Tubulin as an Antiprotozoal Drug Target

Karl A. Werbovetz*

Division of Medicinal Chemistry and Pharmacognosy, College of Pharmacy, Ohio State University, 332 Lloyd M. Parks Hall, 500 West 12th Avenue, Columbus, OH 43210, USA

Abstract: Since tubulin is a known anticancer and anthelmintic drug target, the investigation of protozoal tubulin could lead to the development of new antiparasitic drugs. This review outlines the current state of knowledge concerning drug-mammalian tubulin interactions, the effects of antimicrotubule agents on parasites and parasite tubulin, and our current hypotheses regarding the development of selective ligands for protozoal tubulin as antiparasitic drug candidates.

INTRODUCTION

Diseases caused by parasitic protozoans place an enormous burden on health care systems in the developing world. Unfortunately, the development of drugs to treat diseases such as malaria, African trypanosomiasis, Chagas' disease, and leishmaniasis has not kept pace with the need. Of the approximately 1450 new chemical entities registered from 1972 - 1997, only 13 (< 1%) were intended for use against tropical diseases [1], with only nine of these intended for use against the four diseases listed above. The reality of the current situation is that cheap, effective antimalarial chemotherapy is being compromised by drug resistance, and that the chemotherapy of leishmaniasis and trypanosomiasis remains unsatisfactory. If the desperate need for new tropical medicines is to be met, novel approaches to discovery, development, and production are required. Significant contributions are required from scientists and policy makers outside of the pharmaceutical industry. Fortunately, there is hope that considerable progress will be made against these diseases in the near future. Aggressive programs designed to combat malaria have been implemented [2], and new drugs against malaria and leishmaniasis are making their way through the development process [3,4].

Most antiparasitic agents have been discovered by the traditional lead-based approach. A more recent strategy for antiparasitic drug discovery is to seek specific inhibitors for critical biological targets within the organism aided by protein crystal structures and/or target-based assays [5]. In this review, I will present an overview of tubulin, a prospective drug target in protozoan parasites. I will also give a brief synopsis of what is known on the molecular level concerning the binding of different drug classes to the more well-characterized mammalian tubulin protein. Knowledge of the molecular details of ligand-tubulin interactions is required for the design of compounds that selectively target protozoal tubulin. In most cases, however, interactions between ligands and tubulin from protozoans remain undefined. Thus, for each class of antimicrotubule agents I will describe what is known about the effects of these compounds on parasites and/or parasite tubulin. I will conclude with a short description of our current view of tubulin as a target for new antiparasitic drugs and outline some of our thoughts on future possibilities for exploiting this target.

*Address correspondence to this author at the Division of Medicinal Chemistry and Pharmacognosy, College of Pharmacy, Ohio State University, 332 Lloyd M. Parks Hall, 500 West 12th Avenue, Columbus, OH 43210, USA; Phone: (614) 292-5499, Fax: (614) 292-2435, E-mail: werbovetz.1@osu.edu

TUBULIN AND MICROTUBULES

The Vital Cellular Roles of Tubulin

Tubulin is an essential protein in all eukaryotes. This heterodimeric protein assembles under appropriate conditions *in vivo* and *in vitro* to form microtubules: long, hollow cylindrical filaments with a 25 nm outer diameter. Microtubules possess polarity in that one end (the plus end) grows rapidly, while the other (the minus end) has a tendency to lose subunits (Fig. **1**). Within cells, the minus ends of microtubules are usually found attached to the centrosome, and the plus ends extend outward from this nucleating site. Diverse processes such as chromosomal segregation, organelle transport, and cellular motility all rely on microtubules for execution. The vital role played by tubulin in cells is highlighted by the fact that tubulin is the proposed target for clinically useful anticancer [6] and anthelmintic agents [7]. Such observations suggest that tubulin merits investigation as an antiprotozoal drug target.

Tubulin in Protozoan Parasites

Most of the biochemical work concerning tubulin in protozoan parasites has been conducted with kinetoplastids. Tubulin is a major (if not the major) protein of *L. mexicana* [8], *T. brucei* [9], and the non-pathogenic kinetoplastid *Crithidia fasciculata* [10]. *Crithidia* and *T. brucei* possess at least three cellular pools of tubulin: cytoplasmic, subpellicular, and flagellar [9-11]. Cytoplasmic tubulin likely plays an essential role in chromosomal segregation [12], as in other eukaryotic cells. Subpellicular tubulin lies beneath the cell membrane of kinetoplastids in a skeletal framework. Tubulin is also the major component of the

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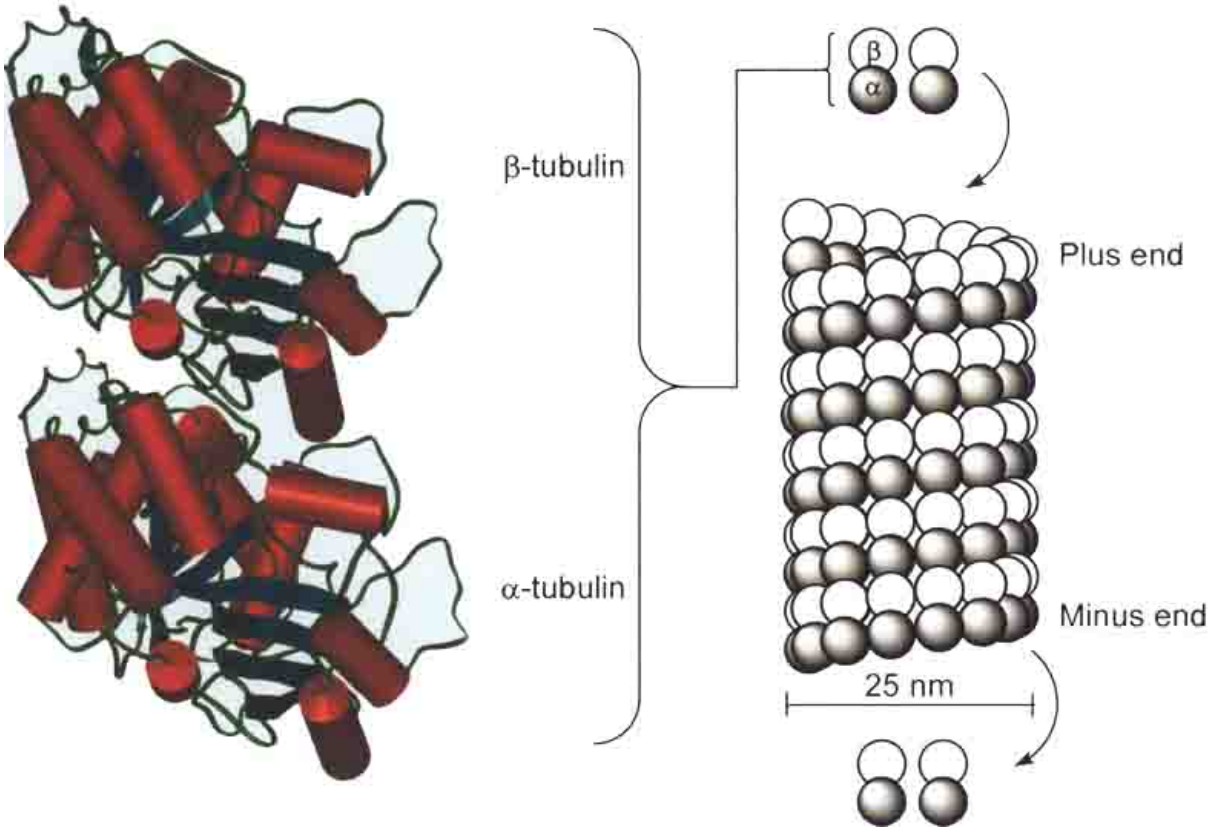

Fig. (1). Assembly of tubulin αβ-heterodimers into microtubules. On the left is a ribbon diagram of the heterodimer generated with the program InsightII (Molecular Simulations, San Diego, CA). The right side of the figure depicts the dynamic instability of microtubules, with tubulin heterodimers adding at the plus end and departing at the minus end of the microtubule.

flagellum present in the motile developmental stages of kinetoplastids. Tubulin is highly homologous among kinetoplastid parasites, while the degree of homology is lower between mammalian and parasite proteins. The amino acid sequence of β-tubulin from *L. mexicana* is 93% identical to the corresponding sequence from *T. brucei*, 94% identical to the *T. cruzi* β-tubulin sequence, and 83% identical to human β-tubulin. The amino acid sequence of α-tubulin from *L. donovani* is 94% identical to the corresponding sequence from *T. brucei*, 93% identical to α-tubulin from *T. cruzi*, and 79% identical to human α-tubulin. Studies describing tubulin in other protozoan parasites, such as *Plasmodium* and *Toxoplasma*, have mainly employed cell biology techniques. This is probably due to the fact that tubulin is traditionally purified by biochemical methods due to difficulties in expressing an assembly-competent protein, and such parasites are difficult to culture on a large scale. Interestingly, the β-tubulin sequence of *P. falciparum* is 85% identical to that of *L. donovani* and 89% identical to human β-tubulin.

CLASSES OF ANTIMICROTUBULE AGENTS AND THEIR EFFECTS ON TUBULIN

Antimicrotubule agents may be broadly categorized as either microtubule stabilizers or microtubule destabilizers. The important anticancer agent taxol (also called paclitaxel) was the first microtubule stabilizer discovered [13], but other natural products such as the epothilones [14] and eleutherobin [15] have been identified with similar properties. Agents that depolymerize microtubules through binding to tubulin can be further subdivided into fairly complex natural products that interact with the protein at the vinca alkaloid domain [16,17] and smaller molecules that bind at the colchicine site [18,19]. The reader is referred to Fig. (**2**) for structures of representative compounds that bind to the taxol site, the vinca alkaloid domain, and the colchicine site. Although the effects of these drugs on cells were once attributed to the polymerization or depolymerization of cellular microtubules, more recent work has indicated that antimicrotubule agents probably act in cells by disrupting normal microtubule dynamics. Interference with microtubule dynamics, or the rate of microtubule assembly and disassembly of microtubules in cells, seems to occur at drug concentrations far below those required to affect the bulk properties of microtubules. Effects on microtubule dynamics are observed at the lowest antimicrotubule agent concentrations that inhibit cell proliferation, accompanying the blockage or slowing of mitosis [20-23].

Taxol-site Agents

Photoaffinity labeling provided the first hint of the location of the taxol binding site on mammalian tubulin. The taxol analog 3'-(*p*-azidobenzamido)taxol labeled the β-subunit of tubulin upon photolysis, and the radiolabeled polypeptide was identified as β-tubulin residues 1-31 ([24],

see Fig. (**2**) for appropriate numbering and labeling of taxol). Tritiated 2-(*m*-azidobenzoyl)taxol, where the photoaffinity label was located off the B-ring of the taxoid core, reacted with a peptide containing amino acids 217-231 of β-tubulin [25]. Despite intense interest in tubulin as a chemotherapeutic target, the information obtained through biochemical studies was severely limited due to the lack of a crystal structure for this protein. In 1998, an electron crystallography-derived atomic model of bovine brain tubulin was obtained in the presence of zinc ions and taxol, providing a springboard toward the greater understanding of the molecular nature of tubulin-drug interactions [26]. The taxane ring of taxol is well defined in this structure, and its main interaction is at β275Leu. In agreement with an earlier mutagenesis study [27], βPhe272 interacts with the C3' taxol phenyl ring [28]. This model also concurs with the earlier photoaffinity labeling experiments placing the C2 side chain in proximity to β217-231 and the C3' group close to β1-31. A taxol/epothilone/eleutherobin pharmacophore has been advanced that provides support for the hypothesis that these compounds bind to the same site on mammalian tubulin [29]. Recently, a more refined three-dimensional structure of the taxol-bound mammalian tubulin heterodimer was published [30].

Studies with cultured kinetoplastid parasites indicate that taxol blocks the replication of both *L. donovani* and *T. cruzi* [31-34]. Numerous abnormal parasites were observed after parasite cultures were treated with taxol, including organisms with multiple nuclei and kinetoplasts [31-33]. Analysis of taxol-treated *L. donovani* by flow cytometry indicated that these cells accumulated in the G_2 + M phase of the cell cycle [32,33], and that some taxol-treated cells contained four times the amount of DNA present in G_1-phase organisms [32]. Low micromolar concentrations of taxol induced the assembly of tubulin isolated from *Leishmania* [32,34]. In one study, taxol inhibited the growth of *L. donovani* promastigotes at concentrations of 50 nM [34], while in the other studies concentrations of taxol in the micromolar range were employed to observe adverse effects against the organisms [31-33]. One explanation for this difference could be that high intracellular concentrations of taxol are not obtained in the parasites used in the latter studies, perhaps due to the presence of a multidrug resistance protein which removes the drug from treated cells [35,36]. Consistent with this hypothesis, purified leishmanial and mammalian tubulin both assemble in the presence of low micromolar concentrations of taxol, while taxol is toxic to neoplastic cells at low nanomolar concentrations [32,37]. Taxol and taxotere are toxic to malaria parasites at

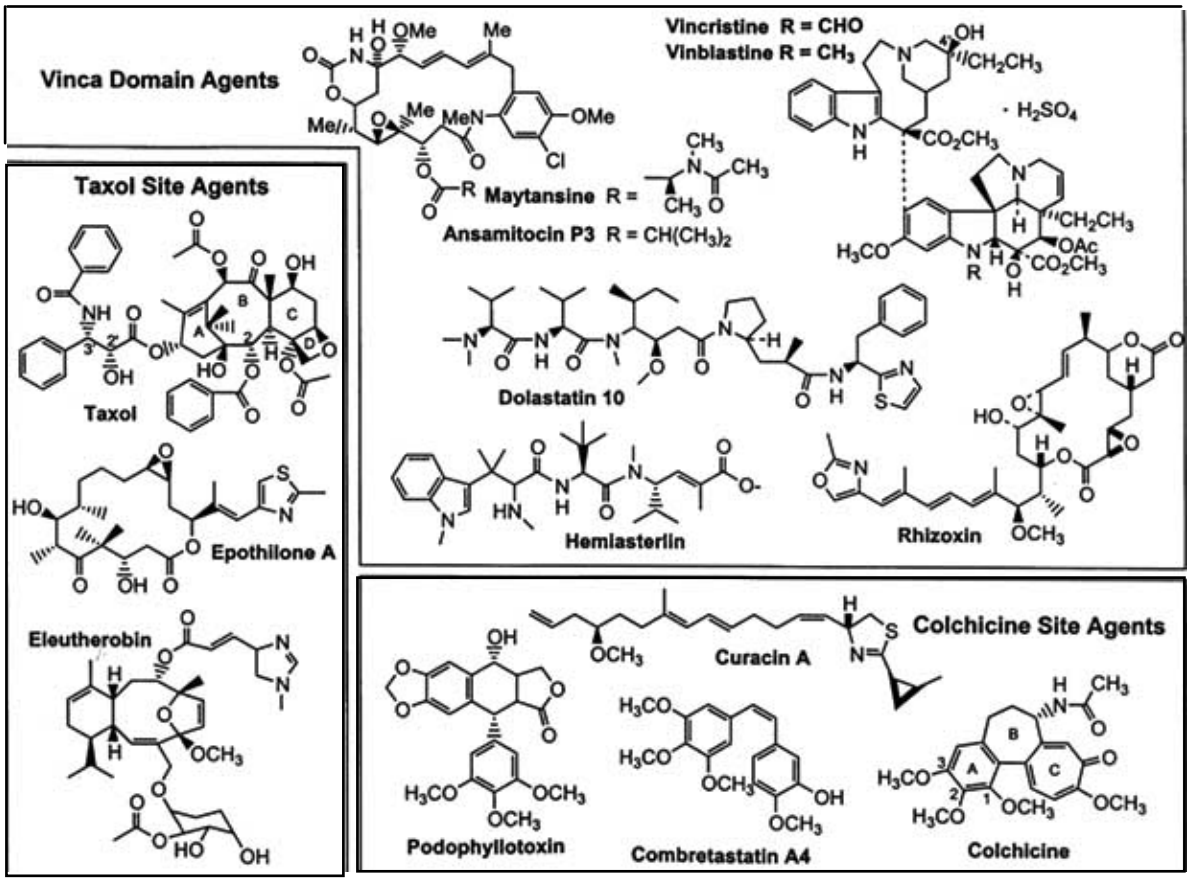

Fig. (2). Structures of representative taxol site, vinca domain, and colchicine site agents that bind to mammalian tubulin.

nanomolar concentrations, and these compounds interfere with mitotic processes in *Plasmodium falciparum* (reviewed in [38]). Antiproliferative effects against *Toxoplasma gondii* were also observed with taxol [39]. However, taxol and related taxoids are generally more toxic to mammalian cells than to parasitic protozoa. The structural complexity of taxol-site agents also precludes the synthesis of new analogs for serious study against protozoan parasites. In our opinion, these compounds are unlikely to be antiparasitic drug candidates in the future.

The Vinca Alkaloid Domain

The antimicrotubule vinca alkaloids vinblastine and vincristine are used in cancer chemotherapy. Several other natural products inhibit the binding of radiolabeled vinca alkaloids to tubulin, such as maytansine [40], the ansamitocins [41], and rhizoxin [42]. A structurally diverse series of peptide natural products also inhibit vinca alkaloid binding to tubulin. Such peptide-containing agents include marine natural products of the hemiasterlin [43], dolastatin [44], and cryptophycin [45] families. Maytansine, the ansamitocins, and rhizoxin are thought to bind to the same tubulin site [46]. Maytansine and rhizoxin have been reported as competitive inhibitors of vinca alkaloid binding to tubulin [40,47], although some disagreement concerning this point exists in the literature [46]. The peptide-containing antimicrotubule agents were shown to be noncompetitive inhibitors of vinca alkaloid binding to this protein [47-49]. Within the peptide class of agents, cryptophycin 1 [48] and hemiasterlin [49] are competitive inhibitors of dolastatin 10 binding to tubulin. These data suggest that the peptide antimicrotubule agents bind at the same site on tubulin, a region termed the peptide site. According to the model proposed by Bai *et al.* [47], binding of these peptide agents to tubulin interferes sterically with binding of vinca alkaloids, maytansine, and rhizoxin at an adjacent vinca alkaloid binding site.

Limited information is available concerning the molecular features of the vinca alkaloid binding domain on tubulin. All vinca alkaloid domain agents examined thus far inhibit the crosslinking of β-tubulin cysteine residues 12 and either 201 or 211 by *N*, *N*'-ethylenebis-iodoacetamide (reviewed in [50]). The fluorescent vinblastine derivative vinblastine-4'-anthranilate, synthesized in an attempt to locate the tubulin-vinca alkaloid binding site, bound to peptide residues 175–213 of β-tubulin after photolysis and proteolytic digestion ([51], see Fig. (**2**) for location of the 4' position of vinblastine). Vinblastine and maytansine both blocked adduct formation, indicating specific binding of this analog to tubulin. A fluorescent photoaffinity analog of rhizoxin has also been prepared where the oxazole ring was replaced by 5'-azidonaphthalene-1'-sulfonylhydrazone [52]. Peptidase digestion of tubulin modified by this analog and subsequent peptide mapping studies led to the conclusion that the modified peptide fragment contained residues 363-379 of β-tubulin. With the availability of the three dimensional model of mammalian tubulin [26,30] and photoaffinity labeling data for rhizoxin and vinblastine, it may soon be possible to determine the molecular details of drug binding in the vinca domain.

Vinca alkaloid domain ligands have profound effects on protozoan parasites. Treatment of *T. brucei* with 2 μM ansamitocin blocked the normal segregation of basal bodies as the organism progresses through the cell cycle [53]. Nanomolar concentrations of rhizoxin induced bizarre morphological changes in procyclic *T. brucei* parasites [54] and blocked formation of the mitotic spindle in these organisms [12]. Studies examining vinblastine- and vincristine-treated *T. cruzi* at drug concentrations ranging from 3 to 50 μM [55] and ansamitocin P3- and hemiasterlin-treated *Leishmania* at drug concentrations ranging from 2 to 10 μM [32] showed that both nuclear division and cytokinesis could be affected by vinca domain agents without blocking nucleic acid or organelle synthesis. In *L. donovani* promastigotes, aberrant cell types observed after drug treatment included parasites with one nucleus and two geometrically distinct kinetoplasts, parasites with multiple kinetoplasts, and cytoplasts containing a kinetoplast but no nucleus ("zoids"). Flow cytometric analysis of *Leishmania* treated with these vinca domain drugs indicated a dramatic shift toward the G_2 + M phase of the cell cycle, with some cells containing four times the amount of DNA present in G_1. Analogous results were observed in *T. cruzi* epimastigotes treated with vinca alkaloids, as giant cells containing multiple nuclei and kinetoplasts were found. In the *T. cruzi* study, higher concentrations of the vinca alkaloids inhibited both nuclear division and cytokinesis, while lower concentrations of these drugs blocked cytokinesis but not organelle replication [55]. Selective inhibition of cytokinesis was also found in *Leishmania* cultures treated with hemiasterlin at a concentration of 10 μM, as parasites containing two distinct kinetoplasts and nuclei along with a spindle fiber were observed. Less published data is available concerning the effects of vinca domain drugs in other parasites, but vinblastine and vincristine block the growth of erythrocyte-stage *Plasmodium falciparum* at nanomolar concentrations (reviewed in [38]).

Studies with purified kinetoplastid tubulin confirm that this protein is highly sensitive to vinca domain antimicrotubule agents. Maytansine at a concentration of 20 μM and vinblastine at a concentration of 40 μM completely blocked the assembly of *T. brucei* tubulin as assessed by electron microscopy [9]. Later studies using purified leishmanial tubulin employed a spectrophotometric assembly assay to measure the effects of vinca domain agents on parasite tubulin polymerization [32,56]. All of the vinca domain agents tested (vinblastine, maytansine, ansamitocin P3, rhizoxin, and hemiasterlin) inhibited leishmanial tubulin assembly at low micromolar concentrations. These data indicate that leishmanial tubulin has approximately the same sensitivity to vinca domain agents as mammalian tubulin. Protozoans are thus highly sensitive to vinca domain antimicrotubule agents, probably because parasite tubulin is highly susceptible to such compounds. Little in depth molecular information is available concerning the interaction between vinca domain agents and mammalian tubulin, so it is possible that differences between parasite tubulin and host tubulin may exist that could be exploited for the discovery of parasite-selective tubulin ligands. Although most vinca domain agents are structurally complex, molecules such as the

tripeptide hemiasterlin are simple enough to permit analog synthesis. Given their potent activity against parasites, the further investigation of vinca domain agents as antiprotozoal compounds is warranted.

The Colchicine Site

Many compounds have been described that compete with colchicine for binding to tubulin, including the natural products podophyllotoxin, steganacin, and combretastatin A4 (reviewed in [19]). These compounds all bear structural similarity to colchicine in that each possesses two aromatic rings, one of which is a trimethoxybenzene ring. The marine natural product curacin and its analogs also bind at the mammalian tubulin colchicine site [57,58]. Unlike the other colchicine site tubulin ligands which contain biaryl ring systems, the curacins are unique in that these molecules consist of a long alkene chain, a thiazoline ring, and a cyclopropyl ring. A wide variety of other molecules have also been reported which competitively inhibit colchicine binding to tubulin. For more detailed reviews of these drug classes, see [6,59].

Affinity and photoaffinity labeling experiments and more recent modeling studies have provided much information concerning tubulin's colchicine-binding region, and virtually all of these studies have been performed with colchicine analogs. An azidophenyl-substituted B-ring analog of colchicine reacted with both α- and β-tubulin ([60], appropriate numbering and labeling of colchicine's ring system is given in Fig. **2**). Direct photoaffinity labeling of tubulin by colchicine indicated that peptide fragments 1-46 and 214-241 of β-tubulin were modified by this molecule [61]. A radioactive affinity label with the reactive group on the colchicine A ring, 3-chloroacetyl-3-demethylthiocolchicine (3CTC), reacted mainly at Cys354 of β-tubulin, while some labeling of tubulin occurred at Cys239 [62]. Using the closely related radioactive affinity label 2-chloroacetyl-2-dimethylthiocolchicine, reaction with β-tubulin occurred predominantly at Cys239, with some modification at Cys354 [63]. In the atomic model of tubulin obtained by electron crystallography [26], the two β-tubulin cysteine residues identified by affinity labeling as being close to the C3 oxygen atom of the colchicine A ring, 239 and 354, are separated by about 8Å. Using the three dimensional structure for mammalian tubulin and energy minimization modeling, two potential colchicinoid binding sites were identified. One of these sites was entirely encompassed within β-tubulin and was consistent with the reactivity of the chloroacetyl-containing colchicine analogs [63]. Taken together, these results suggest that colchicine binds to β-tubulin with its A ring in a pocket containing β-tubulin cysteine residues 239 and 354 and its B-ring side chain pointing toward α-tubulin.

In contrast to the mammalian protein, tubulin from protozoan parasites is relatively insensitive to colchicine. Studies in which colchicine is reported to affect kinetoplastid parasites employed drug concentrations of 100 μg/ml (250 μM) or higher [64,65]. Erythrocyte-stage *Plasmodium falciparum* was two to three orders of magnitude less susceptible to the colchicine analog colcemid than to vincristine and vinblastine [66,67]. Tubulin isolated from *Trypanosoma brucei* forms abnormal microtubules when incubated with colcemid at a concentration of 27 μM, but similar concentrations of vinblastine and maytansine totally block the assembly of this protein as assessed by electron microscopy [9]. Drugs that bind to the colchicine site of mammalian tubulin have little effect on tubulin purified from *Leishmania amazonensis* [56]. While vinca site agents blocked assembly of this protein at low micromolar concentrations, IC_{50} values for assembly inhibition were not reached at the highest achievable concentrations of colchicine, combretastatin A4, and podophyllotoxin (50 –100 μM). In addition, *L. donovani* parasite growth was unaffected by such concentrations of the colchicine-site agents [56]. These data indicate that traditional colchicine-site agents have little effect on protozoal tubulin, suggesting significant differences between mammalian and parasite tubulins at the "colchicine site." Burns concluded that residues in the region of amino acid 316 of the β-subunit are critical for tubulin binding [68]. *Leishmania* possesses the sequence ASAL rather than the typical mammalian VAAV/I in residues β313-316. This observation provides further support for the hypothesis that the region around tubulin residue β316 is critical for colchicine binding. Amino acid variation in this region could lead to differences in protein folding among tubulins from distinct species, perhaps altering the geometry of the "colchicine site." Although malarial β-tubulin appears to be more closely related to human β-tubulin than leishmanial β-tubulin in terms of sequence identity, amino acids 313-316 of *P. falciparum* β-tubulin have the sequence ACAM. We would thus also expect tubulin from malaria parasites to possess a unique ligand binding preference at the "colchicine site."

Benzimidazoles

For other classes of anti-tubulin molecules, binding sites have not been characterized in as much detail. Among these agents are the benzimidazoles, which are used clinically as anthelmintic and antifungal agents (see [69] for review and Fig. (**3**) for structures of some important benzimidazole drugs). Although these agents were presumed to be colchicine site agents in the past, a distinct site has recently been proposed for the benzimidazoles that is near the colchicine site [70]. Indirect evidence concerning the benzimidazole binding site has come from mutagenesis studies. When charged-to-alanine mutations were generated in yeast tubulin genes, one of the resultant phenotypes observed was resistance to benomyl [71]. Mutations resulting in resistance to very high concentrations of benomyl (≥ 75 μg/ml) were then mapped onto a homology model for yeast tubulin. Resistant organisms possessed β-tubulin residues (123, 197, 198, and 318) located in the same general region of the protein. The β198 region is also the site of other mutations conferring a high degree of benomyl resistance, including a mutation at β238 [72]. Alterations in β198 have also been observed in several benomyl resistant tubulins from organisms other than *S. cerevisiae* [73,74]. Of special interest for this article, Katiyar *et al*. found that β-tubulin residues 198 and particularly 200 were strong predictors of benzimidazole susceptibility to

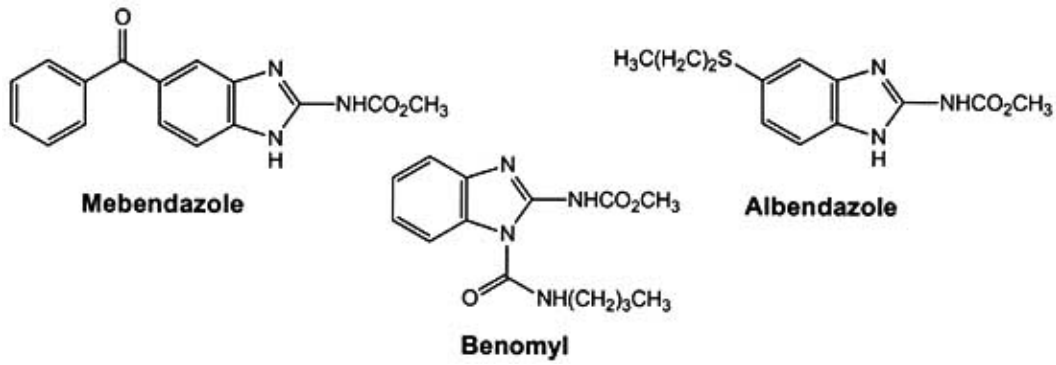

Fig. (3). Structures of the antihelmintic benzimidazoles mebendazole and albendazole and the benzimidazole-containing fungicide benomyl. Benomyl is a prodrug, with the active anti-tubulin form being methyl benzimidazolecarbamate (carbendazim).

protozoan parasites [75]. Parasites possessing both a Lys residue at β198 and a Phe residue at β200, such as *Giardia lamblia* and *Trichomonas vaginalis*, were highly susceptible to various benzimidazoles in culture. However, organisms such as *Entamoeba histolytica* and *Leishmania major*, which have substitutions at β198 and β200, respectively, are much less sensitive to the benzimidazoles. Data such as these have led several researchers to speculate that residues such as β198 and β200 are involved in the binding of benzimidazoles to tubulin.

A series of benzimidazoles possessed activity against erythrocyte-stage *Plasmodium falciparum* at mid-micromolar concentrations [67]. Albendazole was later found to be inactive against *P. berghei* in mice [76] and toxic to *P. berghei*-infected rats [77]. *In vitro* activity in the low micromolar range was observed against *T. brucei* and *T. cruzi* with benzimidazoles such as albendazole, fenbendazole, and mebendazole, but this activity was ascribed to inhibition of parasite fumarate reductase [78]. We have found that benzimidazoles have little effect on leishmanial tubulin assembly or on the growth of *Leishmania* parasites ([56] and K. Werbovetz, unpublished results). Work with purified leishmanial tubulin, together with examination of β-tubulin sequences of *Plasmodium*, *Trypanosoma*, and *Leishmania*, suggest that the tubulin from these parasites is not susceptible to benzimidazoles. Any activity displayed by benzimidazoles against these parasites is likely due to inhibition of other target(s). Based on *in vitro* susceptibilities and β-tubulin sequences, parasites such as *Giardia lamblia* and *Trichomonas vaginalis* probably do possess tubulin that is sensitive to the benzimidazoles.

Antimitotic Herbicides

Several classes of herbicides selectively inhibit the assembly of plant tubulin (see [79] for review and Fig. (**4**) for structures). Treatment of susceptible plants with dinitroanilines such as trifluralin and oryzalin causes morphological abnormalities in the root tip, a region of the plant exhibiting high levels of cell division. Oryzalin inhibits the rapid phase of taxol-induced polymerization of rose tubulin at low micromolar concentrations, but does not inhibit taxol-induced bovine brain tubulin assembly at similar concentrations [80]. The phosphoric thioamide herbicide amiprophos-methyl (APM) also inhibits the taxol-induced assembly of rose tubulin *in vitro* while having no effect on the taxol-promoted assembly of bovine brain tubulin [81]. Thus far, no biochemical attempts have been reported to probe the binding site for these herbicides on plant tubulin. Mutations in α-tubulin have been shown to confer herbicide resistance in the weed *Eleusine indica* and in transgenic maize calli [82,83], leading to the speculation that these mutations could increase the stability of microtubules and/or destabilize the binding of herbicide to tubulin [83].

Of particular interest are reports concerning the activity of dinitroanilines against *Leishmania* parasites and leishmanial

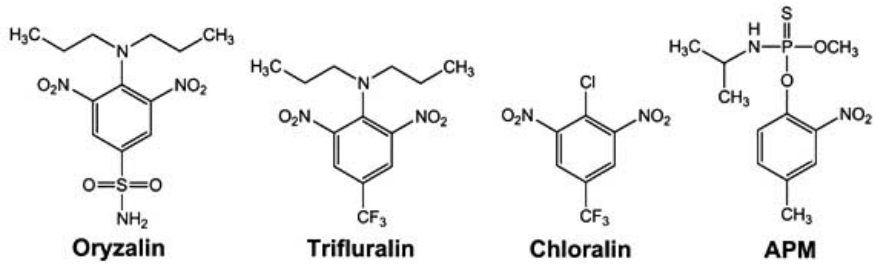

Fig. (4). Structures of the dinitroaniline herbicides oryzalin and trifluralin, the phosphoric thioamide herbicide APM, and the trifluralin precursor chloralin. Chloralin impurities have been observed in commercial trifluralin preparations, and chloralin has displayed activity against both leishmanial tubulin assembly and *Leishmania* parasite growth *in vitro*.

tubulin [84-86]. Radiolabeled trifluralin was shown to bind better to partially purified *Leishmania* tubulin than to rat brain tubulin [84]. Trifluralin was also reported to possess activity against bloodform *T. brucei* [85] and has displayed activity in animal models of leishmaniasis [85] and Chagas' disease [87]. Other reports describing the effects of trifluralin and related compounds on protozoan parasites have appeared [88-90], but effects on purified parasite tubulin were not examined. Based on differences between amino acid sequences for plant and kinetoplastid tubulin versus mammalian tubulin, Traub-Cseko *et al.* concluded that trifluralin probably binds to α-tubulin [91].

Although these reports identify dinitroanilines such as trifluralin as prospective antimicrotubule antiparasitic agents, it is difficult to determine from the studies described above whether trifluralin is a selective lead compound against protozoal tubulin. The synthetic precursor of trifluralin, termed chloralin (see Fig. **4**), was shown to be an impurity in commercial trifluralin preparations, and chloralin was much more toxic to *Leishmania* than trifluralin [92]. Chloralin was also much more potent against *L. infantum* and *L. donovani* axenic amastigotes than trifluralin [56,93]. When chloralin and trifluralin were tested for their ability to block the assembly of purified leishmanial tubulin *in vitro*, only chloralin was found to inhibit this process [56]. In order to resolve these apparent inconsistencies, we felt that more work with purified parasite tubulin and pure herbicide preparations was needed to define the susceptibility of parasite tubulin to antimitotic herbicides.

TUBULIN AS A DRUG TARGET IN *LEISHMANIA* – ONE LAB'S PERSPECTIVE

Tubulin – An Attractive But Poorly Characterized Drug Target in Protozoan Parasites

Given the dire need for new drugs against protozoan parasites of the developing world, the demonstrated value of tubulin as a drug target in eukaryotes, and hints that kinetoplastid tubulin possesses a drug susceptibility that is unique from mammalian tubulin, it is surprising that little drug discovery work has been done to explore this protein as a drug target. There are two likely reasons for this. First, it may have been perceived that the quantities of parasite tubulin needed for drug studies were inaccessible. Until very recently, there were no published reports demonstrating the expression of assembly-competent tubulin. It is thought that tubulin does not fold properly when expressed, preventing the preparation of large quantities of recombinant, assembly-competent tubulin. The recent published reports described

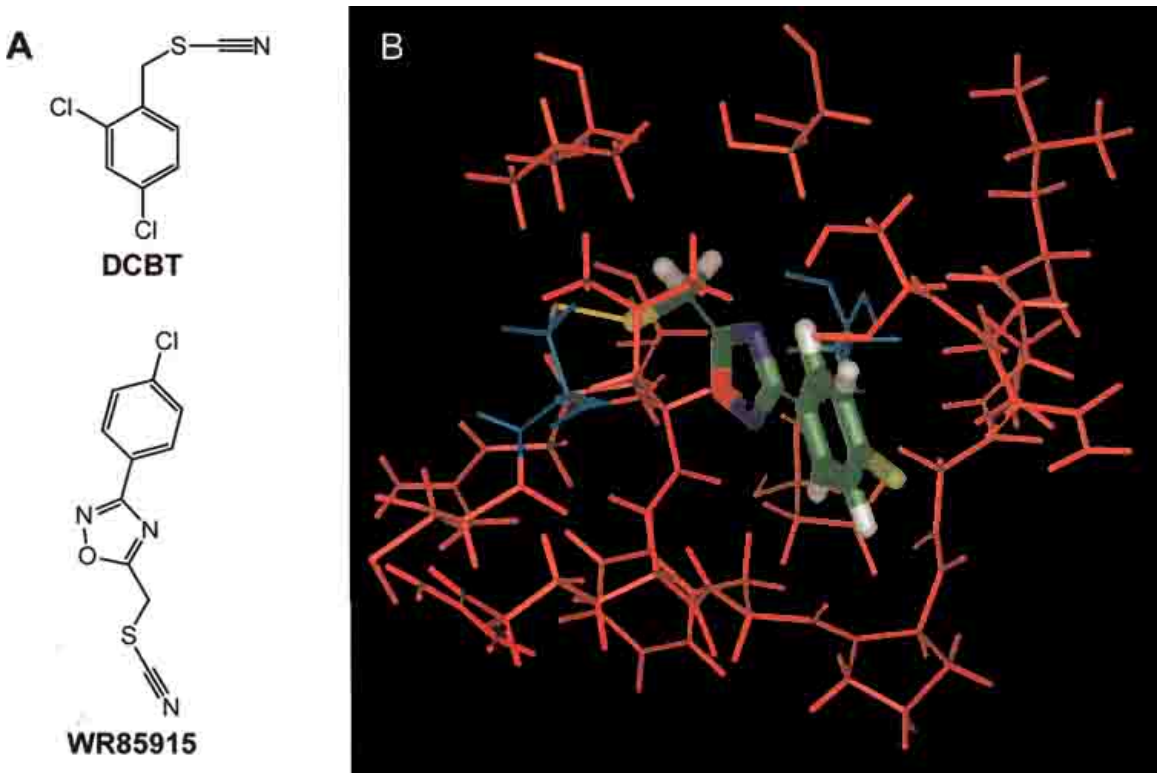

Fig. (5). Aromatic thiocyanates affect tubulin assembly. In Panel A, structures of the two aromatic thiocyanates DCBT and WR85915 are given. Both of these compounds inhibit the assembly of purified tubulin *in vitro*. Panel B shows a model for the proposed covalent interaction between WR85915 and leishmanial β-tubulin which results in tubulin assembly inhibition (generated using the program InsightII, Molecular Simulations, San Diego, CA). In this model, we hypothesize that attack of the sulfhydryl group of Cys239 displaces cyanide ion from WR85915 to give the mixed disulfide adduct, as occurs when DCBT reacts with mammalian tubulin [96]. See text for further details. Residues within 6Å of the biaryl bond in WR85915 are shown in red, except for Cys239 and Cys356, which are in cyan. The putative disulfide bond between Cys239 and WR85915 is shown in yellow.

the expression of assembly competent tubulin on the microgram scale from rabbit reticulocyte lysate [94] and the larger scale preparation of recombinant tubulin from *Haemonchus contortus* [95]. In the latter report, abnormal assembled tubulin structures were observed by electron microscopy. Thus, although tubulin can be purified on the hundred milligram scale from mammalian brain, it is much more difficult to obtain quantities of parasite tubulin needed for drug studies. Second, medicinal chemists may not have been interested in exploring this target in the past. Over the last few years, our laboratory has purified tubulin on a large scale from *Leishmania* promastigotes [56] and has described the effect of known antimicrotubule agents against *Leishmania in vitro* [32]. We have recently begun to explore tubulin as a target for new candidate antimicrotubule agents against kinetoplastids. We are optimistic that such knowledge may ultimately lead to the discovery of new candidates for antiprotozoal chemotherapy.

Examination of the "Colchicine Site" of Mammalian and Leishmanial β-tubulin by Computer Modeling

Since leishmanial β-tubulin is 83% identical to human β-tubulin, the construction of an accurate homology model of leishmanial β-tubulin from the coordinates of the mammalian protein was possible. Our model of leishmanial β-tubulin, constructed with the aid of InsightII software (Molecular Simulations, Inc.), is quite similar to the crystal structure of bovine β-tubulin. However, since we have shown that colchicine site agents have little or no effect on leishmanial tubulin [56], a comparison of the mammalian colchicine site with the corresponding region of leishmanial tubulin was warranted. Colchicine was docked with mammalian β-tubulin with colchicine's C3 methoxy oxygen close to Cys239 and Cys354 as warranted by the biochemical data [62,63], and two binding orientations were determined that are consistent with these data. One of these orientations for colchicine, the one that we favor, is quite similar to the site located entirely within β-tubulin described in the recent paper by Bai *et al.* [63]. The corresponding "colchicine site" in the parasite protein is more restrictive to colchicine. In its lowest energy conformation docked with leishmanial β-tubulin, colchicine is displaced outward from the corresponding cysteine residues in the parasitic protein (data not shown).

Dichlorobenzylthiocyanate (DCBT) reacts specifically with the sulfhydryl residue of Cys239 in mammalian β-tubulin [96]. We later showed that the aromatic thiocyanate WR85915 also blocks the assembly of leishmanial tubulin *in vitro* ([32], see Fig. (**5a**) for the structures of DCBT and WR85915). We then performed a docking experiment where the energy-minimized structure of WR85915 was placed at the "colchicine site" of leishmanial β-tubulin in an orientation where a disulfide bond could form between WR85915 and Cys239 of the parasite protein. We observed interaction energies over two orders of magnitude less than those found when colchicine was manually docked in the same region of modeled leishmanial β-tubulin. A covalent bond was then created between the sulfur atom of WR85915 and the sulfur atom of Cys239, and this covalent complex was minimized for 1000 iterations. This energy minimized structure indicates that the "colchicine site" of leishmanial tubulin is large enough to accommodate the biaryl ring system of WR85915 (Fig. **5b**). Thus, the "colchicine site" of leishmanial tubulin may permit binding of a selective set of ligands, providing an avenue for the development of selective agents against kinetoplastid parasites.

Activity of Oryzalin and Amiprophos-methyl (APM) Against Purified Leishmanial Tubulin

As mentioned earlier, the dinitroaniline herbicide trifluralin was reported to bind leishmanial tubulin and not rat brain tubulin [84]. We were initially discouraged by the lack of activity of trifluralin in our hands against leishmanial tubulin assembly and against axenic *L. donovani* amastigotes [56]. Our own work [56] and other studies

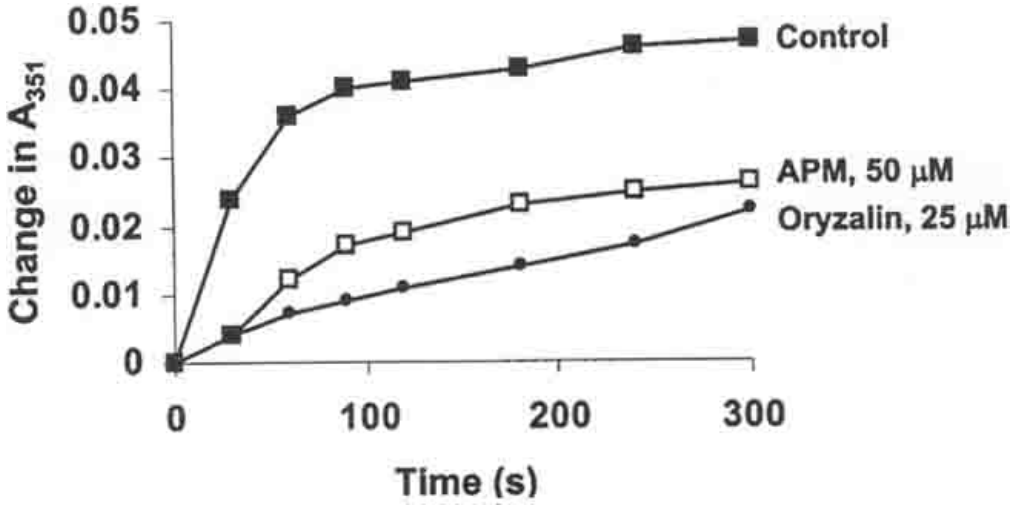

Fig. (6). Inhibition of leishmanial tubulin assembly by oryzalin and APM. Samples containing oryzalin, APM, or a DMSO control were incubated with 0.5 mg/ml leishmanial tubulin under the same conditions as described previously [32]. The change in turbidity of each sample was then monitored at 351 nm after the addition of GTP and DMSO to initiate assembly.

[92,93] had demonstrated that chloralin, the chemical precursor of trifluralin and thus an impurity in some commercial trifluralin preparations, possessed antileishmanial activity superior to trifluralin. Moreover, chloralin itself inhibits tubulin assembly, probably through reaction with tubulin sulfhydryl groups [56]. We therefore pursued other leads in the search for selective antiparasitic antitubulin agents. However, our modeling studies described earlier suggested that small aromatic molecules could be effective ligands for leishmanial tubulin, prompting us to reexamine the antimitotic herbicides. Since trifluralin possesses limited solubility in aqueous solution [79], we decided to examine the effect of the more soluble sulfonamide-containing dinitroaniline, oryzalin, against our purified leishmanial tubulin *in vitro*. Oryzalin did in fact inhibit the assembly of purified leishmanial tubulin, displaying approximately 50% inhibition of assembly at a concentration of 25 μM. Another antimitotic herbicide, amiprophos-methyl (APM), also inhibited the assembly of purified leishmanial tubulin at a concentration of 50 μM (see Fig. (**4**) for structures of oryzalin and APM and Fig. (**6**) for the inhibition of tubulin assembly by these compounds). Previous studies concerning trifluralin [84] and oryzalin [97] with leishmanial tubulin used only partially purified protein and did not employ the assembly assay to examine drug-tubulin effects. Our recent studies verify the activity of small aromatic antimitotic herbicides on leishmanial tubulin and confirm that the selective inhibition of leishmanial tubulin is possible. Presumably we could not demonstrate antitubulin activity with trifluralin due to the insolubility of this compound [79]. More importantly, our recent experiments indicate that oryzalin and APM are promising lead structures for drug discovery.

FUTURE DIRECTIONS

Although tubulin from kinetoplastid parasites has been recognized as a selective target for dinitroaniline herbicides for some time, there has been little follow-up on this initial observation. Now that the parasite protein can be purified on a relatively large scale, it is possible for the first time to develop a structure-activity relationship between analogs of the dinitroaniline herbicides and activity against parasite tubulin. This structure-activity relationship should provide insights into the synthesis of more potent ligands for parasite tubulin. Our early results indicate that several new analogs of oryzalin are more effective ligands for leishmanial tubulin than the parent compound (G. Bhattacharya and K. Werbovetz, unpublished observations).

Recently, two new phenylcyclohexenes containing a biaryl trimethoxybenzene ring were found to inhibit tobacco root elongation at sub-micromolar concentrations ([98], see Fig. (**7**) for structures). These compounds also weakly inhibited bovine brain tubulin assembly at a concentration of 200 μM, and one of the compounds was a competitive inhibitor of colchicine binding to bovine brain tubulin. In addition to providing another potential lead against parasite tubulin, these results also give credence to our hypothesis that the "colchicine site" of leishmanial tubulin is a target for ligands, specifically the antimitotic herbicides. Further support for this hypothesis could come from affinity labeling studies with analogs of the antimitotic herbicides, just as affinity labeling experiments provided information regarding the mammalian tubulin taxol site, colchicine site, and vinca alkaloid domain. It is likely that the binding site of the antimicrotubule herbicides may also accept a structurally diverse series of ligands, a hopeful possibility for future antiparasitic drug discovery efforts.

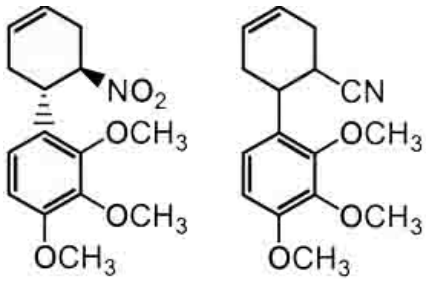

Fig. (7). Structures of new phenylcyclohexene antimitotic herbicides that also bind weakly to the colchicine site of mammalian tubulin [98].

FINAL THOUGHTS

Drug discovery and development research for protozoal parasitic diseases based on tubulin, a proven chemotherapeutic target, will benefit from the fact that this protein can now be isolated on a fairly large scale, appropriate assays and model systems are available for drug testing, a reasonably accurate homology model of the protein can be constructed, and candidate compounds should be accessible through straightforward routes. The latter point could be very important in the future, considering that drugs used to treat disease in the developing world must be inexpensive so that they can be made available to those who need them most. With these thoughts in mind, it is likely that tubulin will be an important target for much-needed antiparasitic agents in the future.

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