Antimitotic Natural Products Combretastatin A-4 and Combretastatin A-2: Studies on the Mechanism of Their Inhibition of the Binding of Colchicine to Tubulin†

Chii M. Lin,‡ Holly H. Ho,‡ George R. Pettit,§ and Ernest Hamel*,‡

Laboratory of Biochemical Pharmacology, Developmental Therapeutics Program, Division of Cancer Treatment, National Cancer Institute, National Institutes of Health, Bethesda, Maryland 20892, and Cancer Research Institute and Department of Chemistry, Arizona State University, Tempe, Arizona 85287

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ABSTRACT: Combretastatin A-4 (CS-A4), 3,4,5-trimethoxy-3′-hydroxy-4′-methoxy-(*Z*)-stilbene, and combretastatin A-2 (CS-A2), 3,4-(methylenedioxy)-5-methoxy-3′-hydroxy-4′-methoxy-(*Z*)-stilbene, are structurally simple natural products isolated from the South African tree *Combretum caffrum*. They inhibit mitosis and microtubule assembly and are competitive inhibitors of the binding of colchicine to tubulin [Lin et al. (1988) *Mol. Pharmacol. 34*, 200–208]. In contrast to colchicine, drug effects on tubulin were not enhanced by preincubating CS-A4 or CS-A2 with the protein. The mechanism of their binding to tubulin was examined indirectly by evaluating their effects on the binding of radiolabeled colchicine to the protein. These studies demonstrated rapid binding of both compounds to tubulin even at 0 °C (binding was complete at the earliest times examined), in contrast to the relatively slow and temperature-dependent binding of colchicine. Although the binding of the *C. caffrum* compounds to tubulin was quite tight, permitting ready isolation of near-stoichiometric amounts of drug–tubulin complex even in the absence of free drug, both CS-A4 and CS-A2 dissociated rapidly from tubulin in the presence of high concentrations of radiolabeled colchicine. Apparent rate constants for drug dissociation from tubulin at 37 °C were 3.2×10^{-3} s^{-1} for CS-A4, 4.8×10^{-3} s^{-1} for CS-A2, and 2.9×10^{-5} s^{-1} for colchicine (half-lives of 3.6, 2.4, and 405 min, respectively). Thus, the effectiveness of the *C. caffrum* compounds as antimitotic agents appears to derive primarily from the rapidity of their binding to tubulin. A new model for the colchicine site on tubulin is proposed which envisages A-ring and C-ring subsites in homologous locations on the two tubulin subunits, with phenyl rings binding as well as or better than the tropolone ring of colchicine in the C-ring subsite.

Tubulin, the major protein component of microtubules, is the target of a large number of small molecules. Since the mitotic spindle is composed predominantly of microtubules, the major cellular effect of such compounds is the accumulation of cells arrested in metaphase. Generally, this antimitotic effect correlates well with inhibition of microtubule assembly. The large majority of the most potent antimitotic compounds effective against animal cells are derived from plants; and, in addition, most well-described antimitotic agents appear to bind to tubulin competitively with colchicine, implying a common binding site, despite highly variable structures [reviewed in Hamel (1989)].

Recently, a large number of closely related compounds derived from the South African tree *Combretum caffrum* have deen described by Pettit and his collaborators (Pettit et al., 1982, 1987, 1988, 1989; Pettit & Singh, 1987). Many of them are potent antimitotic agents, inhibiting both tubulin polymerization and the binding of colchicine to tubulin (Lin et al., 1988). The *C. caffrum* agents are of particular interest not only for their antineoplastic potential but also because they are structurally the simplest natural products yet described with potent antitubulin effects. Two of the most active stable compounds isolated from the plant extracts are presented in Figure 1. Combretastatin A-4 (CS-A4)[1] is a *cis*-stilbene, and combretastatin A-2 (CS-A2) is a close analogue presumably formed in plant tissues by fusion of vicinal methoxy groups into a methylenedioxy bridge. CS-A4 and CS-A2 have obvious structural analogies to, respectively, colchicine and cornigerine [a highly active colchicine analogue derived from *Colchicum cornigerum*; see El-Hamidi and Santavy (1962), Rosner et al. (1981), and Hamel et al. (1988)], whose structures are also presented in Figure 1.

Our initial studies with CS-A4 and CS-A2 (as well as with active but unstable analogues with a second hydroxyl substituent at position 2′) documented impressive inhibition of the binding of radiolabeled colchicine to tubulin by both compounds. With equimolar inhibitor and colchicine, binding of the latter was consistently inhibited by over 90% by CS-A2 and by over 95% by CS-A4 (Lin et al., 1988). CS-A4, in fact, is the most effective inhibitor of colchicine binding that we have examined, and we are not aware of any more potent agent described in the literature. The studies presented here were undertaken to determine the mechanisms by which these two agents exert their inhibitory effects on colchicine binding and thus gain insight, indirectly, into their mechanism of binding to tubulin.

Materials and Methods

Materials. Electrophoretically homogeneous bovine brain tubulin freed of unbound guanine nucleotide and containing

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* Address correspondence to this author at Building 37, Room 5B22, National Institutes of Health, Bethesda, MD 20892.

‡ National Institutes of Health.

§ Arizona State University.

[1] Abbreviations: CS-A4, combretastatin A-4; CS-A2, combretastatin A-2; MTPT, 2-methoxy-5-(2′,3′,4′-trimethoxyphenyl)tropone.

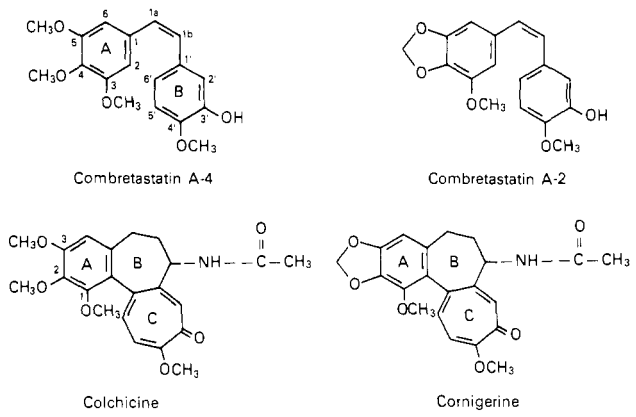

FIGURE 1: Structural formulas of CS-A4, CS-A2, colchicine, and cornigerine.

approximately 1 molar equiv each of GTP and GDP was prepared as described previously (Hamel & Lin, 1984a). Nonradiolabeled colchicine, GTP, and monosodium glutamate were obtained from Sigma, [*ring C methoxy*-^{3}H]colchicine was from Amersham, and [8-^{14}C]GTP was from Moravek Biochemicals. Both GTP preparations were repurified by triethylammonium bicarbonate gradient chromatography on DEAE-Sephadex A-25. Stock 2 M monosodium glutamate solutions were adjusted to pH 6.6 with HCl. CS-A4 and CS-A2 were prepared as described previously (Pettit & Singh, 1987; Pettit et al., 1989). MTPT was a generous gift of Dr. T. J. Fitzgerald, Florida A&M University.

Methods. Protein concentrations were determined by the method of Lowry et al. (1951), with bovine serum albumin as standard. Tubulin polymerization was followed turbidimetrically (Gaskin et al., 1974) at 350 nm in Gilford Model 2400S spectrophotometers equipped with electronic temperature controllers (0 to 37 °C temperature jump in about 75 s). GTP hydrolysis was measured by following the formation of [8-^{14}C]GDP from [8-^{14}C]GTP by thin-layer chromatography on poly(ethylenimine)–cellulose (Hamel & Lin, 1984b). Separation of tubulin–drug complexes from free drug with Sephadex G-50 (superfine) was accomplished either by column chromatography or by centrifugal gel filtration in 1.0-mL syringes (Penefsky, 1977). Details of the latter procedure have been described previously (Hamel & Lin, 1984b).

The binding of radiolabeled colchicine to tubulin was followed by the DEAE-cellulose filter assay (Borisy, 1972). We have used a reaction condition which results in virtually complete stabilization of the colchicine binding activity of tubulin, permitting prolonged assay times (Hamel & Lin, 1981). Each reaction mixture (100 μL) contained 0.1 mg/mL (1.0 μM) tubulin, 0.5 mg/mL bovine serum albumin, 1.0 M monosodium glutamate, 0.1 M glucose 1-phosphate, 1.0 mM GTP, 1.0 mM $MgCl_2$, and [*ring C methoxy*-^{3}H]colchicine, as indicated. The radiolabeled colchicine was mixed with nonradiolabeled colchicine as required for the desired final concentration. Depending on the specific activity chosen for a particular experiment, the final reaction mixtures contained 0.5–1.6% (v/v) ethanol, the solvent of the radiolabeled colchicine. The effect, if any, of the ethanol on the reaction has not been evaluated. In addition, nonradiolabeled drugs were dissolved in dimethyl sulfoxide. This solvent did not appear to have a significant effect on the binding reaction; but all reaction mixtures in an experiment contained identical concentrations of dimethyl sulfoxide as well as of ethanol. After the desired incubation at 37 °C the reaction was stopped with 2 mL of ice-cold water. Reaction mixtures were poured onto a stack of two DEAE-cellulose filters, the test tube was rinsed with three 2-mL washes of ice-cold water, and the total volume was aspirated under weak suction. The stack of filters was then rapidly washed in succession with three 2-mL portions of ice-cold water and counted in a liquid scintillation spectrometer. Once the reaction mixture was diluted with water, the radiolabel retained by the filters did not change for at least 2 h. We have found no significant difference in radioactivity retained if buffer solutions were used to wash the filters instead of water or if the washing procedure was more extensive than that described here. Data points represent the average of triplicate samples, which were generally within 10% of each other.

Table I: Comparison of CS-A4 and CS-A2 to Podophyllotoxin and MTPT as Inhibitors of the Binding of Colchicine to Tubulin[a]

drug added	% inhibition
CS-A4	97
CS-A2	94
podophyllotoxin	89
MTPT	69

[a] Each reaction mixture contained the components described in the text, 0.1 mg/mL (1.0 μM) tubulin, 5 μM radiolabeled colchicine, and the indicated inhibitor at 5 μM. Dimethyl sulfoxide concentration was 5% (v/v). Incubation was for 10 min at 37 °C. In the control reaction, 31 pmol of colchicine was retained by the DEAE-cellulose filters.

Results

Table I presents an experiment which compares the inhibitory effects of CS-A4 and of CS-A2 to those of podophyllotoxin and MTPT (Fitzgerald, 1976) on the binding of colchicine to tubulin.[2] The inhibitors and colchicine were present in equimolar amounts, and both of the *C. caffrum* compounds were more effective than podophyllotoxin. With CS-A4, in particular, binding of radiolabeled colchicine to the protein was negligible. MTPT was substantially less inhibitory than the other agents.

Detailed studies were performed with CS-A4 to establish the type of inhibition of colchicine binding which was obtained with the *cis*-stilbene. Lineweaver–Burk analysis of the data demonstrated that inhibition was competitive in nature, while Dixon analysis yielded an apparent K_i value of 0.12 μM (data not presented). Analysis of the effect of podophyllotoxin under identical reaction conditions also demonstrated competitive inhibition, with an apparent K_i value of 0.5 μM (data not presented). Although the inhibitory effect of CS-A2 was not examined in such detail, the experiments we have performed with this drug, too, are most consistent with a competitive type of inhibition.

At present radiolabeled CS-A4 and CS-A2 are not available, and therefore, the binding of these agents to tubulin can only be examined indirectly. We have previously noted that both colchicine's inhibitory effects on tubulin polymerization and its stimulatory effects on tubulin-dependent GTP hydrolysis (David-Pfeuty et al., 1977) are markedly enhanced by a drug–tubulin preincubation, provided the colchicine is present at relatively low concentrations (Hamel & Lin, 1982; Hamel

[2] The short time (10 min) chosen for the incubation in this study results in submaximal amounts of radiolabeled colchicine bound to the tubulin (see below), ranging from about 30% to 70% of the plateau value. With our tubulin preparations, stoichiometry of binding ranges from about 0.5 to 0.7 mol of colchicine/mol of tubulin in reaction mixtures containing 1 μM tubulin and 5 μM colchicine and incubated for 40–60 min at 37 °C. We have found that the shorter incubation time yields superior data for analysis of inhibitors (i.e., Lineweaver–Burke and Dixon plots). In addition, as will become clear below, the short incubation time results in especially impressive inhibitory effects with agents that bind rapidly and/or reversibly at the colchicine site.

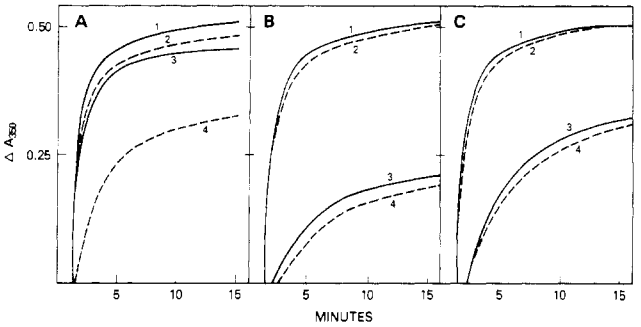

FIGURE 2: Effect of a drug–tubulin preincubation on subsequent tubulin polymerization. Each 0.24-mL reaction mixture contained (all values refer to final concentrations in 0.25 mL) 1.0 mg/mL (10 μM) tubulin, 1.0 M monosodium glutamate, 4% (v/v) dimethyl sulfoxide, and, if present, either 3 μM colchicine (panel A), 2 μM CS-A4 (panel B), or 2 μM CS-A2 (panel C). Reaction mixtures were either left on ice (solid curves) or incubated for 10 min at 37 °C and then chilled on ice (broken curves). GTP (10 μL; final concentration, 0.1 mM) was added to each reaction mixture, and tubulin polymerization was followed spectrophotometrically at 37 °C. In all panels, curves 1 and 2 represent the reactions without drug; curves 3 and 4, the reactions with drug as indicated above.

Table II: Effect of a Drug–Tubulin Preincubation on Drug-Dependent GTP Hydrolysis by Tubulin[a]

drug added	nmol of GDP formed	
	not preincubated	preincubated
none	16.1	14.6
colchicine	20.9	35.6
CS-A4	41.0	37.1
CS-A2	23.0	24.1

[a] Each 50-μL reaction mixture contained 1.0 mg/mL (10 μM) tubulin, 100 μM [8-^{14}C]GTP, 1.0 M monosodium glutamate, and the indicated drug at 5 μM. Dimethyl sulfoxide concentration was 4% (v/v). With the nonpreincubated samples incubation was for 20 min at 37 °C. With the preincubated samples, all components except the GTP were mixed in a 45-μL volume. They were incubated for 10 min at 37 °C and then chilled on ice. The radiolabeled GTP was added in a 5-μL volume, and the 37 °C incubation was performed as described above.

et al., 1988). Figure 2 presents a polymerization study comparing the effects of CS-A4 and CS-A2 to that of colchicine. As before, preincubation significantly increased inhibition of polymerization obtained with 3 μM colchicine (Figure 2A), but the inhibitory effects of 2 μM CS-A4 (Figure 2B) and CS-A2 (Figure 2C) were not affected by preincubation of either drug with tubulin prior to the addition of GTP.

Similarly, the stimulatory effect of 5 μM colchicine on tubulin-dependent GTP hydrolysis was substantially enhanced by preincubation of drug and tubulin prior to addition of radiolabeled GTP to the reaction mixture (Table II). With the *C. caffrum* compounds, preincubation had little effect on the amount of GTP hydrolyzed.[3] It should be noted that the relatively feeble stimulation of GTP hydrolysis observed with 5 μM CS-A2 was also observed with higher concentrations of this agent, as was the lack of a preincubation effect. For example, in the experiment presented in Table II, the drugs were also compared at 100 μM. With the higher concentration of CS-A2, 21.4 nmol/mL GDP was formed in the nonpreincubated sample, while in the preincubated sample 22.2 nmol/mL GDP was formed.[4]

Table III: Binding of CS-A4 and CS-A2 to Tubulin Is Not Affected by Incubation Temperature or Time[a]

drug in preincubation	% inhibition (±SE) at preincubation temp of	
	0 °C	37 °C
colchicine	3 (±4.0)	65 (±4.1)
CS-A4	65 (±3.5)	68 (±0.7)
CS-A2	52 (±2.9)	52 (±2.4)

[a] In the preincubation, 0.34-mL reaction mixtures contained 2.0 mg/mL (20 μM) tubulin, the indicated drug at 20 μM, and 1.0 M monosodium glutamate. The dimethyl sulfoxide concentration was 4% (v/v). Control reaction mixtures contained dimethyl sulfoxide but no drug. Reaction mixtures preincubated at 0 °C were mixed on ice and immediately applied to the 1.0-mL syringe columns and processed in a cold room. Reaction mixtures preincubated at 37 °C were left at that temperature for 10 min, chilled on ice, applied to syringe columns and processed in a cold room. In most experiments aliquots (0.1 mL) of each reaction mixture were applied to three separate syringe columns. These were processed as described previously (Hamel & Lin, 1984b). GTP (final concentration, 1 mM) was added to each filtrate (to enhance stabilization of the tubulin), and protein concentrations in the filtrates were determined. Binding of radiolabeled colchicine by the filtered protein was then measured. Each 0.1-mL reaction mixture contained the components described in the text, 0.1 mg/mL (1.0 μM) tubulin or tubulin–drug complex, and 5.0 μM radiolabeled colchicine. Incubation was for 10 min at 37 °C. The control reaction mixtures in the 0 °C experiments bound an average of 24 pmol of radiolabeled colchicine and an average of 23 pmol in the 37 °C experiments. The values presented in the table represent the averages of values obtained in five to nine independent experiments. SE, standard error.

We have interpreted the preincubation effect of colchicine in these assays to result from the relatively slow, temperature-dependent binding of colchicine to tubulin (Hamel & Lin, 1982; Hamel et al., 1988). It seems likely that the lack of a preincubation effect with the *C. caffrum* compounds reflects rapid binding to tubulin, and this rapid binding may occur at low temperatures. To explore this possibility, we undertook the isolation of tubulin–drug complexes by centrifugal gel filtration (Table III), assuming that failure of the recovered tubulin to bind radiolabeled colchicine indicated formation of a tubulin–drug complex.[5] In all experiments, tubulin which had been preincubated with nonradiolabeled drug was directly compared to tubulin which had been preincubated under the same conditions without drug. In one set of experiments, 20 μM tubulin was mixed with 20 μM drug on ice, and the reaction mixtures were immediately processed in a cold room at 4 °C. As would be predicted, there was no evidence for significant formation of a tubulin–colchicine complex, as subsequent binding of radiolabeled colchicine differed little from that obtained with the control tubulin. With both CS-A4 and CS-A2 there was substantial subsequent inhibition of colchicine binding (CS-A4 being somewhat more effective than CS-A2; cf. Table I), indicating significant formation of complexes of tubulin with these agents in the cold.

[3] It should be emphasized that in the control reaction mixtures GTP hydrolysis is associated with a tubulin polymerization reaction, while in the drug-treated samples stimulation of GTP hydrolysis occurs despite inhibition of polymerization (David-Pfeuty et al., 1977; Hamel & Lin, 1982; Hamel et al., 1988). Further, in time-course studies, most drugs inhibit GTP hydrolysis at early time points, with stimulation most marked at later time points (Hamel et al., 1988; Duanmu et al., 1989).

[4] We have examined effects of CS-A4 and CS-A2 on tubulin-dependent GTP hydrolysis under a number of reaction conditions. At low ionic strength in the absence of microtubule-associated proteins, the stimulatory effect of CS-A4 was similar to that of colchicine and cornigerine [see Hamel et al. (1988)], while that of CS-A2 was only slightly stimulatory. At low ionic strength in the presence of microtubule-associated proteins, the stimulatory effects of CS-A4, colchicine, and cornigerine were again similar [cf. Hamel et al. (1988)], while CS-A2 was slightly inhibitory. We have also examined the effects of about 15 other highly active *C. caffrum* derived and synthetic compounds on tubulin-dependent GTP hydrolysis. A wide range of patterns was observed, with no structure–activity correlation apparent.

[5] These experiments were performed in 1.0 M glutamate because of its potent stabilization of tubulin (Hamel & Lin, 1981).

If tubulin and drug were preincubated for 10 min at 37 °C prior to centrifugal gel filtration (at 4 °C), however, there was apparent substantial formation of a complex of tubulin with nonradiolabeled colchicine, for subsequent binding of radiolabeled colchicine was inhibited as much as occurred when the protein was preincubated with CS-A4 (Table III). With both CS-A4 and CS-A2 there was no apparent enhancement of subsequent inhibition of colchicine binding when the preincubation was performed at 37 °C as compared to 0 °C. In summary, these studies yielded the results predicted for colchicine and are consistent with such rapid binding of both CS-A4 and CS-A2 to tubulin that no effect of temperature or length of incubation could be detected within the limits of the assay.

Nevertheless, the inhibition of radiolabeled colchicine binding observed with the *C. caffrum* compounds following centrifugal gel filtration (Table III) was substantially less than that observed in unfiltered samples (Table I). Although the initial drug to tubulin ratio was lower (1:1) in the experiments of Table III as opposed to that of Table I (5:1), this suggested that the binding of both CS-A4 and CS-A2 to tubulin might be reversible. We attempted to document this by column gel filtration, as opposed to the small (1.0 mL) columns used in the centrifugal gel filtration technique. In this series of experiments, tubulin (30 μM) was incubated at 37 °C with a 10-fold molar excess of nonradiolabeled drug to ensure saturation of the protein with drug.[6] The reaction mixture was then chilled on ice and subjected to column gel filtration, following which the tubulin was assayed for its ability to bind radiolabeled colchicine (in comparison to a tubulin sample incubated without drug and processed in the same manner). A series of experiments was performed on Sephadex G-50 (superfine) columns (1.5 × 45 cm) with CS-A4 and CS-A2, as well as with colchicine and MTPT (Ray et al., 1981) as controls, respectively, for irreversibly and reversibly formed tubulin–drug complexes. The controls yielded the predicted results: preincubation with colchicine resulted in almost complete elimination (96% inhibition) of subsequent ability to bind radiolabeled colchicine, and preincubation with MTPT did not affect subsequent binding of radiolabeled colchicine following column gel filtration (consistent with completely reversible formation of the MTPT–tubulin complex). The two *C. caffrum* compounds yielded intermediate results, and in fact, the extent of inhibition obtained with CS-A4 and CS-A2 in this series of experiments (65% and 49%, respectively) was almost identical with that obtained with the much smaller syringe columns (Table III). At first glance, this might suggest irreversible formation of CS-A4–tubulin and CS-A2–tubulin complexes, but the drug:tubulin ratio prior to gel filtration was much higher in the column experiments than in those of Table III, while apparent drug–tubulin complex formation only increased with colchicine.

Evidence for reversible formation of drug–tubulin complexes was therefore sought by altering the conditions of column chromatography, with only colchicine and CS-A4 evaluated (data not presented). Changing column length had no appreciable effect on subsequent binding of radiolabeled colchicine, consistent with irreversible binding of both CS-A4 and colchicine to tubulin. Increasing the temperature at which gel filtration was performed from 4 to 37 °C led to small decreases in subsequent inhibition of radiolabeled colchicine binding both with nonradiolabeled colchicine and with CS-A4 (implying reversible drug–tubulin complex formation), but substantial experimental variation was observed.

An unequivocal result was finally obtained when the tubulin–drug complexes were isolated by gel filtration chromatography and subsequently incubated for varying lengths of time with an excess of radiolabeled colchicine. Such experiments clearly demonstrated displacement of CS-A4 and CS-A2, as well as nonradiolabeled colchicine, from tubulin by radiolabeled colchicine (Figure 3).

A series of three experiments was performed, differing only in the amounts (5, 15, or 40 μM) of radiolabeled colchicine added in the final incubation (the experiment of Figure 3 presents the last of these three experiments). First, 30 μM tubulin was incubated at 37 °C alone or with 300 μM colchicine, CS-A4, or CS-A2. The protein solutions were then subjected to gel filtration chromatography at 4 °C. Finally, 1 μM tubulin or tubulin–drug complex (based on the tubulin concentration) was incubated with radiolabeled colchicine at 37 °C. In all three experiments, radiolabeled colchicine bound to the protein in the tubulin–drug complex solutions more slowly than to the control tubulin, with binding in the tubulin/colchicine solution occurring much more slowly than in the solutions with the *C. caffrum* compounds (compare panel B to panel A of Figure 3). Binding of radiolabeled colchicine in the tubulin/CS-A2 solution occurred more rapidly than in the tubulin/CS-A4 solution (Figure 3A). (Figure 3C compares the slower binding of 5 μM and the more rapid binding of 40 μM radiolabeled colchicine to the uncomplexed tubulin.)

The data of these experiments (Figure 3A,B and experiments with lower concentrations of radiolabeled colchicine) were analyzed according to the following scheme:

$$\text{tubulin–nonradiolabeled drug} \rightleftharpoons \text{tubulin} + \text{nonradiolabeled drug} \quad (1)$$

$$\text{tubulin} + [^3\text{H}]\text{colchicine} \rightleftharpoons \text{tubulin–}[^3\text{H}]\text{colchicine} \quad (2)$$

Since the binding of colchicine to tubulin is only slowly reversible, the reaction represented by eq 1 becomes essentially irreversible in the presence of a sufficient concentration of radiolabeled colchicine. Thus, formation of tubulin–[^{3}H]-colchicine complex represents an equivalent disappearance of tubulin–nonradiolabeled drug complex, which should be readily analyzed as a first-order decay process. Panels D–F of Figure 3 represent, respectively, evaluation of loss of colchicine, CS-A4, and CS-A2 in the presence of 40 μM [^{3}H]colchicine (i.e., the data of Figure 3A,B). Overall, in the three experiments with 5, 15, and 40 μM [^{3}H]colchicine, such analyses indicated biphasic modes of decay, but the apparent half-lives with all three drugs became progressively shorter and the onset of the apparent second mode of decay progressively earlier as the concentration of [^{3}H]colchicine increased. We therefore believe that we have not been able to include sufficient radiolabeled colchicine in the reaction mixtures to make decay of the tubulin–nonradiolabeled drug complex truly rate limiting (except, perhaps, with the CS-A2 complex in the presence of 40 μM colchicine—Figure 3F).[7] The apparent half-lives we have obtained from the faster decay processes must therefore be taken to represent only upper limits for these values.

The apparent half-lives of the tubulin–drug complexes were as follows: with CS-A4 (Figure 3E), 3.6 min (dissociation rate

[6] Addition of radiolabeled colchicine to the prefiltered reaction mixtures demonstrated no residual colchicine binding activity in the reaction mixtures containing CS-A4, CS-A2, or colchicine. With MTPT there was about 20% residual colchicine binding activity.

[7] We did not attempt studies with higher radiolabeled colchicine concentrations because lower specific activity of the colchicine resulted in too few counts retained by the filters in the presence of tubulin, while higher specific activity of the colchicine resulted in unacceptable background levels on the DEAE-cellulose filters in the absence of tubulin.

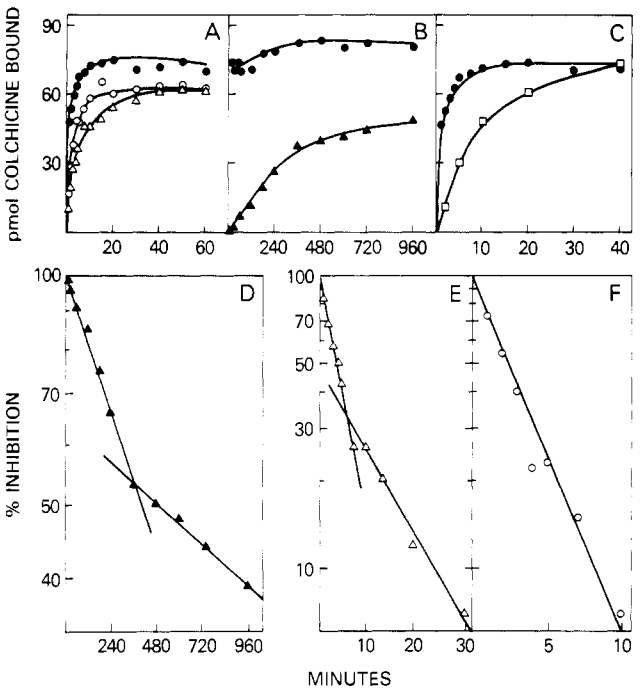

FIGURE 3: Time course of binding of radiolabeled colchicine to tubulin preincubated with CS-A4, CS-A2, or nonradiolabeled colchicine and separated from unbound drug by gel filtration chromatography. Initially, preincubation reaction mixtures (1.0 mL) contained 3.0 mg/mL (30 μM) tubulin, 1.0 M monosodium glutamate, and either no drug or CS-A4, CS-A2, or nonradiolabeled colchicine at 300 μM. The dimethyl sulfoxide concentration was 4% (v/v). The reaction mixtures were incubated for 15 min at 37 °C and chilled on ice. They were applied to columns of Sephadex G-50 (superfine) (1.5 × 20 cm) in a cold room. The columns were equilibrated and developed with 1.0 M monosodium glutamate. Fraction volume was about 1 mL. After quantitation of protein in the peak fractions, colchicine binding was measured in 0.1-mL reaction mixtures containing the components described in the text, 0.1 mg/mL (1.0 μM) tubulin or tubulin–drug complex, and 40 μM radiolabeled colchicine. Incubation was at 37 °C for the indicated times. Symbols are as follows: (●) tubulin preincubated without drug; (□) tubulin preincubated without drug, except that 5 instead of 40 μM radiolabeled colchicine was added to the reaction mixtures; (○) tubulin preincubated with CS-A2; (△) tubulin preincubated with CS-A4; (▲) tubulin preincubated with nonradiolabeled colchicine. (A) Comparison of the colchicine binding reaction of tubulin preincubated without drug to the tubulin preincubated with CS-A4 or CS-A2 (followed for 1 h). (B) Comparison of the colchicine binding reaction of tubulin preincubated without drug to tubulin preincubated with nonradiolabeled colchicine (followed for 16 h). (C) Comparison of the initial time course of binding of either 5 or 40 μM radiolabeled colchicine to tubulin preincubated without drug. (D) Kinetic analysis of the binding of radiolabeled colchicine to tubulin preincubated with nonradiolabeled colchicine. The analysis assumes that binding of radiolabeled colchicine is equivalent to dissociation of nonradiolabeled colchicine from tubulin (i.e., percent inhibition of binding of the radiolabeled drug), with complete dissociation (i.e., 0% inhibition) taken as the average of the control values obtained at 6, 8, 10, 12, and 16 h. (E and F) Kinetic analyses of the binding of radiolabeled colchicine to tubulin preincubated with either CS-A4 (panel E) or CS-A2 (panel F). Analysis as described for panel D, except that the plateau values with each drug (average of the 40-, 50-, and 60-min time points) were taken as representing complete dissociation, rather than the control plateau values. This assumes minor variations in the protein determination as the cause for the lower plateaus obtained with the drug-treated tubulin preparations. This assumption was made because it yielded more linear curves. Moreover, in other experiments the plateau values obtained for the binding of radiolabeled colchicine with drug-treated tubulin equaled or exceeded those obtained with control tubulin.

constant, 3.2 × 10^{-3} s^{-1}); with CS-A2 (Figure 3F), 2.4 min (dissociation rate constant, 4.8 × 10^{-3} s^{-1}); and with colchicine (Figure 3D), 405 min (dissociation rate constant, 2.9 × 10^{-5} s^{-1}).

Finally, these observations of reduced rates of binding of radiolabeled colchicine to tubulin–drug complexes (Figure 3), which result in a progressive reduction of the extent of inhibition as a function of time, led us to reevaluate drug inhibitory effects in reaction mixtures containing unliganded tubulin and excess inhibitor concentrations. An experiment identical with that presented in Table I (1 μM tubulin, 5 μM colchicine, 5 μM inhibitor) was performed, but in addition to the 10-min incubation (which yielded similar data to that presented above), samples were incubated for longer periods of time. As in the experiment presented in Figure 3 [1 μM tubulin, 40 μM colchicine, 1 μM (maximum) inhibitor], inhibition of colchicine binding declined as incubation time increased, although less dramatically. At the final time point (4 h), CS-A4 inhibited colchicine binding 80%; CS-A2, 53%; podophyllotoxin, 75%; and MTPT, 13%.

Discussion

The studies presented here were undertaken to examine the interactions of the new antimitotic agents CS-A4 and CS-A2 with tubulin to obtain greater insight into their mechanism of cytotoxic action. Our initial evaluation of these compounds demonstrated that they were impressive inhibitors of the binding of colchicine to tubulin (Lin et al., 1988), and here we have indirectly examined the interactions of the *C. caffrum* agents with tubulin by studying their effects on the binding of radiolabeled colchicine to the protein.

Standard analytical methods demonstrated that CS-A4 is a competitive inhibitor of the binding of colchicine to tubulin, and on the basis of its close structural homology to colchicine (Figure 1), it is reasonable to conclude that the A and B phenyl rings of CS-A4 must bind respectively in subsites on tubulin which bind the A and C rings of colchicine. Although we have not examined the effects of CS-A2 on colchicine binding as thoroughly, it seems likely that it must bind in the identical sites, on the basis of its structural homologies to CS-A4 and to cornigerine (Figure 1).

The experiments presented here demonstrate that CS-A4 and CS-A2 bind to tubulin with high avidity. There was no enhancement of binding of either agent by raising the reaction temperature or by prolonging the incubation time (Table III). Despite rapid displacement of both compounds from tubulin by excess colchicine (Figure 3), isolation of complexes of tubulin with both *C. caffrum* agents was readily achieved by gel filtration chromatography. Although we cannot precisely quantitate the amounts of drug–tubulin complex surviving gel filtration chromatography in the absence of free CS-A4 or CS-A2, the analyses presented in Figure 3E,F, showing linear disappearance of the complexes on a logarithmic scale as a function of time (i.e., first-order kinetics), were based on the assumption of total occupancy of the drug binding site at zero time (see legend to Figure 3). Consequently, it is probable that most of the active tubulin recovered by gel filtration remained liganded to drug. CS-A4 and CS-A2 thus rapidly bind to and dissociate from tubulin, but the binding reactions are much faster than the dissociation reactions. During gel filtration chromatography release of the drug must be followed by rapid rebinding—i.e., passage of protein through the column is much slower than the drug binding reaction. In addition, it should be noted that MTPT, which does appear to dissociate completely from tubulin during gel filtration chromatography,[8]

[8] Even though colchicine binding data indicated complete dissociation of MTPT from tubulin, the MTPT-treated sample was deficient in polymerization, indicating the presence of residual drug.

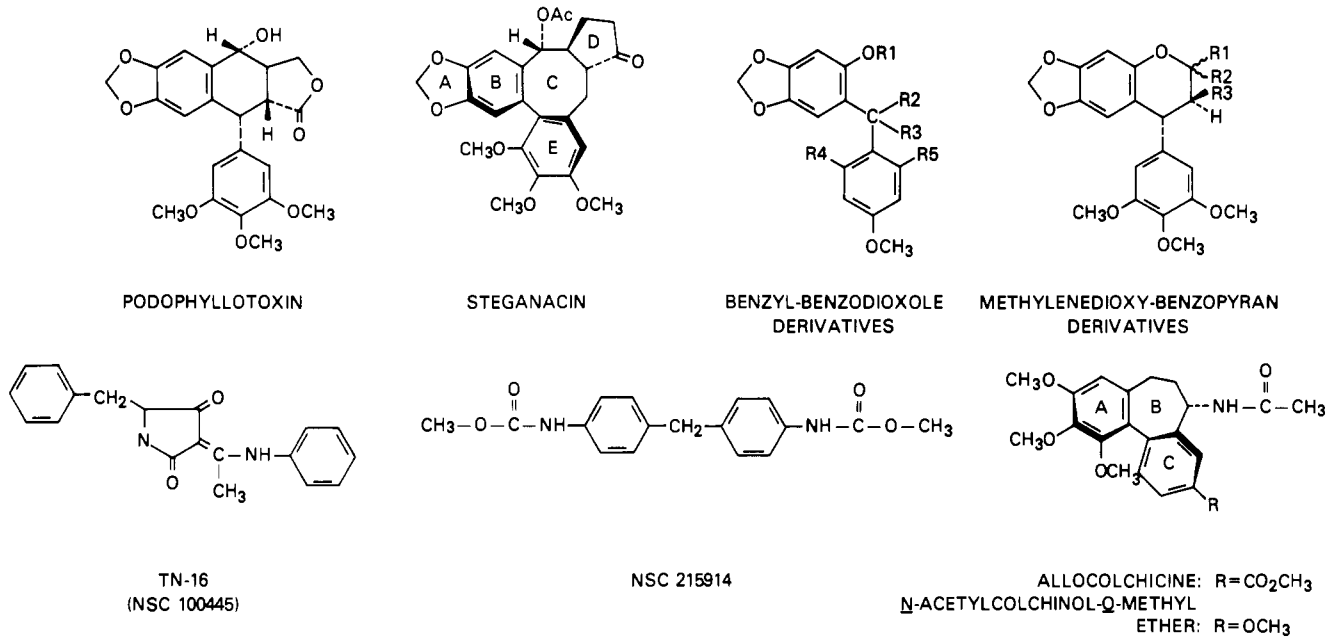

FIGURE 4: Structural formulas of podophyllotoxin, steganacin, benzylbenzodioxole derivatives, (methylenedioxy)benzopyran derivatives, TN-16, NSC 215914, allocolchicine, and *N*-acetylcolchinol *O*-methyl ether. Configuration of steganacin taken from Taafrout et al. (1983). Configurations of allocolchicine and *N*-acetylcolchinol *O*-methyl ether based on the colchicine configuration described by Yeh et al. (1988).

has a significantly higher dissociation rate constant than those of the *C. caffrum* compounds: three laboratories (Bane et al., 1984; Banerjee et al., 1987; Engelborghs & Fitzgerald, 1987) have reported values of (4–6) × 10^{-2} s^{-1}, corresponding to a half-life of 12–17 s, about $^1/_{10}$ to $^1/_{15}$ the half-lives of the complexes of tubulin with CS-A4 and CS-A2.

In comparison to colchicine, both the tubulin binding and the tubulin dissociation reactions of CS-A4 and CS-A2 are much faster—CS-A4 and CS-A2 almost seem to snap into place. Although we are not yet able to quantitate the binding reactions, it is undoubtedly their rapidity which is responsible for the nearly total inhibition of colchicine binding by the *C. caffrum* agents at short incubation times. Dissociation of CS-A4 from tubulin is over 100 times as fast as the dissociation of colchicine (half-lives of 3.6 and 405 min, respectively, at 37 °C), while dissociation of CS-A2 is faster still (half-life of 2.4 min at 37 °C). It is likely its faster dissociation from tubulin relative to CS-A4 that accounts for the weaker inhibition of colchicine binding by CS-A2, and perhaps for its significantly lower cytotoxicity (Lin et al., 1988).

The colchicine binding reaction has been extensively studied, and many proposals have been made to account for the slow, relatively irreversible, and temperature-dependent interaction of the drug with tubulin [reviewed in Hamel (1989)]. The kinetics of the reaction appear to be biphasic and most consistent with a mechanism in which a conformational change in the tubulin–colchicine complex is the rate-limiting step (Garland, 1978; Lambeir & Engleborghs, 1981). Several studies have focused on the binding of the tropolone C ring to tubulin, for enhanced fluorescence (Bhattacharyya & Wolff, 1974) and circular dichroic changes (Detrich et al., 1981) attributable to binding of this moiety to tubulin have been described. Andreu and Timasheff (1982a,b) have proposed a detailed model for the binding of colchicine to tubulin which involves first a slow binding of the tropolone C ring to the protein followed by a change in tubulin conformation which allows rapid binding of the trimethoxybenzene A ring. The rapid binding of the *C. caffrum* compounds and their potent inhibitory effects on colchicine binding appear to be inconsistent with this model. Assuming the A rings of CS-A4 and colchicine are analogous structures [and that the (methylenedioxy)benzene rings of CS-A2 and cornigerine are functionally equivalent], then the colchicine A-ring subsite must be fully available on the tubulin molecule prior to the protein–drug interaction. Although this would then suggest that it is the tropolone C ring of colchicine which is responsible for the drug's slow binding to tubulin, this latter conclusion must be tempered by the observation of rapid binding and dissociation of MTPT (Ray et al., 1981; Bane et al., 1984; Banerjee et al., 1987; Engelborghs & Fitzgerald, 1987), as well as by evidence that colchicine B-ring substituents strongly affect the binding reaction (Bhattacharyya et al., 1986; Banerjee et al., 1987).

Nevertheless, CS-A4 and CS-A2 are only two, albeit the most potent, inhibitors of colchicine binding which contain either two phenyl rings or a phenyl ring and a benzodioxole ring system. These compounds (structures presented in Figure 4) include podophyllotoxin, steganacin,[9] benzylbenzodioxole derivatives, (methylenedioxy)benzopyran derivatives, TN-16, the bis-carbamate NSC 215914, and the synthetic biphenyl colchicinoids allocolchicine and *N*-acetylcolchinol *O*-methyl ether [for detailed references, see Hamel (1989)]. [In studies currently under way in our laboratory, we have observed extensive binding of the latter compound to tubulin at 0 °C, although the binding reaction is slower than that with the *C. caffrum* agents. *N*-Acetylcolchinol *O*-methyl ether also dissociates rapidly from tubulin compared to colchicine (G. J. Kang and E. Hamel, unpublished data).] Most, if not all, of these compounds appear to bind to tubulin more rapidly than colchicine. It thus seems possible that the tropolone C ring binds in a subsite on tubulin that more readily accommodates a benzene ring. The slow binding of colchicine to tubulin might

[9] The configuration of steganacin presented in Figure 4 is adapted from Taafrout et al. (1983), who argued in its favor over earlier, alternate representations. This relationship between the B and E rings is identical with that proposed by Yeh et al. (1988) between the A and C rings of colchicine for the active configuration of the latter (the "*a*S"-biaryl configuration). Consequently, the same configuration is used for allocolchicine and *N*-acetylcolchinol *O*-methyl ether in Figure 4, as both are derived synthetically from colchicine.

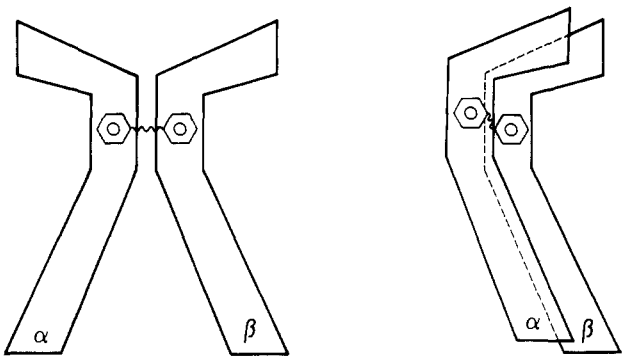

FIGURE 5: Schematic models of the colchicine binding site spanning both subunits of tubulin. The A ring binds to one subunit; the C ring (or a second phenyl ring) binds to the other subunit. The two subsites are located in homologous regions of the two polypeptide chains.

result from the poorer fit of the C ring in this site, perhaps involving a conformational change in the tropolone ring as suggested by Detrich et al. (1981).

Many efforts have been made to localize the colchicine binding site to one of the tubulin subunits, but apparently contradictory results have been obtained. Reactive colchicine analogues, derivatized at the nitrogen atom of the B ring, form specific covalent links predominantly with α-tubulin (Schmitt & Atlas, 1976; Williams et al., 1985). In addition, a proteolytic fragment of α-tubulin binds colchicine with low stoichiometry (Serrano et al., 1984). On the other hand, genetic evidence favors a major role for β-tubulin in the colchicine binding site, for most mutants in tubulin genes which confer resistance to drugs binding at the colchicine site are in β-tubulin genes [Sheir-Neiss et al., 1978; Cabral et al., 1980; but see also Keates et al. (1981)]. Moreover, colchicine and other drugs binding at the colchicine site have major inhibitory effects on the alkylation of the cysteine residues of β-tubulin by sulfhydryl-reactive agents (Luduena & Roach, 1981; Roach & Luduena, 1984; Roach et al., 1985, 1987).

We would like to propose a model for the colchicine binding site of tubulin that spans both subunits and takes into account their substantial sequence homology (Ponstingl et al., 1981; Krauhs et al., 1981). We envisage a subsite on each subunit for a substituted phenyl ring (i.e., an A-ring subsite and a C-ring subsite) in homologous locations of the two polypeptide chains. Figure 5 presents two schemes where such subsites are brought into close proximity with the subunits arranged either in tandem or face-to-face. We are presently attempting to develop appropriate affinity analogues of CS-A4 with reactive groups in the A or B rings, or of colchicine with reactive groups in the A or C rings, to provide experimental evidence for this model.

Finally, we should briefly comment on the relatively short half-life (about 7 h) we have described for the tubulin–colchicine complex (Figure 3D). It was measured here (for comparison to results obtained with CS-A4 and CS-A2) by following the binding of radiolabeled colchicine to tubulin saturated with nonradiolabeled colchicine. When the dissociation of radiolabeled colchicine from tubulin was directly measured, half-lives of 24 (McClure & Paulson, 1977), 36 (Garland & Teller, 1975; Garland, 1977), and 77 h (Sherline et al., 1975) were obtained.[10] We are not able to explain the difference between our results and these earlier studies, nor the wide range of values previously reported. Most likely it results from the very different reaction conditions we have used, which include as a reaction component, most notably, 1 M monosodium glutamate. These reaction conditions were chosen because of the high degree of stability they confer upon tubulin (Hamel & Lin, 1981), but they also appear to result in a higher rate of binding of colchicine to the protein (the time course presented in Figure 3C documents faster binding than is described in most reports in the literature). Colchicine may also dissociate more rapidly from tubulin in 1 M glutamate than in lower ionic strength solutions.

[10] Borisy and Taylor (1967a,b) have, however, reported relatively short half-lives (1–5 h) for the tubulin–colchicine complex when cell extracts, as opposed to purified tubulin, were used.

Registry No. CS-A_4, 117048-59-6; CS-A_2, 111394-44-6; GTP, 86-01-1; colchicine, 64-86-8.

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The Length of a Junction between the B and Z Conformations in DNA Is Three Base Pairs or Less†

Zhongyi Dai, Gerald A. Thomas, Elisabeth Evertsz, and Warner L. Peticolas*
Department of Chemistry and Institute of Molecular Biology, University of Oregon, Eugene, Oregon 97403
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ABSTRACT: Recently it has been suggested that double-helical complexes formed between the DNA sequences $(CG)_n(A)_m$ and their conjugates, $(T)_m(CG)_n$, would be candidates for the formation of a B–Z junction in aqueous solution at high salt concentrations [Peticolas et al. (1988) *Proc. Natl. Acad. Sci. U.S.A. 85*, 2579–2583]. The junction was predicted to occur between a B-type helix in the $d(A)_m \cdot d(T)_m$ section and a Z-type helix in the self-complementary $(CG)_n \cdot (CG)_n$ sequence. In this paper we report Raman experiments on the deoxyoligonucleotides d(CGCGCGCGCGAAAAA) and d(CGCGCGAAAAA) and their complements. It is found the latter compound cannot be induced into the Z form in saturated salt solution but that the former sequence goes into a B–Z junction at 5.5 M salt. From a comparison of the relative intensity of the Raman conformational marker bands for B and Z DNA for both the A–T and C–G base pairs, it is shown that in 5.5 M NaCl solution none of the A–T base pairs are in the Z form, but nine of the C–G base pairs are in the Z form. The remaining three C–G base pairs are either in the junction or in the B form. Thus, the junction is formed from three or less C–G base pairs. If the solution is made 95 μM with $NiCl_2$, then the entire duplex goes into the Z form and the Raman bands of the adenine are completely changed into those of the Z form. A similar three base pair B–Z junction has recently been reported by Sheardy and Winkle [(1989) *Biochemistry 28*, 720–725], who used different base sequences for both the B and Z tracts. This indicates that the short B–Z junction length may be independent of the base sequence.

DNA–protein interaction appears to be governed by the sequence of hydrogen-donating and -accepting groups that occur along the major and minor grooves of the DNA (Seeman et al., 1976; Berg & von Hippel, 1987). When DNA is in its canonical B form, the distances between the hydrogen-bond-donating and -accepting portions of the helix can be obtained from the coordinates of the atoms in the base pairs along the chain. It is becoming increasingly evident that DNA exhibits polymorphism and can possibly go into forms other than the B form. Since it is unlikely that much cellular DNA is in a form different from the canonical B form, it is necessary that unusual conformations such as the Z form begin and end with a junction. Even the B form may be subject to change de-

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