Antimicrotubule Properties of Benzophenanthridine Alkaloids

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ABSTRACT: Chelidonine, sanguinarine, and chelerythrine are natural benzophenanthridine alkaloids that inhibit taxol-mediated polymerization of rat brain tubulin in the micromolar range. Chelidonine is a weak, competitive inhibitor of colchicine binding to tubulin but does not inhibit podophyllotoxin binding. On the other hand, sanguinarine inhibits both colchicine and podophyllotoxin binding to tubulin with I_{50} values of 32 and 46 μM, respectively, and chelerythrine inhibits with I_{50} values of 55 and 60 μM, respectively. The inhibition by these two agents is of the mixed type. Tubulin forms an acid-reversible pseudobase with the imminium ion of sanguinarine, probably through several of its sulfhydryl groups, as shown by the loss of the yellow color of sanguinarine and its 596-nm fluorescence emission peak. Chelidonine, on the other hand, cannot undergo such pseudobase formation, and we conclude that it acts by a different mechanism. A number of previously described pharmacologic effects of these agents may be due to their inhibition of microtubule function.

The interaction of podophyllotoxin at the colchicine-binding site of tubulin has been shown to result from overlap of the trimethoxyphenyl moiety common to both drugs, whereas the benzodioxole moiety of podophyllotoxin and the tropolone moiety of colchicine are thought to occupy distinct binding domains (Cortese et al., 1977). In the case of colchicine, it has been possible to define these domains by the use of single-ring analogs of the A and C rings (Cortese et al., 1977; Andreu & Timasheff, 1982; Engelborghs et al., 1993), but analogs of the benzodioxole moiety have not been available. For this reason, we have tested the benzophenanthridines chelidonine, sanguinarine, and chelerythrine. While substantial chemical information exists for these alkaloids, there is little information regarding their interactions with defined cell components.

Chelidonine, derived from *Chelidonium majus* (greater celandrine) and sanguinarine, obtained from the roots of *Sanguinarius canadensis* (bloodroot), had been mentioned as antimitotic agents before the discovery of tubulin (Eigsti & Dustin, 1955; Lettré, 1952). In addition, they have significant cytotoxicity (Simánek, 1985), and chelidonine was recently found to have moderate activity against reverse transcriptase (Sethi, 1981) as do sanguinarine and chelerythrine. Numerous modifications of the benzophenanthridine structure have been made, but very few of them have been tested as antimitotics and even fewer are available for testing (Sethi, 1981; Shamma, 1972; Simánek & Preininger, 1977). As not all antimitotic agents interact directly with the microtubule system, and because the benzodioxole attached to a nonaromatic ring resembles, to a certain extent, the benzodioxole arrangement of podophyllotoxin, steganacin, the insect sterilants of the benzyl-1,3-benzodioxole series, combretastatin A_2 and the colchicine analog called cornigerine, we have studied the interaction of chelidonine with purified rat brain tubulin. While chelidonine is not, strictly speaking, a benzophenanthridine, we have compared its effects with those of the true benzophenanthridines sanguinarine and chelerythrine. These compounds are reputed to have antimicrobial, DNA intercalating, and antitumor activity, show phototoxicity, and are believed to have specific inhibitory effects on certain enzymes (Sethi, 1981; Tuveson et al., 1989; Arnason et al., 1992; Cohen et al., 1978; Walterová et al., 1981; Herbert et al., 1990). The structures of these alkaloids are compared in Figure 1. We show here that these three alkaloids are indeed antimicrotubule agents and that the actions of the true benzophenanthridines, sanguinarine and chelerythrine, differ from the congener, chelidonine.

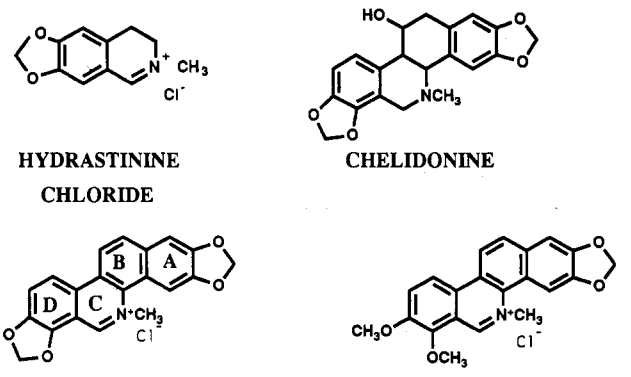

FIGURE 1: Benzophenanthridines and related compounds. The ring numbering is shown for sanguinarine according to Shamma (1972).

MATERIALS AND METHODS

Sanguinarine chloride, hydrastinine chloride, and berberine hemisulfate were from Sigma, chelidonine was obtained from Koch-Light Ltd., and chelerythrine was from LC Laboratories. These compounds were used without further purification. 2,3-(Methylenedioxy)naphthalene was from the Sigma-Aldrich Library of Rare Chemicals. It was recrystallized from *n*-heptane. [^{3}H]Colchicine was from New England Nuclear, and [^{3}H]podophyllotoxin was purified and used as described (Cortese et al., 1977). Taxol was the kind gift of Dr. Matthew Suffness, National Cancer Institute. Rat brain microtubule protein was prepared from frozen rat brains as previously described (Sackett et al., 1991). Pure tubulin was prepared from this material as described (Sackett et al., 1990) and was pelleted and stored in liquid nitrogen at a concentration of 25 mg/mL, where it was stable for extended periods. All polymerization studies were carried out in a MES (2-

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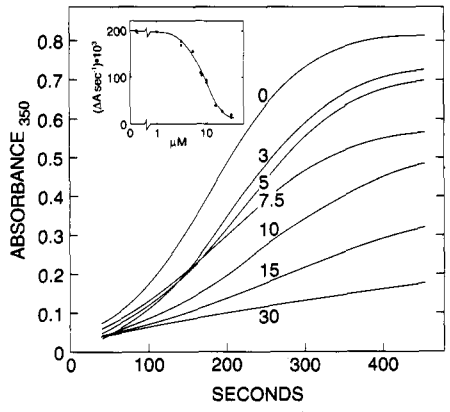


FIGURE 2: Effect of chelidonine on taxol-mediated tubulin polymerization. Rat brain tubulin (4.9 mg/mL) in assembly buffer with 0.67 mM GTP, 20 μM taxol, 1% dimethylsulfoxide, polymerized at 35.5 °C. A_{350} reading started at 40 s. Numbers over the curves are micromolar chelidonine concentrations. Inset: Initial rate of polymerization taken from the above experiment plus another with a different tubulin preparation.

morpholinoethanesulfonic acid) assembly buffer composed of 0.1 M MES, pH 6.85, 1.0 mM EGTA (ethylene glycol bis(β-aminoethyl ether)-*N,N'*-tetraacetic acid), and 1.0 mM $MgCl_2$. Chelidonine was kept as a concentrated stock solution of dimethyl sulfoxide; sanguinarine, chelerythrine, hydrastinine, and berberine were diluted from stock solutions in water. Controls contained the same concentrations of dimethyl sulfoxide, where indicated. Tubulin polymerization was assayed by measurement of the absorbance at 350 nm and was determined in masked 100-μL Hellma cuvettes, thermostated at 36 °C, in a Cary 219 double-beam spectrophotometer. Colchicine and podophyllotoxin binding were assayed after 30-min incubation at 36 °C by the (diethylamino)ethane-impregnated filter paper method (Cortese et al., 1977). The disks were counted in 5 mL of Ultima Gold scintillation fluid to <2% counting error. Fluorescence excitation and emission spectra were determined at room temperature in a Perkin-Elmer MPF 66 fluorometer operating in the (corrected) ratio mode using 5 nm slits and 5 mm path length cells.

RESULTS

Chelidonine. Taxol-induced polymerization of pure rat brain tubulin was inhibited in a concentration-dependent manner (Figure 2). The concentration of chelidonine required for 50% inhibition of the *initial rate* (I_{50}) was 8–9 μM (Figure 2, inset). The *extent* of tubulin polymerization also varied inversely with the chelidonine concentration (Figure 2), but since plateau inhibitions were not obtained at the higher chelidonine concentrations, it was not possible to determine I_{50} values for this parameter. Similar inhibition of microtubule assembly was also seen when 10% dimethyl sulfoxide ($I_{50} \sim$ 20 μM) was used instead of taxol, but the results showed greater variation and are not shown. Microtubules formed in the presence of dimethyl sulfoxide and chelidonine were completely depolymerized by cooling to 4 °C, and polymerization recommenced upon rewarming at a rate only slightly lower than that during the first polymerization cycle (data not shown).

Depolymerization of microtubules (preassembled in 20 μM taxol) by addition of 30 μM chelidonine led to a rapid decrease of ~20% in absorption at 350 nm. This exceeds the decrease expected from the 1% dilution of the sample. The initial decrease was followed by a nearly stable polymer concentration with a loss of 12% of the OD_{350} over the next 1400 s (Figure 3). Similar curves have been observed after addition of podophyllotoxin to steady-state tubulin polymers (Margolis, 1982; Ringel & Horwitz, 1991). The basis for this partial disassembly is not known at present. It may reflect depolymerization of a less stable form of the polymer (ribbons etc.), followed by a slow decay of more stable microtubules by treadmilling or dynamic instability, or it may indicate the interaction with a tubulin isotype. In any event, the binding site for chelidonine behaves *as if* it were not accessible in the bulk of the polymer.

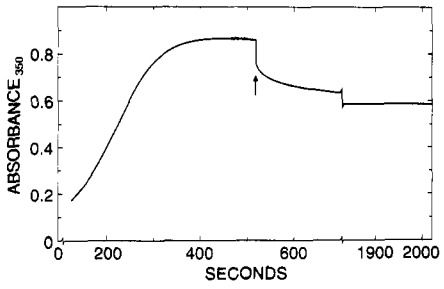


FIGURE 3: Depolymerization of preformed microtubules by chelidonine. Incubation conditions are as in Figure 2 except that chelidonine was added at the arrow. Recording starts at 120 s and is interrupted at 720 s. A control with a 1% volume change showed a much smaller initial drop (not shown).

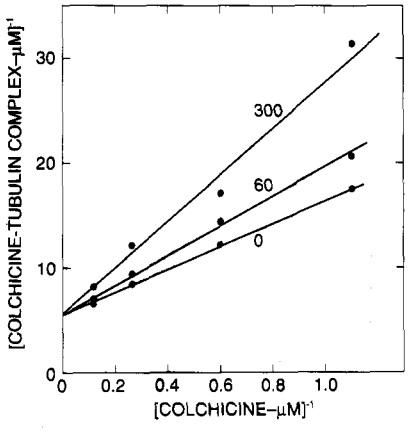


FIGURE 4: Double-reciprocal plot of chelidonine inhibition of colchicine binding. 2.5 μM rat brain tubulin was incubated at 36 °C for 30 min and then assayed by the (diethylamino)ethane filter paper method. Numbers on lines indicate chelidonine concentrations. Free colchicine concentrations are depicted on the abscissa. They were calculated from specific activities: total colchicine – bound colchicine = free colchicine.

Because excitation and emission maxima of chelidonine (λ_{max} = 294.4 and 328.8 nm, respectively) were rather similar to those of the tryptophan spectrum of tubulin, it was difficult to measure chelidonine binding to tubulin directly. Therefore, attempts were next made to study the possible interference by chelidonine with the binding of colchicine and podophyllotoxin to rat brain tubulin. This was carried out by the DEAE filter paper method (Cortese et al., 1977). Chelidonine inhibited [^{3}H]colchicine binding to tubulin in a competitive manner as shown in Figure 4. It is apparent that considerably higher concentrations of the alkaloid were required to achieve half-maximal inhibition of binding ($K_i \sim$ 250–300 μM in different experiments) than were effective against polymerization. By contrast, under otherwise identical assay conditions, the binding of [^{3}H]podophyllotoxin was, to our surprise, entirely insensitive to chelidonine up to concentrations of 450 μM in numerous experiments. A comparison of the colchicine and podophyllotoxin effects of chelidonine is presented in Table I.

Table I: Concentrations for 50% Inhibition by Benzophenanthridines and Related Compounds

drug	colchicine binding[a] (μM)	podophyllotoxin binding[a] (μM)	microtubule assembly[b] (μM)
chelidonine	240 (K_i)	≫450	8–9
sanguinarine chloride	32	46	12–13
chelerythrine chloride	55	60	37
2,3-(methylenedioxy)-naphthalene	≫1000	≫1000	≫341
hydrastinine chloride	≫1000	≫1000	≫333

[a] Concentrations of colchicine and podophyllotoxin were 1 or 2 μM; that of tubulin was 2.5 μM. [b] Tubulin concentrations were 49 μM (see Figures 2 and 5).

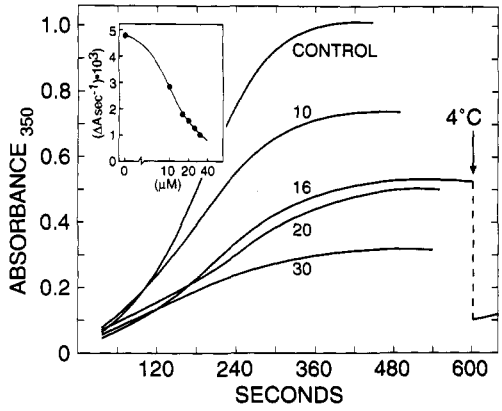


FIGURE 5: Effect of sanguinarine on taxol-mediated tubulin polymerization. Conditions are as for Figure 2. Numbers over curves indicate micromolar sanguinarine concentrations. Inset: Initial rates of polymerization plotted against sanguinarine concentration.

Sanguinarine. As shown in Figure 5, sanguinarine interferes with tubulin polymerization in a concentration-dependent manner that is not very different from that seen with chelidonine. The inset shows that half-maximal inhibition (I_{50}), based on initial rates of polymerization, is 13 μM. Exposure of the partially inhibited polymer to 4 °C leads to complete loss of absorbance at 350 nm. The depolymerized mixture reassembles, on rewarming, to exhibit approximately the starting absorbance.

In contrast to the inhibition of binding of chelidonine, the binding of colchicine to tubulin was inhibited by sanguinarine with an I_{50} of 30 μM, a value about 8–10-fold lower than exhibited by chelidonine. More importantly, and in marked contrast to chelidonine, sanguinarine readily inhibited [^{3}H]-podophyllotoxin binding to tubulin, yielding an I_{50} value of ~43 μM. Inhibition is of the mixed type; although there is an effect of sanguinarine on the K_m, the sanguinarine-promoted inhibition of the binding of *both* ligands is to a large extent noncompetitive (Figure 6). This type of inhibition thus differs from the competitive inhibition shown by chelidonine (see Figure 4).

The extended conjugation of sanguinarine suggested the feasibility of studying its interaction with tubulin by fluorescence. The emission spectra of sanguinarine are depicted in Figure 7. At fixed concentrations of the drug, increasing concentrations of rat brain tubulin caused a progressive decrease in the low-energy (596 nm) peak. The loss of the low-energy peak is accompanied by an increased intensity of the 436-nm peak with a blue shift to 415 nm and then to 410 nm with substantial tubulin excess. Abolition of the red emission occurred at substoichiometric tubulin concentrations (Figure 7, inset). This suggested that more than one reactive group in tubulin might be responsible. Because there are 18

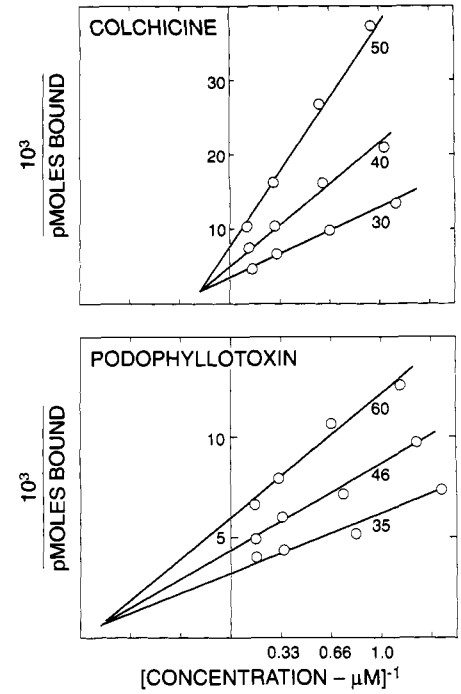


FIGURE 6: Double-reciprocal plots of sanguinarine-mediated inhibition of colchicine and podophyllotoxin binding to rat brain tubulin: upper panel, [^{3}H]colchicine; lower panel, [^{3}H]podophyllotoxin. 2.5 μM tubulin was incubated at 36 °C for 30 min and then assayed by the (diethylamino)ethane filter paper method. Numbers on the lines indicate micromolar sanguinarine concentrations. Free cholchicine or podophyllotoxin concentrations are depicted on the abscissas. They were calculated from specific activities: total ligand – bound ligand = free ligand.

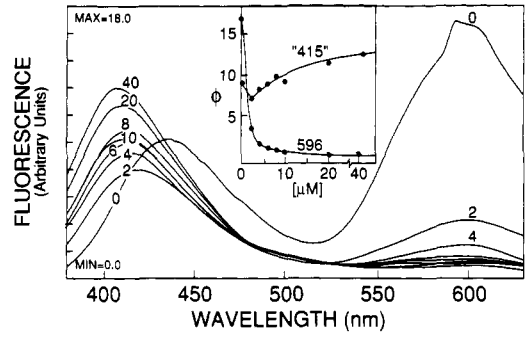


FIGURE 7: Effect of tubulin on the fluorescence emission spectra of 5 μM sanguinarine. Conditions: excitation at 326.5 nm, 5-nm slits, corrected ratio mode in assembly buffer. Inset: Fluorescence intensities (Φ) in arbitrary units at the 596-nm maximum and at the blue maximum (433-410 nm) plotted as a function of tubulin concentration.

sulfhydryl groups in the tubulin dimer (Krauhs et al., 1981), we added excess mercaptoethanol to sanguinarine; this also led to abolition of the yellow color of the solution and abolished the 596-nm emission peak.

Although we initially thought that this loss of the 596-nm emission peak might indicate a thiol-induced reduction of the drug, it has been pointed out (Simánek & Preininger, 1977; Walterová et al., 1981; Walterová et al., 1980; Nandi et al., 1985), that in aqueous solution the imminium ion of sanguinarine can take up OH^-, covalently, leading to pseudobase formation and a decrease in the conjugation; this is accompanied by loss of the yellow color and the emission at ~596 nm. We have confirmed this reaction with OH^- and the fact that addition of acid reverses the pseudobase formation with return of the yellow color and the 596-nm emission. Other nucleophiles such as mercaptoethanol, dithiothreitol, neu-

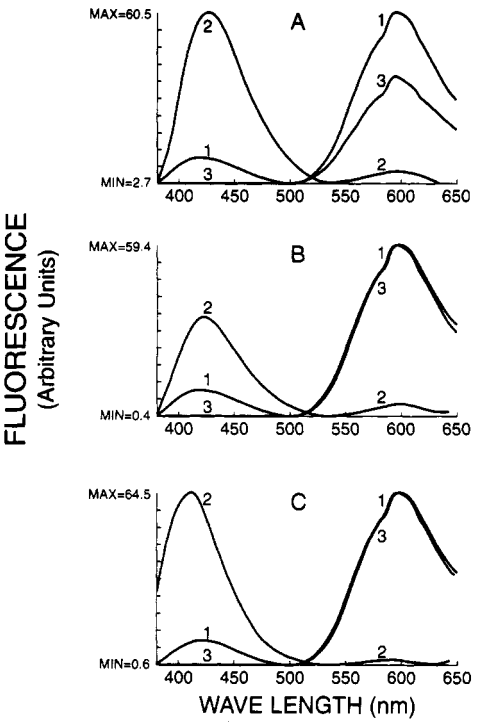


FIGURE 8: Reversal of the "bleaching" of the red emission peak of sanguinarine by acid. Conditions: 10 μM sanguinarine, excitation at 326.5 nm, 5-nm slits, corrected ratio mode. Curves: (1) sanguinarine alone; (2) sanguinarine plus thiol or tubulin; (3) curve 2 plus 20 mM HCl. Note that in the range 400–450 nm the intensities of curves 3 are barely above the baseline. Panels: (A) 1.0 M mercaptoethanol; (B) 10 mM dithiothreitol; (C) 10 μM tubulin.

tralized cysteine hydrochloride and reduced glutathione, and thiosulfate can all bleach the yellow color and the emission at 596 nm; the color can be restored by acid in all cases. It must be pointed out, however, that acid virtually completely abolished the 415–430-nm peak. The presence of this peak in curves 1 (Figure 8) is due to the fact that the pH in this experiment was 6.9. Examples of this are depicted in Figure 8A,B for mercaptoethanol and dithiothreitol. The 596-nm peak is virtually abolished by these thiols (curves 2) but can be restored by acid (curves 3). These results are identical to those obtained when tubulin interacts with sanguinarine; this effect is also reversed by acid (Figure 8C). The greater effect of tubulin on the 596-nm peak is striking.

It seems likely that the interaction between the drug and the protein can be viewed as a nucleophilic attack by tubulin on the imminium ion of sanguinarine. Since –SH groups are stronger nucleophiles than –OH groups, we suggest that the nucleophilic attack by tubulin on sanguinarine is mediated by its –SH groups. We believe this to be a novel class of interactions for tubulin. The same interaction occurs with chelerythrine. Pretreatment with dithiothreitol abolished the inhibitory effect of sanguinarine on microtubule assembly. In agreement with this hypothesis, pretreatment of chelidonine with mercaptoethanol or other thiols had no effect on its ability to block polymerization of tubulin or to interfere with colchicine binding; thus its mechanism of interaction with tubulin is of a different nature.

Whether pseudobase formation is the only mechanism for the sanguinarine effect is not clear at present. Two reports (Tuveson et al., 1989; Arnason et al., 1992) have shown that the phototoxicity of sanguinarine results from the production of hydrogen peroxide and that catalase prevents toxicity. These experiments were carried out at 4–5.8 mW/cm^2 (at wavelengths >290nm), but in our study bleaching of sanguinarine occurred under ordinary laboratory lights (<1 μW/cm^2). As no attempts were made to exclude oxygen, we added catalase to remove the effects of any peroxide that might be generated. The inhibitory effect was not prevented by 17 μg/mL of a highly active crystalline catalase preparation. It seems probable, therefore, that the sanguinarine–tubulin interaction proceeds by a mechanism not dependent on peroxide generation.

Chelerythrine (Figure 1) was recently promoted as a potent and highly specific inhibitor of protein kinase C (Herbert et al., 1990). It possesses two methoxy groups in place of the D-ring-dioxole of sanguinarine (see Figure 1) but is otherwise identical. The p*K* of the imminium ion is higher (8.8 *vs* 7.9) (Nandi et al., 1985), but chelerythrine undergoes reversible pseudobase formation like sanguinarine. Chelerythrine inhibits tubulin polymerization with an I_{50} of 37 μM and is thus a somewhat weaker inhibitor of microtubule assembly than chelidonine or sanguinarine. It inhibits the binding of *both* colchicine and podophyllotoxin to tubulin, as shown in Table I. The decreased potency of chelerythrine may reflect the higher p*K* or may result from the loss of the D-ring-dioxole, since in the benzylbenzodioxole derivatives substitution of methoxy groups for the dioxole lowers inhibitory potency (Batra et al., 1985). Furthermore, the podophyllotoxin analogue, sikkimotoxin, with two methoxy groups in place of the dioxole of podophyllotoxin, is about 20-fold less active than podophyllotoxin (Stähelin & von Wartburg, 1989). It is of interest that chelerythrine is also a weaker inhibitor than sanguinarine for alanine aminotransferase (Herbert et al., 1990).

It has been repeatedly shown that the low-affinity bindings of both the single A and C rings of colchicine to tubulin each contribute to the binding of colchicine to tubulin. In an attempt to learn if a simpler compound forming a pseudobase from an imminium ion might be inhibitory, we tested the effect of hydrastinine chloride (see Figure 1) on tubulin polymerization. As shown in Table I, there was virtually no inhibition of assembly by this analogue at 333 μM, and we could not detect any inhibition of colchicine or podophyllotoxin binding at 1.0 mM concentration. Since the benzodioxole ring of hydrastinine is not isosteric with chelidonine, these results do not allow us to make a decision between this structural difference and the possibility that a dioxole-bearing imminium moiety is not by itself sufficient to inhibit the polymerization of tubulin. By similar reasoning, we have tested the effects of 2,3-(methylenedioxy)naphthalene, a compound equivalent to the dioxole of the A and B rings of sanguinarine and chelerythrine. There was no effect by 1.0 mM concentrations of this compound on the binding of either podophyllotoxin or colchicine to tubulin. Moreover, 341 μM concentrations had no effect on tubulin polymerization (see Table I). While we cannot rule out low-affinity binding because of limited solubility, it would appear that both halves of the benzophenanthridines are required for significant binding to tubulin and must be joined.[1]

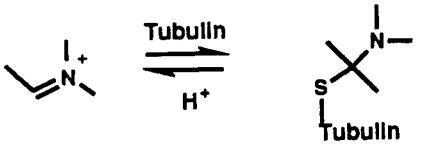
COMMENT

The present study shows that the benzophenanthridines are moderately potent anti-microtubule-assembly agents,

[1] We have also tested the benzodioxole-containing alkaloid berberine, a folk remedy that has various antibiotic activities and binds DNA (Hahn & Ciak, 1975), one of whose tautomeric forms resembles a portion of the chelidonine or sanguinarine molecule. Its nitrogen atom is, however, in a bridgehead rather than a ring position. We failed to see significant inhibition of tubulin polymerization at 100 μM.

whereas they are less potent inhibitors of colchicine binding. This potency discrepancy between the effect on assembly and binding is typical for many antimicrotubule drugs such as colchicine (Farrell & Wilson, 1980), rotenone (Marshall & Himes, 1978), podophyllotoxin (Farrell et al., 1979), and the benzylbenzodioxoles (Batra et al., 1985), for example. Such substoichiometric inhibition of assembly, shared by chelidonine, sanguinarine, and perhaps chelerythrine, most probably reflects drug–tubulin complex inhibition at the growing end of the microtubule that prevents further addition of tubulin dimers or an alteration in the microtubule lattice. However, in comparisons of assembly inhibition with the (stoichiometric) inhibition of colchicine binding by such drugs, it must be remembered that the pseudoirreversibility of colchicine binding may well complicate the interpretation of displacement assays and may contribute to the discrepancy in the potencies.

It has previously been shown that colchicine has an effect on the properties of certain cysteine residues of tubulin (Luduena & Roach, 1981) and the drug binds in the vicinity of these residues (unpublished observations). Moreover, it has been proposed that the methyltropolone moiety (ring C) of colchicine interacts with a nucleophile in tubulin, most probably –SH, to form a σ-complex (Lacey, 1988). This would suggest a superficial resemblance to the pseudobase formation when tubulin binds sanguinarine. However, such a mechanism would entail a marked reduction of the aromaticity of the tropolone, would produce a hypsochromic shift in the absorption spectrum of colchicine that is not observed, and would prevent the fluorescence observed upon binding of colchicine to tubulin (Bhattacharyya & Wolff, 1974) since colchicine requires an intact tropolone moiety to fluoresce. Hence we think that the σ-complex is an improbable structure for colchicine binding. By contrast, sanguinarine and chelerythrine form reversible adducts with tubulin through a nucleophilic attack by the protein (probably several –SH groups) on the imminium group of these compounds as follows:



The greater nucleophilicity of tubulin as compared to small thiols is interesting. This type of interaction is new for tubulin but is not unique, since alanine aminotransferase (Walterová et al., 1981) and the Na/K ATPase (Herbert et al., 1990) are inhibited by sanguinarine > chelerythrine by similar interactions. Furthermore, we have observed bleaching of the yellow color of sanguinarine also with alcohol dehydrogenase and aldolase, whereas proteins lacking a free –SH group such as ribonuclease A, lysozyme, insulin, or mercaptalbumin had no such effect. This finding also suggests that the –OH groups of serine or threonine in proteins are not likely to be sufficiently strong nucleophiles (under our concentration conditions) to interact with the imminium group of the drug.

Chelidonine cannot form pseudobases, yet it inhibits microtubule assembly. Thus other types of interactions with tubulin must also occur, and this probably involves the extended ring system or two attachment points as shown by the negative results with hydrastinine (see Table I). Because of the virtual absence of chelidonine-promoted inhibition of podophyllotoxin binding while inhibition of colchicine binding persists, albeit weakly, we speculate that pseudobase formation most probably occurs at the podophyllotoxin-binding domain *not* shared by colchicine (Cortese et al., 1977). A dissociation between colchicine and podophyllotoxin binding has also been observed with carrot tubulin—i.e., colchicine binding is not inhibited by podophyllotoxin (Okamura et al., 1984). It must be remembered that, in contrast to sanguinarine and chelerythrine, chelidonine is nonplanar, with both the B and C rings (see Figure 1) in the half-chair conformation (Sugiura et al., 1986); hence steric factors may also contribute to the discrepancy between its effects on colchicine and podophyllotoxin bindings.

Finally, whatever the precise interaction locus of the benzophenanthridines on tubulin may be, it is important to remember that these agents are not specific for the various enzymes or macromolecules inhibited (Simánek, 1985; Sethi, 1981; Cohen et al., 1978; Walterová et al., 1981; Herbert et al., 1990) and that antitubulin effects must be considered when these drugs are added to intact tissues. Inhibitions may result from nucleophilic interactions of tubulin or another target macromolecule with the alkaloid, in addition to any antitumor effects (Lettré, 1952; Simánek, 1985; Shamma, 1972) that may be ascribed to intercalation into DNA.

ACKNOWLEDGMENT

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