)	4-5	35	9.9	–
36 (S)	Trans Form of #35	0.4	61 (3)	20-25	51	15	–
37 (S)	$R_1 = R_2 = OH$	4	40 (20)	2-3	50	6.3	–
38 (S)	#37, but Bridge Saturated	8	16 (15)	5-7.5	–	16	–
39 (S)	$R_1 = R_2 = OCH_3$	40	–	>100	–	–	62

Fig. 6. Effects of compounds with a benzodioxole ring system on cell growth, tubulin polymerization, and colchicine binding. Experimental conditions were as described for Fig. 1. *NP*, natural product; *S*, synthetic.

ing that steric factors may be of less importance at the 1a position.

Alterations introduced in the final compounds presented in Fig. 2 greatly reduced the inhibitory activity of this class of agents. In particular, placement of the hydroxyl at position 1b as opposed to 1a (compound **18**) and reversal of the 3′- and 4′-substituents in the B phenyl ring (compound **20**) yielded compounds with little activity.

Fig. 3 presents data with three compounds that differ only in the length of the bridge between the two phenyl rings, increasing from two to four carbons. Each additional methylene group resulted in a progressive loss of activity in all assays.

A total of seven natural products with a saturated two-carbon bridge linking two phenyl rings have been isolated from the *C. caffrum* extracts. Our data with these, together with data obtained with six synthetic analogs, are summarized in Fig. 4. The most active inhibitors in the *in vitro* tubulin assays (polymerization and colchicine binding) were described above (compounds **3** and **7**). Single modifications of the substituents on the two phenyl rings of compound **3** occur in six agents, whereas the other six compounds have two or more alterations in these substituents. As with the (*cis*)-stilbenes, a second hydroxyl group at position 2′ (compound **7**) did not reduce (and perhaps enhanced—note the colchicine assay) the *in vitro* activity of this class of agents, whereas acetylation (compound **5**) or methylation (compound **12**) at the 3′-hydroxyl almost eliminated *in vitro* inhibition of tubulin-dependent reactions. Similarly, demethylation of the 4′-hydroxyl (compound **25**) resulted in a nearly inert compound. The remaining two single modifications (demethoxylation at position 4—compound **23**; demethylation at the hydroxyl at position 5—compound **10**) yielded compounds of intermediate activity. Five of the six agents with multiple substituent changes were nearly inert, but compound **24** was partially active despite demethoxylation at position 4 and demethylation at the 4′-hydroxyl.

For the group of compounds presented in Fig. 4, the correlation between cytotoxicity and inhibition in *in vitro* tubulin assays was not notable. The reasons for this are not known but

probably are related to drug uptake and/or metabolism by L1210 cells.

Four natural products were isolated in which the two phenyl rings are fused into phenanthrene-like structures, with one agent, compound **31**, being a true phenanthrene derivative (Fig. 5). These compounds all have little inhibitory activity in the *in vitro* tubulin assays and relatively little cytotoxicity, whether their molecular structures are completely rigid (compound **31**) or somewhat more flexible (compound **32**). The most active member of this group is compound **31**, but it is very feeble relative to compound **1** (as well as several other agents described here). These two agents are structurally identical, except for fusion in compound **31** of the carbons at the 2 and 5′ positions of compound **1**.

Compound **35** (Fig. 6) differed significantly in structure from the agents described above in bearing a methylenedioxy bridge on the A phenyl ring (forming a benzodioxole ring system) in place of two vicinal methoxy groups. In compound **35** the B phenyl ring and the benzodioxole ring system are linked by an unsaturated two-carbon bridge in a *cis*-configuration, as is the case with the two phenyl rings of compound **1**. Compound **35** is about 1/14th as cytotoxic as compound **1**, and it is also somewhat less effective as an inhibitor of tubulin polymerization and of the binding of colchicine to tubulin. Among the natural products, only compounds **1**, **9**, and **15** are more cytotoxic, whereas only compounds **1** and **6** are more potent inhibitors of the *in vitro* tubulin-dependent reactions.

The activity of compound **35** led to the chemical synthesis of four related compounds (Fig. 6). Structure-activity relationships among these agents essentially corresponded to those obtained above with the biphenyls. The *trans*-isomer of compound **35** (compound **36**) was less active in all assays than was compound **35**, although there was only a minor difference between the two isomers as inhibitors of colchicine binding. A second hydroxyl at position 2′ yielded an unstable compound (compound **37**) with apparent loss of cytotoxicity but enhancement of inhibition of tubulin polymerization and of colchicine binding (but only with colchicine:inhibitor = 1:1, as was the case with compound **6** versus compound **1**). Reduction of the bridge double bond of compound **37** (to produce compound **38**) led to loss of activity in all assays, but compound **38** still has substantial *in vitro* inhibitory effects on tubulin polymerization and colchicine binding. Addition of methyl groups at the 2′- and 3′-hydroxyls (compound **39**) virtually eliminated activity in all systems (cf. compound **29** with compounds **3** and **7**; Fig. 4).

Discussion

In this report we have summarized our findings with a large new group of antimitotic agents, 17 natural products derived from extracts of the stem bark of *C. caffrum* and 22 structurally similar synthetic compounds. A wide range of cytotoxicity against L1210 murine leukemia cells in culture was observed, as well as a wide range of antitubulin activity. The most potent agents described here are highly active in these assay systems. Although in the studies reported here we described only inhibition of the glutamate-induced polymerization of purified tubulin, no significant differences have been observed when microtubule assembly dependent on microtubule-associated proteins was examined (14–16).[3] If we arbitrarily choose an IC_{50} value of 10 μM (i.e., a drug concentration equimolar with the tubulin concentration used in the reaction mixtures) for the polymerization assay as indicating "significant" inhibition, six natural products (compounds **1**, **6**, **7**, **9**, **15**, and **35**) and eight synthetic compounds (compounds **2**, **3**, **4**, **11**, **16**, **17**, **37**, and **38**) fulfill this criterion. Similarly, if we define "significant" cytotoxicity by an IC_{50} value of 1 μM or less for inhibition of cell growth, then eight natural products (compounds **1**, **6**, **9**, **15**, **23**, **24**, **31**, and **35**) and 11 synthetic agents (compounds **2**, **3**, **4**, **5**, **8**, **11**, **16**, **17**, **18**, **21**, and **36**) fulfill this criterion.

The overlap between these groups is obviously incomplete, but compounds **7**, **37**, and **38** have limited cytotoxicity, whereas compounds **8**, **21**, **23**, **24**, and **36** have a partial ability to interact with tubulin *in vitro*. As judged by their negligible inhibition of polymerization and colchicine binding, three agents (compounds **5**, **18**, and **31**) with reasonable cytotoxicity have at best feeble *in vitro* interactions with tubulin. Nonetheless, these three compounds do cause the accumulation of cells in mitotic arrest at cytotoxic drug concentrations and thus appear to attack the microtubule system. Although compound **5** may be deacetylated to the active compound **3**,[4] mechanisms by which compounds **18** and **31** exert their effects are presently unknown.[5]

Five synthetic derivatives [acetylated analogs (compounds **4**, **5**, and **8**), the (*trans*)-stilbene compound **2**, and the racemic compound **16**] and four natural products (compounds **1**, **9**, **15**, and **35**) had outstanding cytotoxicity, inhibiting the growth of L1210 cells by 50% at concentrations of 0.1 μM or less. Compounds **1** and **35** were also among the most active agents as inhibitors of both tubulin polymerization and colchicine binding to tubulin. Compound **1** was the best overall inhibitor in this series of drugs. Structurally, compound **1** is a derivative of (*cis*)-stilbene, consisting of an unsaturated two-carbon bridge with two substituted phenyl rings in a *cis*-relationship (Fig. 1), and compound **35** is a close analog because two vicinal methoxy groups attached to the A phenyl ring have been replaced by a methylenedioxy bridge to form a benzodioxole ring system (Fig. 6).

This relationship between compounds **1** and **35** also exists in the colchicine family of drugs (1–3) in which the vicinal 2- and 3-methoxy groups of the A ring of colchicine have been substituted in the drug cornigerine with a methylenedioxy bridge, as in compound **35**. The functional relationship of the two *Colchicum* compounds differs from that of the two *C. caffrum* compounds. Cornigerine is more potent than colchicine both as a cytotoxic agent and as an *in vitro* inhibitor of tubulin (3). Compound **35**, however, is less active than compound **1** in all assays.

The only structural modification that enhanced the activity of either compound **1** or compound **35** as a tubulin inhibitor was addition of a second hydroxyl substituent on the B phenyl ring at position 2′. The compound **1** analog (compound **6**) is a

[3] E. Hamel, unpublished observations.

[4] Similarly, compound **4** is probably deacetylated either in the culture medium or by the L1210 cells to the more active compound **1**. Although it has significant *in vitro* activity against tubulin as an inhibitor of polymerization and colchicine binding, compound **4** is disproportionately cytotoxic.

[5] It should be noted, however, that in terms of relative activity in the polymerization and cytotoxicity assays [i.e., IC_{50} (polymerization)/IC_{50} (cytotoxicity)], compounds **18** and **31** are not that different from compound **1**. This ratio is about 100 for compounds **18** and **31** as compared with 360 for compound **1**.

natural product whereas the compound **35** analog (compound **37**) is synthetic. Even though they are potent *in vitro* inhibitors of tubulin polymerization and of the binding of colchicine to tubulin, both compound **6** and compound **37** had much reduced cytotoxicity and were unstable both in solution and stored in solid form. Acetylation of the hydroxyls of compound **6** greatly enhanced its cytotoxicity, and acetylation of the single hydroxyl group of compound **1** did not alter its potency against the leukemia cells. Although both acetylated compounds were less active as *in vitro* inhibitors of tubulin polymerization, the observation of unchanged or enhanced cytotoxicity in the esters may have utility in designing an optimum analog for detailed *in vivo* antineoplastic studies.

It should also be stressed that the (*cis*)-stilbenes, compounds **1** and **6**, are the most potent inhibitors of the binding of colchicine to tubulin yet described. Compound **1** is a competitive inhibitor, binding at the colchicine site, with an apparent K_i of 0.14 μM. Although compound **6** is also probably a competitive inhibitor, its instability precluded an adequate determination of a K_i value.

Without exception, *trans*-isomers were less active than the corresponding *cis*-isomers, and reduction of the double bond also yielded agents with less activity. Only with the ring substituent pattern observed in compound **1** was a complete set of *cis*, *trans*, and saturated analogs available (compounds **1**, **2**, and **3**) for comparative studies. In addition, similar phenyl substituents were available in two natural products in which fusion of the 2′- and 5′-carbons, together with the two-carbon bridge, gave a third six-member ring interposed between the A and B rings (Fig. 5). Superficially, this yielded two agents structurally very close to colchicine, with the new ring reminiscent of the B ring of colchicine. In compound **31**, the equivalent of the original bridge is unsaturated, producing a true substituted phenanthrene. In compound **32**, the original bridge is saturated, producing a structure presumably less rigid than compound **31**. Compounds **31** and **32** have only limited cytotoxicity and feeble interactions with tubulin. Thus it is tempting to postulate that this represents an inability of the A and B phenyl rings to assume proper orientations for binding to tubulin. This may also explain the partially reduced activity of the saturated compound **3** relative to the (*cis*)-stilbene compound **1**. It is more difficult to rationalize the surprisingly good, albeit reduced, activity of the (*trans*)-stilbene compound **2**. More cytotoxic than the reduced compound **3** (possibly as a consequence of better uptake into cells), compound **2** was less active as an inhibitor of tubulin polymerization but virtually identical to compound **3** as an inhibitor of colchicine binding to tubulin. One possible simple explanation for the activity of compound **2** (as well as of compound **36**, the *trans*-form of compound **35**) is that *cis-trans* isomerization occurs in the reaction mixtures (also see below).

Finally, we should note the structural similarity of compound **2** to diethylstilbestrol, for both are (*trans*)-stilbene derivatives. Diethylstilbestrol and related compounds have been reported by many laboratories to cause mitotic arrest and inhibit *in vitro* tubulin polymerization both with and without microtubule-associated proteins (25–31). Moreover, diethylstilbestrol inhibits the binding of radiolabeled colchicine to tubulin (28). The published data (25–30) and our own preliminary experiments indicate that compound **2** is more active than diethylstilbestrol. Nevertheless, the major structural differences between the substituents on compounds **1** and **2** and on diethylstilbestrol (hydroxyl groups at the 4- and 4′-positions on the two phenyl rings, an ethyl group at both bridge carbons) suggest that compound **1**, despite its excellent cytotoxicity and antitubulin activity, may be improved upon by further modifications in the phenyl rings, in the bridge, or in both simultaneously. The *cis*-analog of diethylstilbestrol would be an interesting compound with which to begin such additional structure-activity studies.

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