Antimitotic Natural Products and Their Interactions with Tubulin

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I. INTRODUCTION

Antimitotic natural products have long been of interest to scientists and physicians, even before their precise mechanism of action could be articulated. These compounds, or preparations derived from the organisms that produce them, have a long history both as poisons and in the treatment of human diseases. The first antimitotic agent to be characterized, colchicine, was instrumental in the purification of the major component of microtubules,[1] cellular organelles that oscillate between forming the mitotic spindle and acting as the superstructure of the interphase cytoskeleton.[2] This protein was subsequently named tubulin, and it consists of two 50 kDa subunits (α- and β-tubulin) with

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two tightly bound molecules of guanine nucleotide, usually in the form of GTP. Half the GTP, considered bound to the nonexchangeable site, cannot be removed from tubulin without denaturing the protein. The other half is hydrolyzed during microtubule assembly and, although tightly bound to the exchangeable site, can be readily displaced by exogenous GDP or GTP.

Almost invariably antimitotic agents have been found to interact specifically with tubulin, rather than with other components of the microtubule (generically referred to as "microtubule-associated proteins" or MAPs) or other proteins involved in mitosis. While antimitotic agents have significant roles in the treatment of inflammatory (colchicine), fungal (griseofulvin), and parasitic (benzimidazole carbamates) diseases, the greatest current interest in these compounds derives from their role in the treatment of cancer. While the *Catharanthus* alkaloids vincristine and vinblastine have been used for almost 30 years, particularly in the treatment of childhood neoplasms and adult lymphomas, excitement with this class of agent has been renewed by promising results obtained with the taxoids paclitaxel (Taxol®) and its semisynthetic analog docetaxel (Taxotere®).[3,4]

As recently as 1984 one could generalize that virtually all antimitotic natural products had been derived from higher plants. Since then, however, a large number of new agents have been described. These have had an extraordinary range of chemical structures and have been discovered among many different classes of organism, including mammals. (However, it should be noted that the true species of origin may not be that from which a given compound was nominally extracted.) The purpose of this review is to summarize the known antimitotic natural products, provide a single source for salient aspects of their molecular structures, and outline key elements in their interactions with tubulin and microtubules. The reader is referred to recent reviews of colchicine,[5] podophyllotoxin–steganacin–combretastatin,[6] *Catharanthus* (vinca) alkaloids,[7] taxoids,[8–12] and vinca domain drugs[13] for further detail and more comprehensive references to the literature.

II. INHIBITORS OF ASSEMBLY INTERACTING AT THE COLCHICINE SITE

A. Colchicine

Colchicine is the major alkaloid obtained from the higher plant *Colchicum autumnale* and related species (for recent reviews, see Refs. 5 and 14). A representation of its active biaryl configuration (designated *aS,7S;* see Ref. 15) is presented in Fig. 1. Most natural analogs are modified in the C-7 side chain substitutent, but allocolchicine, with an aromatic 6-member C ring and a $COOCH_3$ substituent at C-10, and cornigerine (structure in Fig. 1) have both been obtained from *C. cornigerum.* (Both allocolchicine and cornigerine have activity *in vitro* as inhibitors of cell growth and tubulin polymerization comparable to that of colchicine.) Plant extracts were known since antiquity for both

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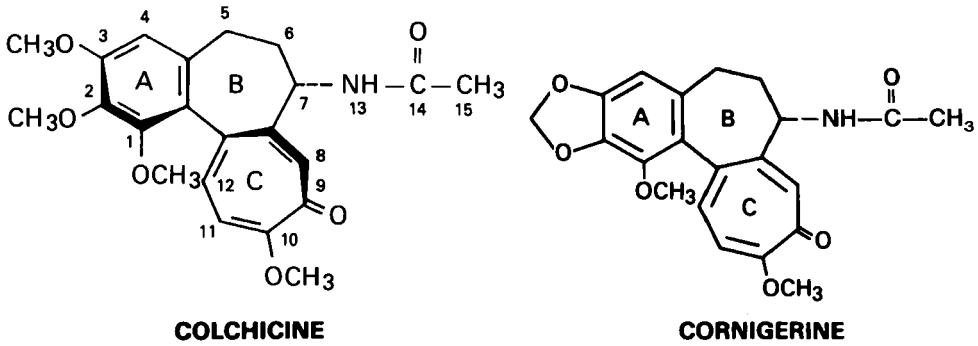


COLCHICINE **CORNIGERINE**

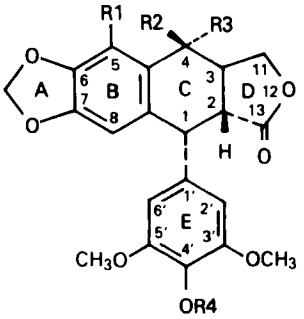
Figure 1. Structures of colchicine and cornigerine.

their therapeutic properties in gout and their toxicity. Colchicine was originally purified early in the 19th century, and its antimitotic activity noted at the end of that century. The availability of radiolabeled colchicine permitted the original purification of tubulin from brain tissue,[1] and the tubulin–colchicine interaction has been extensively studied for nearly three decades.

Colchicine (and most colchicinoids, but not allocolchicinoids) are unique among antimitotic agents in binding relatively slowly to tubulin, in a reaction that occurs at a negligible rate at 0°C, and in binding so tightly that the reaction has frequently been incorrectly described as being irreversible. Upon binding to tubulin there is a substantial increase in the fluorescence of tubulin, and this has permitted careful analysis of the kinetics of the binding reaction,[16] which is biphasic. Analysis of the fast phase was most consistent with a model in which more rapid binding of the drug to tubulin was followed by a slower conformational change in the protein.[16,17] Recent evidence indicates that the biphasic kinetic pattern is a consequence of tubulin heterogeneity.[18] Upon binding to tubulin, colchicine induces hydrolysis of GTP bound in the exchangeable site that, while uncoupled from microtubule assembly, still seems to require tubulin–tubulin interactions.[19]

Although a photoaffinity analog of colchicine primarily labeled α-tubulin,[20] direct photoaffinity labeling of tubulin resulted in colchicine binding primarily to β-tubulin,[21] as did binding of chemically reactive colchicine analogs modified in the A ring.[22] The direct photoaffinity technique yielded radiolabel derived from colchicine in peptides containing amino acid residues 1-36 and 214–241. Binding of colchicine, as well as other colchicine site agents, results in inhibition of formation of a cross-link between cys-239 and cys-354 of β-tubulin by the divalent sulfhydryl reactive agent *N,N'*-ethylenebis(iodoacetamide).[23] Alkylation of cys-239, however, does not appear to inhibit colchicine binding to tubulin.[24]

The net effect of binding of colchicine to tubulin is complete inhibition of tubulin assembly, whether MAPs are present or not. Complete inhibition is often observed at colchicine concentrations much lower than the tubulin concentration, a phenomenon known as substoichiometric poisoning, which also occurs with most of the other agents described below. This occurs even though there is small but measurable incorporation of tubulin–colchicine complex into polymer[25] and may result from suppression of the dynamic properties of microtubules.[26] At high magnesium concentrations[27,28] and with the GTP analog guanosine 5'-[α,β-methylene]triphosphate[29] tubulin polymers with a high colchicine content and highly abnormal morphology have been described.



Podophyllotoxin: R1 = R2 = H
R3 = OH; R4 = CH_3
4'-Demethylpodophyllotoxin: R1 = R2 = R4 = H
R3 = OH
Deoxypodophyllotoxin: R1 = R2 = R3 = H
R4 = CH_3
4'-Demethyldeoxypodophyllotoxin: R1 = R2 = R3 = R4 = H
α-Peltatin: R1 = OH; R2 = R3 = R4 = H
β-Peltatin: R1 = OH; R2 = R3 = H
R4 = CH_3
β-Peltatin A-methyl ether: R1 = OCH_3
R2 = R_3 = H
R4 = CH_3
Epipodophyllotoxin: R1 = R3 = H R2 = H
R4 = CH_3
4'-Demethylepipodophyllotoxin: R1 = R3 = R4 = H; R2 = OH

Figure 2. Structures of podophyllotoxin and related compounds.

B. Podophyllotoxin

Podophyllotoxin and several active analogs, obtained from the higher plant *Podophyllum peltatum* and related species, is shown in Fig. 2 (for a recent review, see Ref. 6). Podophyllotoxins have also been obtained from many unrelated species. Although podophyllotoxin is of limited current therapeutic interest, the medicinal use of the plant extracts has a history comparable to that of extracts containing colchicine, and the compound was originally purified in the late 19th century. Its antimitotic activity was first reported at least a half century ago. The A, B, C, and D rings are almost coplanar, with the E ring oriented almost perpendicular to the plane of the other rings.

The binding of podophyllotoxin to tubulin is more rapid and reversible than that of colchicine, and podophyllotoxin acts as a competitive inhibitor of colchicine binding. Although podophyllotoxin does not interfere with GDP/GTP exchange on tubulin, it generally inhibits tubulin-dependent GTP hydrolysis, unlike any other colchicine site compound yet examined. A recent computer modeling study suggests incomplete overlap of the colchicine and podophyllotoxin binding sites, with the two trimethoxybenzene rings *not* binding in equivalent sites.[30] Podophyllotoxin inhibits tubulin assembly, but the only abnormal polymer yet described when podophyllotoxin is present was formed if the GTP analog guanosine 5'-[α,β-methylene]triphosphate is also included in the reaction mixture.[29] Although its dynamic effects on tubulin assembly have not yet

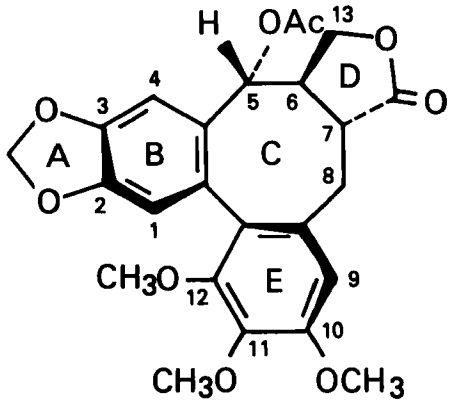


Figure 3. Structure of steganacin.

been studied *in vitro,* podophyllotoxin has effects in cultured cells that suggest the drug suppresses the dynamic instability properties of microtubules.[31]

C. Steganacin

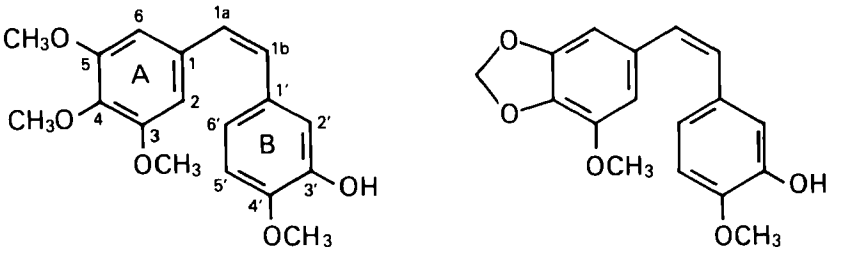
Steganacin and a number of related compounds were first isolated by Kupchan and his colleagues from the stems and stem bark of the East African tree *Steganotaenia araliacea*.[32] The revised structure presented in Fig. 3 is that proposed by Tomioka *et al.*[33] and Robin *et al.*[34] Note that the biaryl configuration is identical to that later proposed by Brossi and collaborators for colchicine,[15] as depicted in Fig. 1. See Ref. 6 for a comprehensive review.

Steganacin inhibits mitosis, competitively inhibits the binding of colchicine to tubulin, and inhibits tubulin polymerization.[35] It resembles colchicine rather than podophyllotoxin in its effects on tubulin-dependent GTP hydrolysis, stimulating a reaction uncoupled from assembly.[36]

D. Combretastatins

The naturally occurring combretastatins were isolated from the stem bark of the South African tree *Combretum caffrum* and characterized by Pettit and his collaborators, beginning in 1982.[37] Among the most potent members of the group are combretastatin A-4[38] and combretastatin A-2,[39] whose structures are presented in Fig. 4. Note the repetition of the colchicine/cornigerine analogy shown in Fig. 1. See Ref. 6 for a comprehensive review.

Soon after its isolation as a cytotoxic agent, the original combretastatin was shown to act as an antimitotic agent and as a competitive inhibitor of colchicine binding and an inhibitor tubulin polymerization. Subsequent studies focused on the more potent combretastatins A-4 and A-2, which display the same biological properties, with combretastatin A-4 acting as an exceptionally potent inhibitor of colchicine binding.[40,41] Typically, when [^{3}H]colchicine and combretastatin A-4 are present in equimolar concentrations and in 5-fold excess to tubulin, greater than 95% inhibition of colchicine binding occurs. However, at prolonged incubation times there is a decline in the extent of inhibition,[41] which is observed with many colchicine site drugs. This and other evidence has indicated that the combretastatins bind rapidly and reversibly to tubulin, with the



Combretastatin A-4 Combretastatin A-2

Figure 4. Structures of combretastatins A-4 and A-2.

dissociation of combretastatin A-2 appearing to be more rapid than that of combretastatin A-4. With the combretastatins in sufficiently high concentration to inhibit assembly completely, a wide variety of effects on tubulin-dependent GTP hydrolysis were observed. With some agents modest inhibition occurred, while with combretastatin A-4 net hydrolysis was substantially stimulated. With combretastatin A-2 GTP hydrolysis differed little from the pattern obtained in a control reaction mixture where hydrolysis was coupled to simultaneous assembly.[41]

E. Curacins

Gerwick and his colleagues.[42,43] described the isolation of curacin A from the marine cyanobacterium *Lynbya majuscula*, together with initial studies that showed it inhibited mitosis, tubulin polymerization, and colchicine binding. The effect on colchicine binding was subsequently shown to be competitive. In addition, small amounts of the nearly equipotent curacins B and C were obtained from the extracts.[44] The structures of these compounds are presented in Fig. 5. These compounds are unique for colchicine site

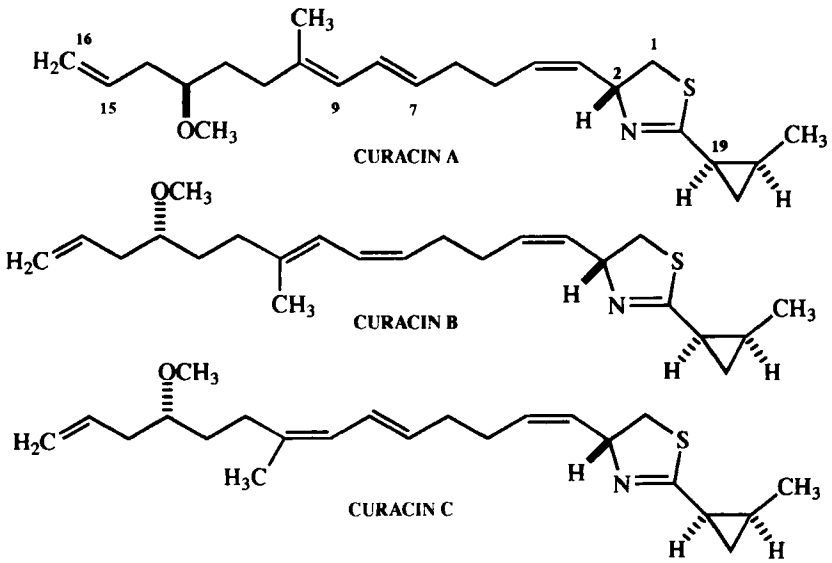


Figure 5. Structures of curacins A, B, and C.

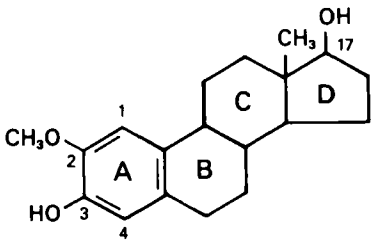


Figure 6. Structure of 2-methoxyestradiol.

agents in that they have no aromatic moiety and only two conjugated olefinic bonds.

The interaction of curacin A with tubulin is characterized by rapid binding, slow dissociation, and induction of GTP hydrolysis uncoupled from normal assembly.[44] While inhibiting microtubule assembly, curacin A induces formation of abnormal polymers;[45] but, as opposed to colchicine, this aberrant polymerization reaction does not require either high magnesium or a GTP analog.

F. 2-Methoxyestradiol

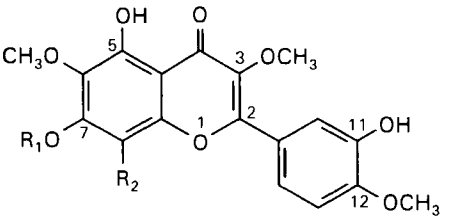
2-Methoxyestradiol (structure in Fig. 6) is the major mammalian metabolite of the primary estrogenic hormone β-estradiol, rising to especially high levels in the third trimester of pregnancy.[46] Mitotic perturbations have been observed for many years in cultured cells treated with both β-estradiol and synthetic estrogens. Seegers *et al.*[47] showed that 2-methoxyestradiol was substantially more potent in this effect than β-estradiol itself, proposing that it was conversion of the latter to 2-methoxyestradiol that was responsible for the cytotoxic properties of β-estradiol.

D'Amato *et al.*[48] found that 2-methoxyestradiol was a weak competitive inhibitor of the binding of colchicine to tubulin and that the agent inhibited the rate but not the extent of tubulin assembly. The polymer formed had morphology that differed little from control polymer but had significantly increased cold stability. These findings raise interesting questions as to whether 2-methoxyestradiol or other steroid molecules may modulate microtubule assembly or function through binding at the colchicine site.

Studies with [4-^{3}H]2-methoxyestradiol[49] have demonstrated relatively rapid binding of the drug to unpolymerized tubulin and its rapid dissociation from the protein. The binding reaction is potently inhibited by colchicine and other colchicine site drugs. The radiolabeled drug also binds to polymerized tubulin in a reaction negligibly inhibited by colchicine site drugs. Thus, the altered properties of polymer formed in the presence of 2-methoxyestradiol may derive from binding of drug to polymer after assembly rather than from participation of a tubulin-drug complex in the polymerization reaction.

G. Flavonols

The two flavonols shown in Fig. 7 have been obtained from higher plants, centaureidin from *Polymnia fruticosa*[50] and "flavonol 2" from a wide variety of plants, including *Zieridium pseudobtusifolium, Acronychia porteri, Polanisia dodecandra* and *P. tachysperma*, and *Guttierrezia microcephala* and *G. sarothrae*.[51,52] These two flavonols are moderately cytotoxic, and centaureidin was shown to arrest cells in mitosis. Both compounds inhibit tubulin polymerization and binding of colchicine to tubulin.[50,52]



centaureidin: $R_1 = R_2 = H$

flavonol 2: $R_1 = CH_3$; $R_2 = OCH_3$

Figure 7. Structures of antimitotic flavonols.

H. Rotenone

Rotenone (structure in Fig. 8) has been derived from several higher plants, including *Lonchocarpus nicou* and *Derris elliptica*. Although primarily known as an inhibitor of mitochondrial respiration, rotenone can cause cells to accumulate in metaphase arrest. The compound has been shown to inhibit tubulin polymerization and colchicine binding, probably competitively. For specific references, see Ref. 11.

I. Griseofulvin (?)

Derived from the mold *Penicillium griseofulvin*, the antifungal agent griseofulvin (structure in Fig. 9) has weak effects on mammalian cell growth and microtubule assembly. Data have been presented both for an interaction with tubulin and for an interaction with MAPs. At least one group[53] observed inhibition of colchicine binding by griseofulvin, but others reported no inhibition of this reaction. For a more comprehensive review and specific references to the literature, see Ref. 11.

III. INHIBITORS OF ASSEMBLY INTERACTING IN THE "VINCA DOMAIN"

A. *Catharanthus* (Vinca) Alkaloids

Vincristine and vinblastine (structures in Fig. 10) have been used in the chemotherapy of neoplastic diseases for about three decades (see Ref. 7 for a recent review). These agents were isolated from the higher plant *Catharanthus roseus* (formerly *Vinca rosea*), and their antimitotic activity was established soon after their discovery.

Besides inhibiting normal microtubule assembly, the *Catharanthus* alkaloids cause an aberrant polymerization reaction, particularly when present at superstoichiometric con-

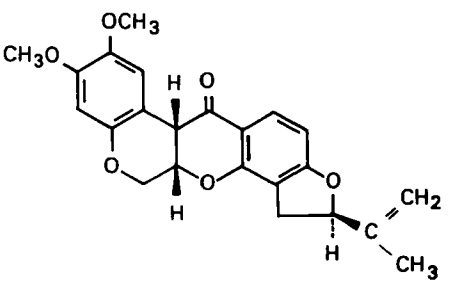


Figure 8. Structure of rotenone.

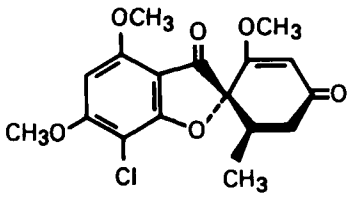


Figure 9. Structure of griesofulvin.

centrations relative to the tubulin concentration. In cell-free systems both with and without MAPs spiral structures are generally formed, but their precise morphology is altered by the MAPs. In cells and under appropriate conditions *in vivo* the abnormal polymer has the appearance of crystalloid inclusions largely composed of tubulin. These have been called tubulin paracrystals. It should be noted that the drug content of these abnormal polymers appears to be stoichiometric with their tubulin content,[54] despite the requirement for higher drug concentrations for their optimal formation.

The binding of vincristine, vinblastine, and related compounds to tubulin has yielded highly divergent quantitative data from different laboratories, probably reflecting differences in experimental techniques and conditions, including, in particular, Mg^{2+} concentration. Often biphasic Scatchard plots were obtained. In an elegant analysis Timasheff and colleagues[55–57] have persuasively argued that such biphasic plots derive from the coexisting aggregation reaction that occurs with *Catharanthus* alkaloid binding to tubulin at a single high affinity site rather than from different classes of binding site. They described the overall process as an isodesmic self-association reaction (multiple steps with identical association constants) with ligand binding stabilizing oligomer, drug binding to both tubulin α-β dimers and oligomers, and self-association of both unliganded tubulin and tubulin-drug complex. Vinblastine has also been shown to bind to microtubules, particularly at their ends, and to cause apparent protofilament unraveling into spiral structures morphologically similar to those observed when drug and un-

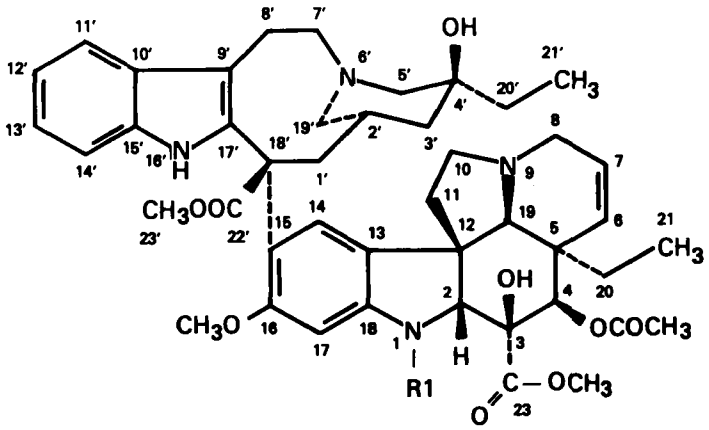


Figure 10. Structures of the *Catharanthus* alkaloids vinblastine and vincristine.

polymerized tubulin are mixed.[58] Low concentrations of vinblastine have been shown to suppress microtubule dynamic instability.[59]

The effects of vinblastine on tubulin-nucleotide interactions has been extensively studied. The drug strongly inhibits tubulin-dependent GTP hydrolysis and weakly inhibits binding of GDP and GTP at the exchangeable nucleotide site.[60,61] Vinblastine does not, however, displace nucleotide bound in the exchangeable site. Almost all the drugs described below are more potent inhibitors of nucleotide exchange than is vinblastine, but, as with vinblastine, none of them appear to displace nucleotide bound to tubulin.

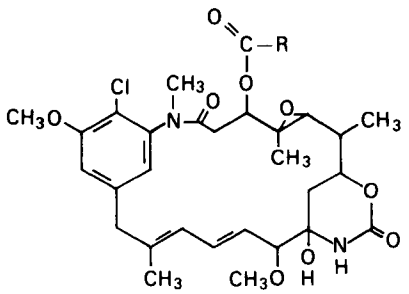
If tubulin is first depleted of exchangeable site nucleotide, *N,N'*-ethylenebis(iodoacetamide) will cause formation of a cross-link between cysteine-12 and either cysteine-201 or cysteine-211 of β-tubulin.[23] Formation of this cross-link is inhibited by GTP and drugs that inhibit nucleotide exchange. There appears to be good quantitative correlation between potency as an inhibitor of exchange and potency as an inhibitor of cross-link formation. Thus, vinblastine weakly inhibits cross-link formation, in tandem with its weak inhibition of exchange. It should also be noted that direct photoaffinity labeling of tubulin with radiolabeled GTP occurs preferentially at cys12 of β-tubulin.[62]

Despite the above findings of vinblastine effects on the exchangeable nucleotide site, which suggest a significant interaction between the drug and β-tubulin, specific labeling of tubulin with a photoreactive vinblastine derivative occurred primarily on α-tubulin (α:β = 3:2).[63]

B. Maytansinoids and Ansamitocins

Maytansine and related compounds have been isolated from the higher plants *Maytenus ovatus*,[64] *M. buchananii, M. serrata, Putterlickia verrucosa,* and *Colubrina texensis* and the structurally similar ansamitocins from a *Nocardia* microorganism[65] (structures in Fig. 11). Their antimitotic activity was established soon after their discovery. See Ref. 13 for a review. Maytansine underwent investigation as an anticancer agent about 15 years ago, but no significant clinical activity was obtained.

Radiolabeled maytansine binds rapidly and reversibly to tubulin in the cold, and at 37°C a K_D value of 0.7 μM was obtained. The binding of maytansine is inhibited by the *Catharanthus* alkaloids, and vincristine was shown to be a competitive inhibitor.[66] In



MAYTANSINE: R= $CH(CH_3)N(CH_3)COCH_3$
ANSAMITOCIN P-3: R= $CH(CH_3)_2$
ANSAMITOCIN P-4: R= $CH_2CH(CH_3)_2$

Figure 11. Structures of maytansine and ansamitocins P-3 and P-4.

turn, most investigators have concluded that maytansine is a competitive inhibitor of the binding of radiolabeled vincristine and vinblastine to tubulin.[61,66,67] However, see Ref. 73 in which a different conclusion was reached in studies on the effects of ansamitocin P-3 on [^{3}H]vinblastine binding. Most studies have indicated maytansine binds more strongly to tubulin than either vinblastine or vincristine.

While inhibiting normal microtubule assembly, maytansine differs from the *Catharanthus* alkaloids in that it does not induce formation of spiral aggregates. Moreover, at concentrations substoichiometric to those of both vinblastine and tubulin, maytansine inhibits vinblastine-induced aggregate formation and causes dissolution of preformed aggregate.[69] This property may account for its strong inhibition of binding of radiolabeled *Catharanthus* alkaloids to tubulin.

Maytansine strongly inhibits nucleotide exchange, particularly at low incubation temperature, tubulin-dependent GTP hydrolysis, and formation of the cys12–cys201/211 cross-link described above.

Only genetic information is available regarding the maytansine binding site. A Chinese hamster ovary cell line with a mutant α-tubulin had increased resistance to the drug,[70] while site-directed mutagenesis at position 100 in β-tubulin increased sensitivity of *Schizosaccharomyces pombe* to ansamitocin P-3.[71]

C. Rhizoxin

A series of structurally related compounds that appear responsible for rice seedling blight have been isolated from fermentation cultures of the fungus *Rhizopus chinensis*.[72] The most potent of these agents is known as rhizoxin (structure in Fig. 12; see Ref. 13 for a recent review). Besides its plant toxicity, rhizoxin has cytotoxic and antifungal properties, with accumulation of cells in mitotic arrest. The compound is currently in clinical evaluation for the treatment of human cancer.[73]

Radiolabeled rhizoxin binds rapidly and reversibly to tubulin at 37°C. Scatchard analysis indicated one high affinity binding site with a K_D value of 0.2 μM. The binding of radiolabeled rhizoxin was competitively inhibited by ansamitocin P-3 and vinblastine (apparent K_i values of 0.1 and 3 μM, respectively).[68] Inhibition of rhizoxin binding also occurs with phomopsin A.[74]

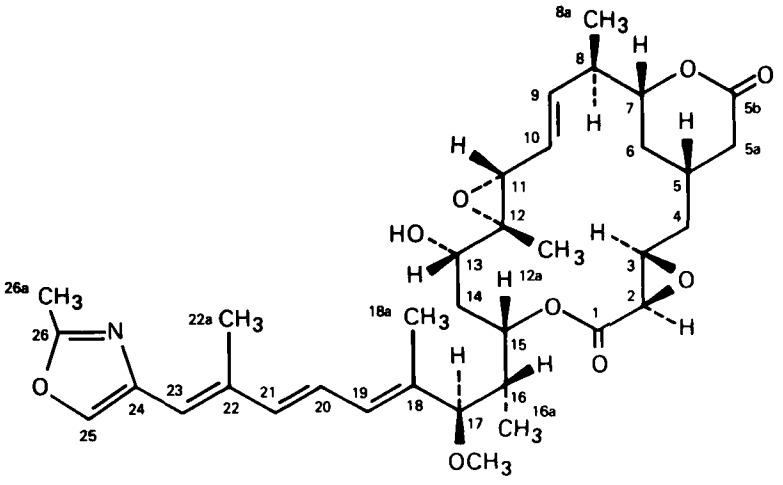


Figure 12. Structure of rhizoxin.

While Takahashi *et al.*[68] reported that inhibition by rhizoxin of [^{3}H]vinblastine binding was not purely competitive and obtained a very low K_i value of 0.01 μM, in my laboratory[61] we have observed competitive inhibition by rhizoxin of [^{3}H]vincristine binding and obtained a K_i value of 12 μM.

Like maytansine, rhizoxin inhibits normal tubulin polymerization and fails to induce the formation of spiral aggregates. Like maytansine, rhizoxin at substoichiometric concentrations to both the tubulin and vinblastine concentrations inhibits vinblastine-induced aggregation.[68] Rhizoxin is less active than maytansine on a molar basis as an inhibitor of tubulin-dependent GTP hydrolysis, nucleotide exchange, and cys12–cys201/211 cross-link formation.[61,75]

Takahashi and coworkers[71,76] have provided impressive genetic evidence that the primary binding site for rhizoxin is on β-tubulin. First, they isolated rhizoxin-resistant mutants of *Aspergillus nidulans* and determined that asparagine-100 had been replaced with isoleucine. They noted that both *Schizosaccharomyces pombe* and *Saccharomyces cerevisiae* were naturally resistant to rhizoxin and lacked asparagine at position 100. When site-directed mutagenesis was used to place asparagine at this position in both species, the new strains were sensitive to rhizoxin. In addition, a photoreactive analog of rhizoxin reacted covalently with β-tubulin, and the reactive portion of the protein was mapped to a peptide fragment containing amino acids 363–379.[77]

D. Phomopsin A

Growing on lupins, the fungus *Phomopsis leptostomiformis* produces toxins that cause livestock to develop lupinosis. One of the characteristic features of this disease is heptocytes arrested in mitosis. The predominant agent in disease-producing extracts is the hexapeptide phomopsin A (structure in Fig. 13; see Ref. 13 for a recent review) composed of modified amino acids. Its structure includes a 13-member ring with an ether linkage.[78] Modestly cytotoxic, phomopsin A causes mitotic arrest in cells growing in culture at micromolar concentrations, with disappearance of intracellular microtubules.

Radiolabeled phomopsin A binds rapidly and stably to tubulin at 37°C, and Scatchard analysis indicated two classes of binding site (K_D values of 10 and 300 nM).[74] These workers reported that no drug-induced tubulin aggregation occurred. However, Tonsing *et al.*[79] described formation of spiral aggregates when phomopsin A was added to polymer; and in my laboratory we have demonstrated formation of phomopsin A-induced aggregate both by electron microscopy[13] and by gel permeation HPLC.[80] This would imply that, as with the *Catharanthus* alkaloids, the biphasic Scatchard plot derives from

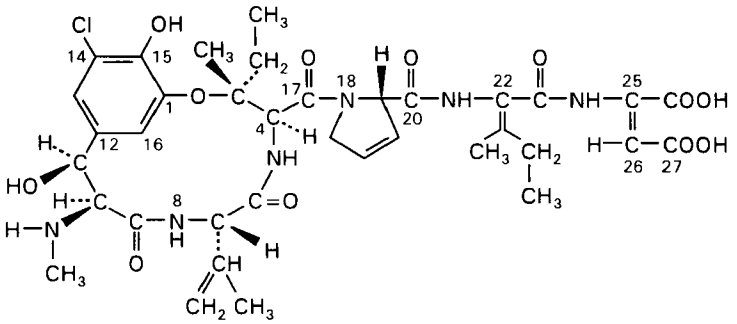


Figure 13. Structure of phomopsin A.

aggregation of drug-protein complex following drug binding at a single high affinity site. Phomopsin A acts as a noncompetitive inhibitor of the binding of [^{3}H]vincristine to tubulin[61] (apparent K_i value, 2.8 μM) and as a competitive inhibitor of the binding of [^{3}H]dolastatin 10.[81] Dolastatin 10 also inhibits the binding of a radiolabeled phomopsin A to tubulin.[74]

Besides inhibiting normal tubulin polymerization reactions, phomopsin A strongly inhibits tubulin-dependent GTP hydrolysis, nucleotide exchange, and formation of the cys12–cys201/211 cross-link.[61,82] Phomopsin A strongly stabilizes tubulin conformation, measured by the protein's prolonged retention of its ability to bind [^{3}H]colchicine and by inhibition of time-dependent binding of bis(8-anilinonaphthalene-1-sulfonate).[61,83]

Tubulin aggregates induced by phomopsin A either from microtubules or from unpolymerized protein have the appearance of rings, spirals, and pinwheels. It is morphologically distinct from the aggregates induced by the *Catharanthus* alkaloids, but very similar in appearance to aggregate induced by dolastatin 10.[13,80,84]

E. The Ustiloxins

Four peptides structurally similar to phomopsin A were isolated from parasitic growths on rice plants caused by the fungus *Ustilaginoidea virens* (structures in Fig. 14).[85] These compounds, like phomopsin A, caused mitotic arrest and cytotoxicity only at micromolar concentrations. They were nevertheless highly potent as inhibitors of micro-

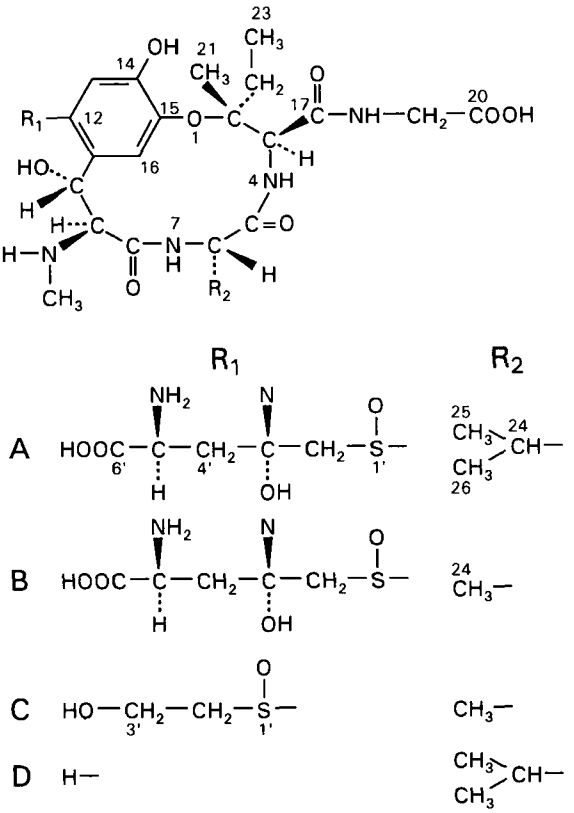


Figure 14. Structures of the ustiloxins.

tubule assembly. Ustiloxin A was shown to inhibit the binding of vinblastine to tubulin, formation of the cys12–cys201/211 cross-link, and to stabilize the conformation of tubulin.[86]

F. Dolastatin 10

The shell-less mollusk *Dolabella auricularia*, a species of sea hare, has yielded a number of cytotoxic and structurally unusual peptides and depsipeptides, the most potent of which is dolastatin 10[87] (structure in Fig. 15; see Ref. 13 for a review). The peptide causes cells to accumulate in mitotic arrest, with the disappearance of intracellular microtubules, and it strongly inhibits *in vitro* tubulin polymerization.

Radiolabeled dolastatin 10 binds rapidly to tubulin both at 0°C and at warmer temperatures. The reaction appears to be reversible. Scatchard analysis of the binding reaction yielded a biphasic plot, and the higher affinity K_D value was 30 nM. The binding of the drug to tubulin was associated with an aggregation reaction, which was readily evaluated by a gel permeation HPLC technique. No binding of drug to α-β-heterodimer (100 kDa tubulin) was observed, with the smallest radiolabeled peak having an apparent mass of 200 kDa.[80] With radiolabeled dolastatin 10 as the ligand, competitive inhibition was obtained with phomopsin A and a chiral isomer of dolastatin 10 and noncompetitive inhibition with spongistatin 1.[81]

Dolastatin 10 noncompetitively inhibits the binding of vincristine (apparent K_i, 1.4 μM) to tubulin, inhibits nucleotide exchange, formation of the cys12–cys201/211 cross-link, and tubulin-dependent GTP hydrolysis.[61,88–90] Dolastatin 10 also inhibits binding of radiolabeled phomopsin A and rhizoxin to tubulin.[91] Moreover, dolastatin 10 stabilizes tubulin conformation, measured both by retention of colchicine binding in its presence and its inhibition of development of binding of bis(8-anilinonapthalene-1-sulfonate).[61,90]

The noncompetitive patterns of inhibition obtained with phomopsin A and dolastatin 10 versus vincristine and the competitive pattern obtained with phomopsin A versus dolastatin 10 implies distinct binding sites on tubulin for the peptide antimitotics and the *Catharanthus* alkaloids. We have proposed a model that rationalizes the inhibitory data in terms of close proximity of the binding sites of the peptides, the alkaloids, and the exchangeable nucleotide.[61] This model proposes that the antimitotic agents interfere with each other's binding and with nucleotide exchange primarily through steric hin-

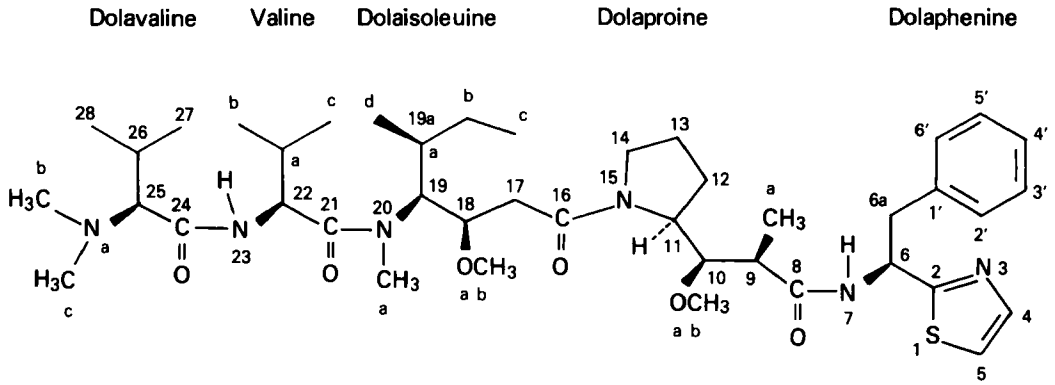


Figure 15. Structure of dolastatin 10.

drance. We have proposed the term "vinca domain" to represent this region of tubulin to distinguish it from the "vinca site" where the *Catharanthus* alkaloids directly bind.

Both with and without MAPs, addition of dolastatin 10 to a reaction mixture causes substantial tubulin aggregation.[80,81,88,91] This causes visible turbidity in tubulin solutions. The morphology of the aggregates consists of rings, spirals, and pinwheel clusters. The appearance is distinct from that of aggregate induced by the *Catharanthus* alkaloids but indistinguishable from that caused by phomopsin A.

G. Dolastatin 15

The depsipeptide dolastatin 15 (structure in Fig. 16; see Ref. 13 for a review) was also originally obtained from *D. auricularia*.[92] Dolastatin 15 is almost as cytotoxic as dolastatin 10 (6-fold less active in L1210 murine leukemia cells), but the depsipeptide is only about 1/20th as potent as the peptide as an inhibitor of *in vitro* tubulin polymerization. Cells treated with dolastatin 15 arrest in mitosis and their microtubules disappear.[93]

The structural analogies between dolastatins 10 and 15 are apparent, and form the basis for including the depsipeptide in this section. The properties of the two agents with tubulin are, however, quite distinct, in that dolastatin 15 does not inhibit the binding of either radiolabeled *Catharanthus* alkaloids or dolastatin 10 to tubulin, does not inhibit nucleotide exchange (although it inhibits GTP hydrolysis), does not stabilize the colchicine binding activity of tubulin, or induce tubulin aggregation.

H. Halichondrins and Halistatins

The lactone polyether halichondrin B and related agents (structures in Fig. 17; see Ref. 13 for a review) have been isolated from a number of marine sponges, including *Halichondria okadai* Kadota, *Axinella* sp., *Axinella carteri*, and *Phakellia carteri*.[94–97] Halichondrin B has been studied in the greatest detail. The agent is highly cytotoxic, causing cells to arrest in mitosis and the disappearance of intracellular microtubules. Halichondrin B inhibits *in vitro* tubulin assembly, tubulin-dependent GTP hydrolysis, nucleotide exchange, and formation of the cys12–cys201/211 cross-link. It acts as a noncompetitive inhibitor of the binding of radiolabeled vinblastine to tubulin (apparent K_i value, 12 μM). Halichondrin B does not stabilize the conformation of tubulin nor does it induce an aggregation reaction.[98–100]

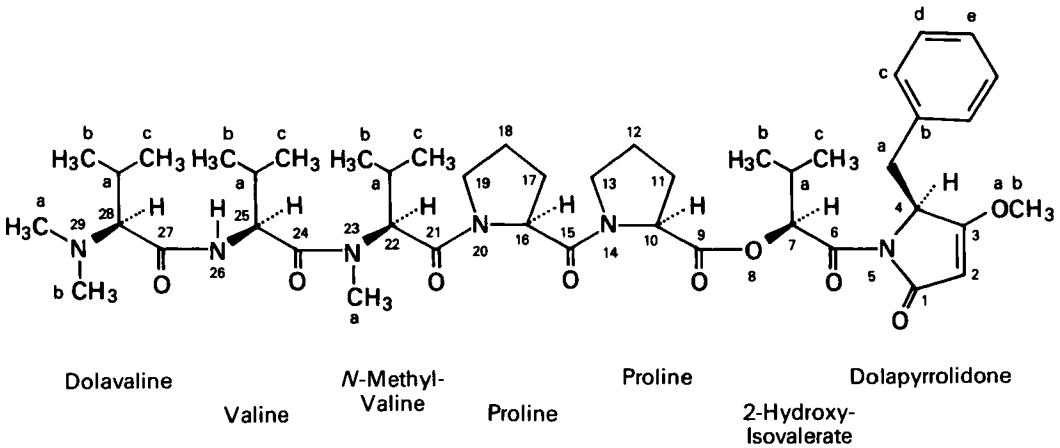


Figure 16. Structure of dolastatin 15.

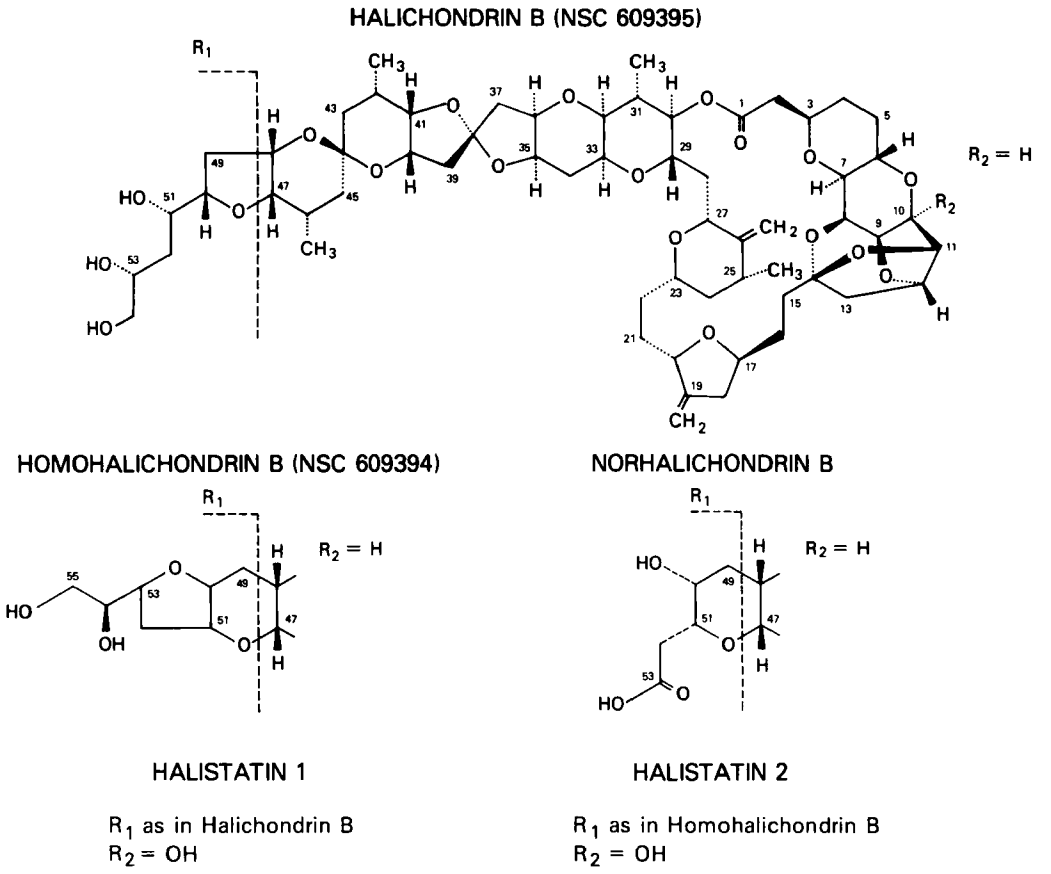
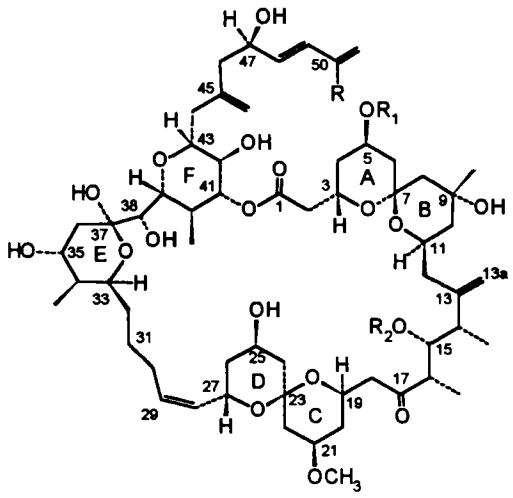


Figure 17. Structures of halichondrin B and related compounds.

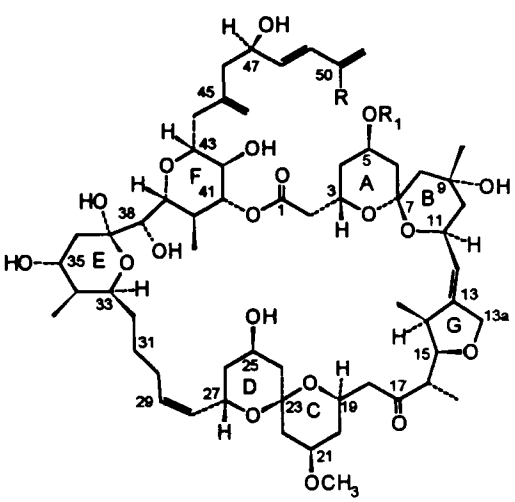
I. Spongistatins

Pettit and colleagues[101–105] isolated a series of nine lactone polyethers termed spongistatins 1–9 (structures in Fig. 18) from the marine sponges *Spongia* sp. and *Spirastrella spinispirulifera* that are highly cytotoxic for human cancer cells in culture. Spongistatin 1 was also isolated from the marine sponge *Hyrtios altum* as altohyrtin A by Kobayashi *et al.*[106] and spongistatin 4 from the marine sponge *Cinachyra* sp. as cinachyrolide A by Fusetani *et al.*[107] Spongistatin 1 was obtained in the largest quantity and is the best studied of these agents. Of all antimitotic drugs studied in my laboratory, spongistatin 1 was the most cytotoxic, having an IC_{50} value of 20 pM with L1210 murine leukemia cells. Cells accumulated in mitotic arrest, and intracellular microtubules disappeared.[108]

Besides inhibiting microtubule assembly *in vitro*, spongistatin 1 strongly inhibits the binding of both vinblastine and dolastatin 10 to tubulin. In both cases the inhibition pattern was noncompetitive, implying a third distinct binding site in the vinca domain.[81] Spongistatin 1 also strongly inhibits nucleotide exchange,[108] while spongistatin 3[100] has been shown to inhibit formation of the cys12–cys201/211 cross-link. The spongistatins do not induce formation of tubulin aggregates, but they inhibit formation of dolastatin 10-induced aggregate when present at concentrations lower than that of both the tubulin and the dolastatin 10.[81]



Spongistatin 1, R = Cl, $R_1 = R_2 = COCH_3$
Spongistatin 2, R = H, $R_1 = R_2 = COCH_3$
Spongistatin 3, R = Cl, R_1 = H, $R_2 = COCH_3$
Spongistatin 4, R = Cl, $R_1 = COCH_3$, R_2 = H
Spongistatin 6, R = H, $R_1 = COCH_3$, R_2 = H



Spongistatin 5, R = Cl, R_1 = H
Spongistatin 7, R = H, R_1 = H
Spongistatin 8, R = H, $R_1 = COCH_3$
Spongistatin 9, R = Cl, $R_1 = COCH_3$

Figure 18. Structures of spongistatins 1-9.

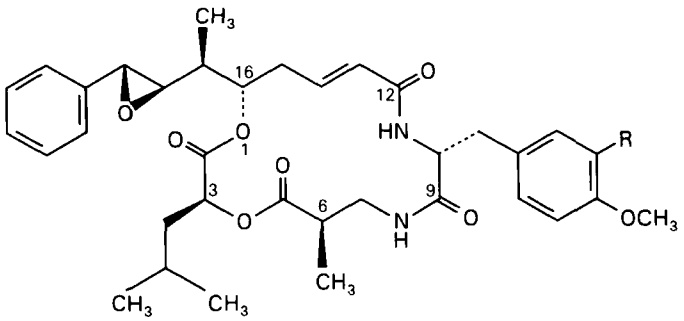
J. Cryptophycins

The isolation of the cyclic depsipeptide cryptophycin A (structure in Fig. 19; originally called cryptophycin) from a terrestrial cyanobacterium, a *Nostoc* sp., was described by Schwartz *et al.*,[109] who reported the agent had antifungal activity and significant toxicity in mice. The compound, together with a series of less abundant congeners (structures in Fig. 19), was reisolated by Trimurtulu *et al.*[110] and shown to have potent cytotoxicity and *in vivo* antitumor activity. Barrow *et al.*[111] described the de novo synthesis of cryptophycins C and D, requiring a revision of the structures of cryptophycins A and C. Smith *et al.*[112] reported an IC_{50} value of 4 pM (cf. spongistatin 1 above) in murine L1210 leukemia cells, which were arrested in mitosis. They also described the disappearance of intracellular microtubules following drug treatment. Although no biochemical studies with tubulin have been published yet, studies in my laboratory have demonstrated that cryptophycin A inhibits microtubule assembly and the binding of *Catharanthus* alkaloids and dolastatin 10 to tubulin. Preliminary studies have indicated that low concentrations of cryptophycin A suppress microtubule dynamic instability.[113]

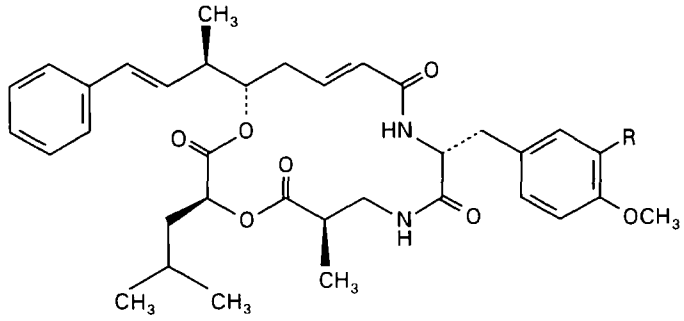
IV. ENHANCERS OF ASSEMBLY/STABILIZERS OF POLYMER

A. Taxoids

Paclitaxel (structure in Fig. 20) was originally isolated from the higher plant *Taxus brevifolia* as a cytotoxic agent,[114] and it has now been obtained from many members of the *Taxus* family, including the common garden yew *T. baccata*.[115] Recently, small amounts of paclitaxel were recovered from a culture of a fungus, *Taxomyces adreanae*, obtained from *T. brevifolia*.[116] In addition, a number of structurally similar compounds with biological activity have been obtained from *Taxus* species, but few detailed studies



A: R = Cl
B: R = H



C: R = Cl
D: R = H

Figure 19. Structures of cryptophycins A-D.

with tubulin have been published. The reader is referred to more extensive reviews of taxoids for more detailed discussion and more comprehensive literature references.[8–12]

Shortly after the antimitotic activity of paclitaxel was first noted,[117] Horwitz and her collaborators[118–120] reported that the compound interfered with microtubule function in cells by enhancing polymer stability and assembly rather than by inhibiting assembly and destabilizing microtubules, as occurs with the compounds described above. These

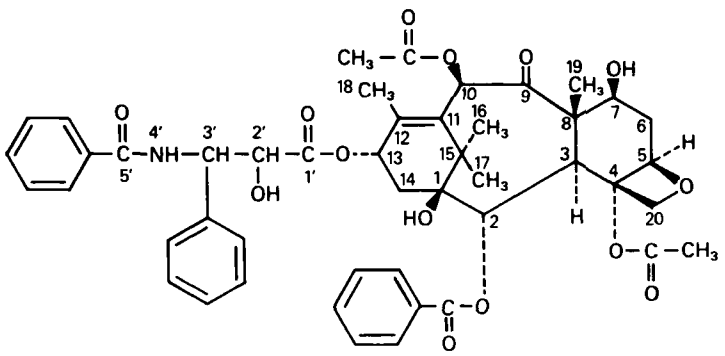


Figure 20. Structure of paclitaxel.

workers and others[121–123] demonstrated that tubulin assembly in the presence of paclitaxel could occur at lower protein concentrations, lower temperatures, without MAPs, and without GTP and that the polymer formed was resistant to cold-, calcium-, and dilution-induced disassembly. Microtubules formed in the presence of paclitaxel are shorter than those formed in control reaction mixtures, and a higher proportion of the protein is polymerized. *In vitro* paclitaxel thus enhances both microtubule nucleation and elongation reactions, and hyperstable polymer forms in its presence. Depending on reaction conditions, including paclitaxel concentration, morphologically abnormal polymers, described as sheets and hoops, can be prominent. Although neither MAPs nor GTP are required for paclitaxel-induced assembly, the drug does not significantly interfere with the interactions of either MAPs or GTP with tubulin. Recent work has shown that low paclitaxel concentrations suppress the dynamic instability properties of microtubules,[124] as has been found with inhibitors of assembly. There is also data that indicate different tubulin isotypes may have different affinities for paclitaxel.[125]

In cells treated with paclitaxel, microtubules persist despite mitotic arrest.[120] The precise appearance of microtubules in treated cells is highly variable, but in some cases paclitaxel induces spectacular abnormal arrays of microtubules. Often thick bundles of microtubules not originating from a microtubule organizing center are seen.[126]

With radiolabeled paclitaxel[127] and 7-acetylpaclitaxel[128] it was demonstrated that stable binding of drug to tubulin only occurs in polymer, with a maximum stoichiometry of about one to one. The binding was reversible with a K_D value of about 1 μM. Binding was inhibited by inhibitors of assembly, but this cannot be readily interpreted in terms of drug binding sites since the radiolabeled drug only binds readily to polymer.

Photoreactive analogs of paclitaxel modified at position C-3′ react specifically or preferentially with β-tubulin.[129,130] The covalent bond was formed with amino acid(s) in the 31 residue amino terminal peptide.

Recent molecular modeling and nuclear magnetic resonance studies[131–133] have indicated that the conformations of paclitaxel and docetaxel in aqueous solution probably differ significantly from the conformation indicated by x-ray crystallography.[134] In the latter the C-2, C-4, and C-13 side chains are widely separated, while in aqueous solution they are close together as a consequence of hydrophobic interactions. This could indicate that the complex of side chains is an important recognition feature for tubulin. However, baccatin III, an analog of paclitaxel lacking the C-13 side chain, was highly active in its interaction with tubulin from the amoeba *Physarum polycephalum*,[135] suggesting that the side chain interactions modify the conformation of the taxoid nucleus.

B. Epothilones

Bollag *et al.*[136] isolated epothilones A and B (structures in Fig. 21) from cultures of the myxobacterium *Sorangium cellulosum* after initial screening indicated the crude extract

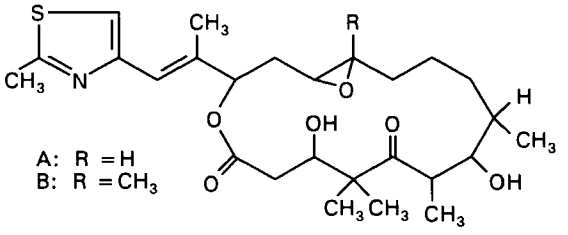


Figure 21. Structures of epothilones A and B.

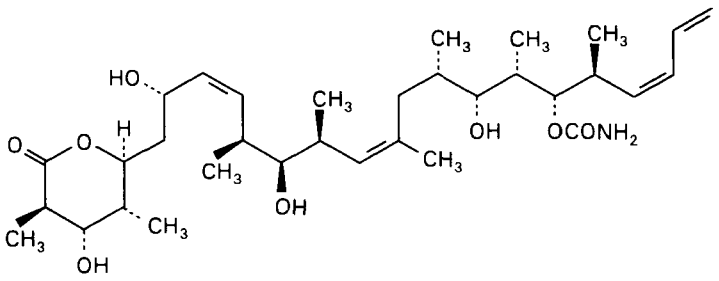


Figure 22. Structure of discodermolide.

contained a component that stabilized microtubules. These agents had previously been isolated by Höfle *et al.*,[137] who described antifungal and cytotoxic activity. Bollag *et al.*[136] showed that the cytotoxic activity was associated with mitotic arrest, cytoskeletal disorganization, and the appearance of microtubule bundles in drug-treated cells. They also found that both epothilones A and B induced *in vitro* microtubule assembly from microtubule protein in the absence of GTP in a concentration-dependent manner that closely resembled the effect of paclitaxel. These polymers were stable to cold and calcium depolymerization. Both epothilone A and epothilone B inhibited the binding of radiolabeled paclitaxel to microtubules.

C. Discodermolide

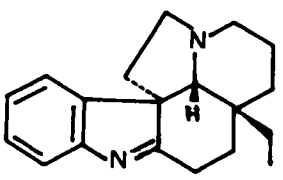
The lactone discodermolide (structure in Fig. 22) was isolated by Gunsakera *et al.*[138] from the marine sponge *Discodermia dissoluta* on the basis of immunosuppresive activity.[139] (Note that the structure shown in Fig. 22 is that of the synthetic compound,[140] which differs in configurations from that originally reported.[138]) The compound is cytotoxic for several cell lines, and, when analyzed by flow cytometry, drug-treated cells accumulated at the G2 + M stage.[139,140] Burkitt lymphoma cells accumulated in mitotic arrest.[141] When human MCF-7 breast cancer cells were treated with discodermolide and examined by indirect immunofluorescence for β-tubulin, extensive bundling of intracellular microtubules was observed. The effect was more dramatic than the binding that occurred following treatment of these cells with paclitaxel.[141]

In studies with purified tubulin[141] discodermolide was found to be more potent than paclitaxel in inducing polymerization under every reaction condition studied: at low temperatures, without MAPs, and/or without GTP. The polymer formed with discodermolide was completely stable to both cold and calcium, while there was some loss of paclitaxel-induced polymer with these depolymerizing agents. The polymer formed with discodermolide when MAPs and GTP were included in the reaction consisted primarily of short microtubules and some sheets. The discodermolide microtubules averaged only 0.7 μm in length, and they were considerably shorter than those formed with paclitaxel (1.7 μm) or in the absence of drug (3.3 μm). Using a reaction condition designed to emphasize quantitative differences between paclitaxel and hyperactive paclitaxel analogs, 50% tubulin assembly was induced by 3.2 μm discodermolide and by 23 μm paclitaxel.

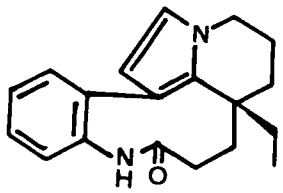
V. MIXED MECHANISM

A. Rhazinilam

Thoisin *et al.*[142] described the isolation of rhazinilam (structure in Fig. 23) from the bark of the Malaysian tree *Kopsia signapurensis* Ridley, noting that the compound had



Dehydroaspidospermidine



Rhazinilam

Figure 23. Structures of rhazinilam and its proposed precursor dehydroaspidospermidine.

been previously purified from a number of closely related plants. These workers also reported inhibition of microtubule assembly by rhazinilam. Thoison *et al.*[142] found variable recovery of rhazinilam in their purification procedure and concluded that this active agent did not occur naturally but was a degradation product derived from dehydroaspidospermidine (structure in Fig. 23).

David *et al.*[143] examined interactions of rhazinilam with cells and tubulin in detail. Although the compound had modest cytotoxicity (IC_{50} values about 1 μM in several cell lines), high concentrations (up to 125 μM) of rhazinilam stabilized cellular microtubules to cold disassembly and caused a rearrangement of cellular microtubules into bundles. Multinucleated cells and multiple asters were also observed. These findings are similar to those obtained following paclitaxel treatment. The persistence of cellular microtubules following rhazinilam treatment was confirmed by electron microscopy, and many of these microtubules had 12 or 14 protofilaments instead of the normal 13.

However, rhazinilam, in contrast to the taxoids, epothilones, and discodermolide, was unable to induce formation of microtubules from either microtubule protein or purified tubulin.[143] When added to microtubule protein or tubulin rhazinilam caused formation of anomalous filamentous structures, most of which were in the form of short, loose spirals. These structures were up to 0.25 μm in length and contained 2–6 parallel filaments. When rhazinilam was added to preformed microtubules, similar loose spirals formed at the microtubule ends, and the spiral filaments appeared to be continuous with the microtubule protofilaments. The drug, however, did not cause a progressive unraveling of the microtubules, but rather stabilized them to cold depolymerization. Formation of the rhazinilam-induced polymer occurred at either 0°C or 37°C and required GTP. Calcium enhanced rhazinilam-induced spiral formation when added to either unpolymerized tubulin or microtubules. Maytansine and vinblastine, but not colchicine, inhibited formation of the anomalous rhazinilam polymer.

[^{3}H]Rhazinilam bound in stoichiometric amounts at a single class of binding site to the spiral polymers with an apparent K_D value of 4.9 μM.[143] There was no significant binding to microtubules. The binding of [^{3}H]rhazinilam to the spirals was inhibited by maytansine and vinblastine, but experiments to determine whether inhibition was competitive were unsuccessful.

In summary, rhazinilam may act in cells by capping microtubules with anomalous spiral structures that result in stabilized microtubules. Such microtubules might then rearrange in a manner similar to that which occurs following treatment with taxoids, epothilones, or discodermolide. The anomalous spiral structures induced by rhazinilam may be related to those induced by either the *Catharanthus* alkaloids or the peptide antimitotics binding in the vinca domain.

VI. CONCLUDING REMARKS

Mammalian tubulin interacts with a wide array of natural products that are remarkable in their diversity in terms of species of origin, molecular structure, and mechanism of interaction with target. In the last decade the number of distinct antimicrotubule compounds from natural sources has easily doubled, and many of them have been extraordinarily cytotoxic. These compounds are of great interest as potential agents for the treatment of human diseases, particularly cancer, and they provide new tools for the study of the roles of microtubules in cell biology.

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