Critical Review

Microtubule Assembly Dynamics: An Attractive Target for Anticancer Drugs

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Summary

Microtubules, composed of $\alpha\beta$ tubulin dimers, are dynamic polymers of eukaryotic cells. They play important roles in various cellular functions including mitosis. Microtubules exhibit differential dynamic behaviors during different phases of the cell cycle. Inhibition of the microtubule assembly dynamics causes cell cycle arrest leading to apoptosis; thus, qualifying them as important drug targets for treating several diseases including cancer, neuronal, fungal, and parasitic diseases. Although several microtubule-targeted drugs are successfully being used in cancer chemotherapy, the development of resistance against these drugs and their inherent toxicities warrant the development of new agents with improved efficacy. Several antimicrotubule agents are currently being evaluated for their possible uses in cancer chemotherapy. Benomyl, griseofulvin, and sulfonamides have been used as antifungal and antibacterial drugs. Recent reports have shown that these drugs have potent antitumor potential. These agents are shown to inhibit proliferation of different types of tumor cells and induce apoptosis by targeting microtubule assembly dynamics. However, unlike vincas and taxanes, which inhibit cancer cell proliferation in nanomolar concentration range, these agents act in micromolar range and are considered to have limited toxicities. Here, we suggest that these drugs may have a significant use in cancer chemotherapy when used in combination with other anticancer drugs. © 2008 IUBMB

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MICROTUBULE DYNAMICS—DYNAMIC INSTABILITY AND TREADMILLING

Microtubules play indispensable roles in cell division, cell motility, cellular transport, maintaining cell polarity, and cell signaling. Impairment in the functioning of microtubules leads to an abnormal morphology of the cells and may even pose a challenge to their survival leading to apoptosis. Microtubule polymerization is a complex process involving a cooperative assembly of $\alpha\beta$ tubulin heterodimers followed by GTP hydrolysis (*1*). Each subunit of the tubulin heterodimer has one GTP molecule bound to it. The α-subunit binds to GTP in an irreversible manner while the GTP bound to β-tubulin is exchangeable (*2*) and it hydrolyzes during polymerization.

Polymerization of microtubules occurs through two important steps; nucleation and elongation. Initially, an oligomer consisting of 6–12 $\alpha\beta$ tubulin dimers is formed in the nucleation step. Further, GTP bound $\alpha\beta$ tubulin dimers add up to the nucleus and lead to its elongation and formation of the protofilament. After a rapid elongation phase, the assembly of microtubules reaches the steady state, where the addition and the dissociation of tubulin subunits at the ends of the microtubules are balanced and there is no net increase in the polymer level. Hydrolysis of GTP introduces unusual equilibrium behaviors in microtubules. Microtubules are labile polymers and they display two types of dynamic behaviors, "treadmilling" and "dynamic instability." **Treadmilling** refers to a net addition of tubulin dimers at the plus end coupled with a net dissociation at the minus end producing a flow of subunits from one end of the microtubule to the other without significantly changing the average length of microtubules (*3, 4*). Microtubule ends also alternate between growing and shortening phases, which is called as **Dynamic instability** (*5*). A transition from a growing phase to a shortening phase is termed as a **catastrophe** while a transition from a shortening phase to a growing phase is termed as a **rescue** (*6*). Using reconstituted microtubules, microtubule dynamics have been investigated extensively *in vitro* by light microscopy. Microtubule dynamics in live cells are also studied by time lapse video microscopy, either by labeling the individual microtubules with fluorescent tubulin or by expressing GFP-tubulin in the cells. Microtubule assembly and activity in the cells is considered to be precisely regulated by several proteins, called as microtubule associated proteins.

Microtubule dynamics play an essential role in the proper orientation and segregation of chromosomes during mitosis (7, 8). The plus ends of the microtubules undergo a series of random elongations and shortenings in the cytoplasm to search and bind the kinetochores of the chromosomes (9). Once the plus ends attach to the kinetochores, the chromosomes are translocated to the equatorial plane. Metaphase microtubules are highly dynamic; however, at the onset of anaphase, microtubule dynamics are stabilized to ensure the successful chromosomal segregation (10). Owing to the shortening of the microtubules and due to the action of motor proteins, a tension is generated in the microtubules, which leads to the poleward movement of the chromosomes (11). A defect in this process blocks the cells at mitosis by activating the spindle checkpoint. The major proteins involved in the spindle checkpoint are Mad1, Mad2, Mad3, Bub1, and Bub3 (12). Since microtubule dynamics play important roles in various cellular functions including mitosis, it is a potential drug target for several diseases including cancer, neuronal diseases, fungal, and parasitic diseases. Several antimicrotubule agents are now used as anticancer drugs (13). In addition, some of the antimicrotubule agents are also under clinical trials. On the basis of their effects on microtubule assembly, microtubule-targeted drugs are generally classified as either a depolymerizing agent or a polymerizing agent.

Depolymerizing Agents

This class of agents inhibits microtubule assembly and depolymerizes microtubules both *in vitro* and *in vivo* (Fig. 1). Most of these agents either bind to the well characterized colchicine or vinblastine site on tubulin. While vinblastine binds close to the exchangeable GTP site on the β-tubulin (14), colchicine site is located at the interface between α and β subunits of the tubulin dimer (Fig. 2) (16). However, several of the depolymerizing agents such as benomyl, estramustine, and LY290181 have been shown not to compete with vinblastine and colchicine for tubulin binding suggesting that these agents bind to tubulin at sites distinct from the colchicine and vinblastine binding sites (18–20). Vinblastine and colchicine inhibit microtubule assembly in low substoichiometric concentrations indicating that these agents inhibit microtubule assembly by end poisoning (13, 21). Vinblastine or tubulin-colchicine complex binds at the ends of microtubules and deter the addition of new subunits. Further, these agents have also been shown to kinetically stabilize the plus ends of microtubules. Present evidence suggests that most of the antimitotic antitubulin agents inhibit cell proliferation by perturbing microtubule dynamics (13, 21).

POLYMERIZING AGENTS

This class of drugs promotes microtubule assembly and stabilizes microtubules. Microtubule polymerizing agents include paclitaxel (Fig. 1), docetaxel, and polyisoprenyl benzophenones.

Paclitaxel is a plant product from *Taxus brevifolia*. It is widely used in the treatment of breast cancer, ovarian cancer, lung cancer, and several sarcomas. Paclitaxel binding site on tubulin is well characterized. It binds on the inner surface of the microtubules in a deep hydrophobic pocket on the β-tubulin (Fig. 2) (15). Paclitaxel binding induces conformational changes in tubulin structure, which stabilizes microtubules by increasing the interaction between the tubulin subunits. Paclitaxel promotes microtubule assembly and suppresses the growing and shortening dynamics of individual microtubules of tumor cells in culture (13).

In this review, we will be mainly focusing on three antimicrotubule agents namely benomyl, griseofulvin, and sulfonamides, which are currently attracting a lot of interest as potential anticancer agents. Benomyl and griseofulvin have been used as antifungal compounds for a long time. However, these agents are also shown to potently inhibit proliferation of various types of cancer cells in culture. An active metabolite of benomyl, carbendazim, is presently undergoing clinical trials for the treatment of advanced solid tumors (clinicaltrials.gov Identifier: NCT00003709, CDR0000066817). Recent reports have shown that griseofulvin specifically targets cells expressing tumor phenotype (22). Several anticancer drugs show high toxicities at their therapeutic doses. These drugs other than inhibiting the cancer cell proliferation also affect the healthy tissues of the body. Many drugs including cisplatin, zoledronate, and mitomycins exhibit significant level of nephrotoxicity (23, 24). Griseofulvin at a concentration where it causes metaphase arrest exhibits less toxicity (25). The use of the combination of griseofulvin and nocodazole in nude mice bearing a colon adenocarcinoma xenograft showed a significant reduction in the tumor size as compared with the treatment with griseofulvin and nocodazole alone (26). Use of low concentrations of nocodazole with griseofulvin may reduce the toxicity of individual agents while synergizing the anticancer effects of both the agents. Considering the fact that griseofulvin has the property of getting accumulated in the keratin layers of the epidermis (27), it could serve as a potential candidate for the treatment of skin cancers either alone or in combination with other drugs. Similar observations have also been reported in case of ketoconazole and nocodazole. Ketoconazole, an antifungal compound, when used in combination with nocodazole reduced the minimum concentration of nocodazole required to cause cell death (28). Further, estramustine in combination with paclitaxel, docetaxel, and carboplatin has been shown to have positive results against hormone refractory prostate cancer, with moderate and reversible toxicities (29–31). Estramustine in combination with epirubicin is presently under phase II clinical trial for the treatment of prostate cancer (clinicaltrials.gov Identifier: NCT00218205). Several sulfonamides such as E7010, E7070 have shown antitumor activity in animal models (32). Further, E7010 and E7070 are currently undergoing clinical trials as anticancer agents against various types of tumors and have shown tolerable toxicity profiles and significant antitumor activity (33, 34).
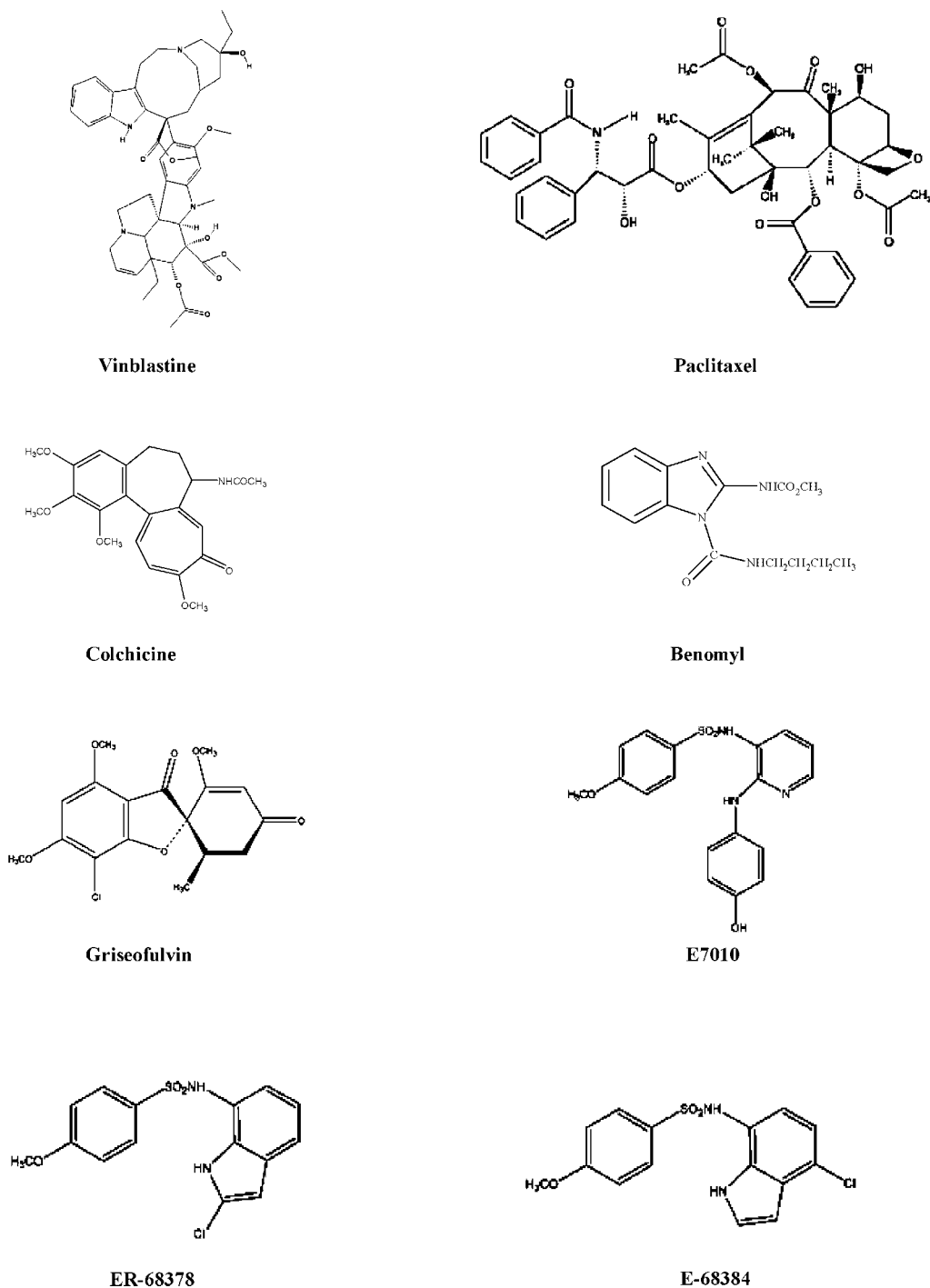
Figure 1. Structures of tubulin-targeted agents.

Benomyl

Benomyl belongs to the benzimidazole group of compounds that are widely used as antifungal agents. Benomyl is selectively toxic to microorganisms and is extensively used in agriculture against a range of fungal diseases of field crops and fruit trees (WHO, report 1993 Environmental Health Criteria number 148: Benomyl). Benomyl is shown to exhibit differential sensitivity against fungal and mammalian tubulin (*35, 36*). The increased toxicity of benomyl to fungi is thought to be due to its higher affinity for fungal tubulin than for mammalian tubulin (*36*). Benomyl binds to mammalian tubulin with a K_d of $\approx 12 \pm 1.2$ μM (*18*) indicating that it binds to mammalian tubulin
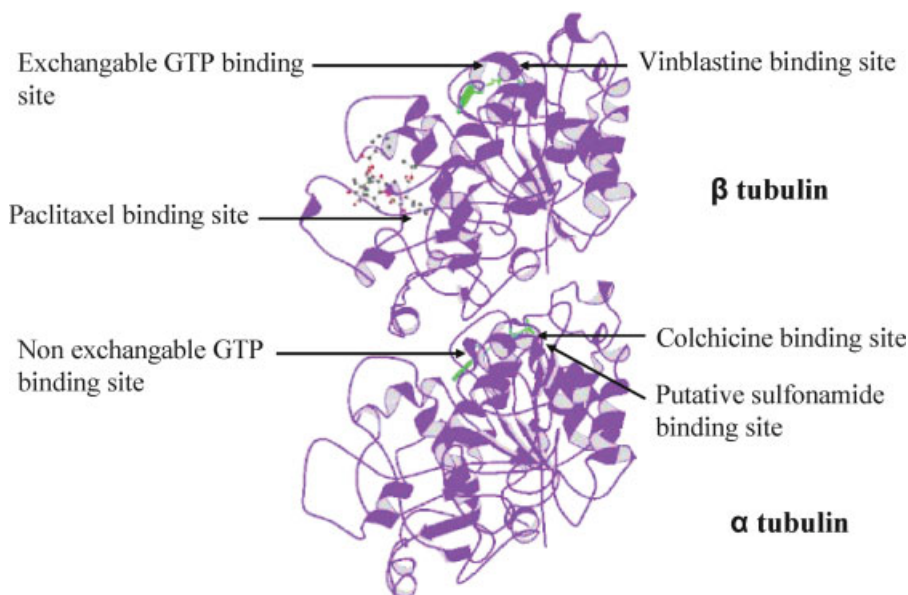
Figure 2. Crystal structure of $\alpha\beta$-tubulin heterodimers (*2*) showing binding sites of different anticancer agents. The binding sites of vinblastine (*14*), paclitaxel (*15*), and colchicine (*16*) are shown. Sulfonamides are thought to bind to the colchicine binding site (*17*).

with a modest affinity rather than a weak affinity as was believed earlier. Mutations in the β-tubulin gene, which either increased the resistance or increased the sensitivity of yeast cells to benomyl, suggested that benomyl targets tubulin in fungal cells (*37, 38*). The exact binding site of benomyl on tubulin is not yet known; however, several members of the benzimidazole group of compounds are shown to inhibit the binding of colchicine both competitively and noncompetitively (*35, 39*). On the basis of the structural similarities of benomyl and other benimidazole analogues, benomyl was thought to bind near or at the colchicine binding site. A study using site-directed mutagenesis of β-tubulin gene in *S. cerevisiae* has indicated that the benomyl binding site is located in the core of β-tubulin at a site distinct from the colchicine site (*40*). Using colchicine fluorescence, Gupta et al. (2004) have found that the preincubation of tubulin with benomyl neither had an affect on the binding of colchicine to tubulin nor did it affect the kinetics of colchicine binding to tubulin. In addition, benomyl was shown to bind to the tubulin-colchicine complex (*18*). These observations lead to the idea that benomyl and colchicine have different binding sites on tubulin. Benomyl also does not bind to the vinblastine site on tubulin (*18*). Further, it alters the far-UV CD spectra of tubulin and also reduces the number of accessible cysteine residues in tubulin, indicating that it induces conformational changes in tubulin (*18*).

Benomyl weakly inhibits the assembly of tubulin subunits into microtubules *in vitro* (*18*). For example, half-maximal inhibition of polymerization occurred in the presence of $\sim$70 μM benomyl. At this concentration nearly 85% of the soluble tubulin is calculated to be bound to benomyl. Thus, in contrast to

colchicine and vinblastine benomyl does not inhibit microtubule polymerization by end-poisoning mechanism; rather, benomyl-tubulin complex copolymerizes along with free tubulin into microtubules. Though benomyl weakly inhibits microtubule assembly, it significantly perturbs the dynamic instability of microtubules *in vitro*. Benomyl reduces the growing and shortening rates at the plus ends of bovine brain microtubules *in vitro*. Additionally, it increases the time spent by each microtubule in the attenuated state and significantly reduces the dynamicity of microtubules. Benomyl-induced conformational changes may reduce the rate of tubulin addition at the plus ends while the copolymerization of benomyl with tubulin into the microtubule lattice may reduce the shortening rate by increasing the stability of the polymer (*18*).

Benomyl inhibits cell-cycle progression at mitosis and also induces apoptotic cell death (a putative mechanism of action of benomyl has been depicted in Fig. 3) (*18, 41*). In the lower inhibitory concentration range of benomyl (at or near half maximal inhibitory concentration range); though the cells form normal spindles, they do not exhibit proper chromosomal segregation. Benomyl suppresses the assembly of microtubule in HeLa cells. Benomyl treated cells also show a decrease in the spindle length and the interpolar distance. Benomyl is shown to perturb the microtubule-kinetochore interactions and reduce the distance between the sister kinetochore pairs indicating that it reduces the tension across the kinetochores. The loss of tension at the kinetochores activates the spindle checkpoint proteins, thereby inducing a mitotic block. Benomyl-induced mitotic arrest in HeLa cells is accompanied by hyper phosphorylation of Bcl-2 and the release of Bax from the Bcl-2-Bax complex indicating
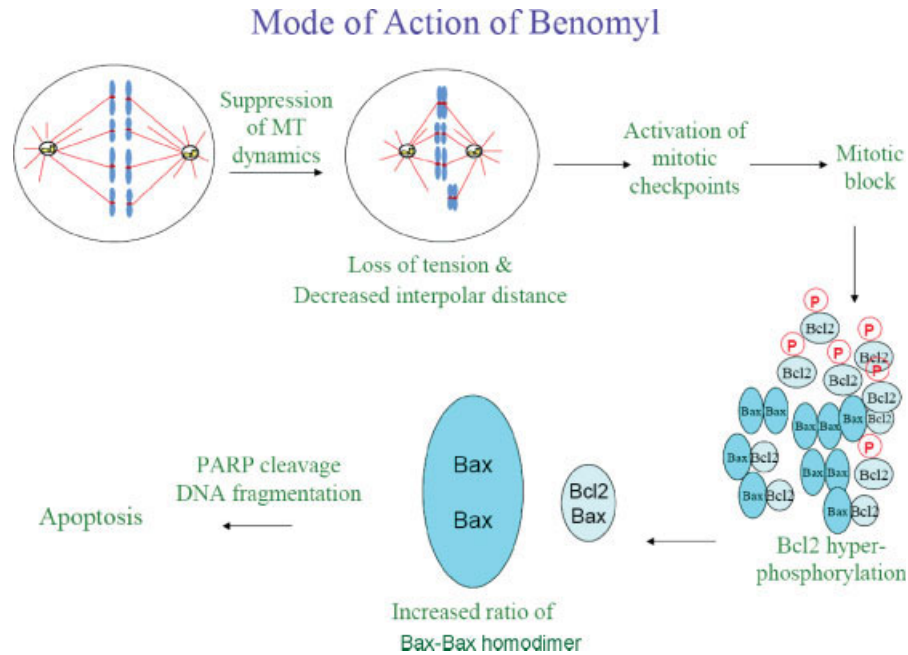
Figure 3. A schematic representation depicting the mechanism of inhibition of cell proliferation by Benomyl. Benomyl suppresses microtubule dynamics causing improper chromosomal segregation and mitotic arrest of cells. Benomyl induces hyperphosphorylation of Bcl-2 and dissociation of Bcl2-Bax complex promoting Bax–Bax homodimerization. An increase in the ratio of Bax–Bax homodimers leads to the execution of apoptotic events like cleavage of PARP and fragmentation of DNA.

that benomyl significantly increased the ratio of free Bax (*41*). The increase in the ratio of free Bax causes the formation of Bax–Bax homodimer, facilitating apoptosis.

Griseofulvin

Griseofulvin is an antifungal antibiotic produced by various species of *Penicillium* (*27*). Griseofulvin has been extensively used for the treatment of ring worm (*Tinea capitis*) and other dermatophyte infections and is considered to be relatively of low toxicity to human beings (*42*). Griseofulvin accumulates in the keratin layers of the epidermis and inhibits the fungal growth (*27*). Although its mechanism of action is not clearly understood it was thought to inhibit mitosis in sensitive fungi by disrupting microtubules (*43*). Griseofulvin, similarly inhibits the proliferation of mammalian cells by targeting microtubules (*44, 45*). Griseofulvin inhibits microtubule assembly concurrent with inhibition of mitosis in echinoderm eggs (*46*) and it inhibits mitosis in 3T3 cells in association with depolymerization of the spindle microtubules (*44*). At lower concentrations, it inhibits mitosis at the metaphase anaphase transition in HeLa cells in the absence of significant depolymerization of spindle microtubules (*47, 48*). Griseofulvin induces cell cycle arrest at G2/M transition; this is characterized by an abnormal spindle formation, increase in the cyclin B1/cdc2 kinase activity and down regulation of myt-1 protein expression (*26*). Additionally, griseofulvin induces apoptosis in cells which is preceded by caspase

3 activation, Bcl-2 hyperphosphorylation and activation of NF-κB pathway (*26, 49*). Cells treated with half maximal inhibitory concentration of griseofulvin have normal bipolar spindles; but they show defects in chromosomal segregation (*48*). Several of the chromosomes in the treated cells fail to align at the equatorial plane during metaphase (*48*). At twice the half maximal inhibitory concentration (2 $\times$ IC$_{50}$), griseofulvin disrupts spindle microtubules and depletes most of the mitotic microtubules at relatively high concentrations. Griseofulvin does not have a distinct effect on the interphase microtubules. Even at twice IC$_{50}$ concentrations of griseofulvin, no detectable change in the interphase microtubules has been observed. However, at higher concentrations (6 $\times$ IC$_{50}$), griseofulvin significantly depolymerizes interphase microtubules of HeLa cells. Griseofulvin has been shown to inhibit the aggregation of multiple centrioles in SCC114 and N115 mouse neuroblastoma cell lines (*22*). These cell lines show an increase in the formation of multipolar spindles in the presence of increasing concentration of griseofulvin (*22*). The cells with bipolar spindles survive in the presence of griseofulvin while the cells with multipolar spindle end up in apoptosis (*22*).

Griseofulvin binds to tubulin *in vitro* with a weak affinity (K_d, 300 μM) (*48*). Griseofulvin does not significantly reduce the polymer mass of tubulin *in vitro*; the inhibition of brain tubulin polymerization requires very high concentrations of griseofulvin (*44, 45, 48*). Griseofulvin does not alter the sedimentable polymer mass of reconstituted microtubules suggesting that

it does not sequester tubulin. In contrast to its weak effect on polymer mass, griseofulvin strongly suppresses the dynamics of reconstituted microtubules *in vitro* (*48*). Griseofulvin (5 μM) reduces the rates of growing and shortening by 50 and 70%, respectively. Further, it strongly reduces the catastrophe frequency and increases the percent of time spent by microtubule in the attenuated state. Griseofulvin even at low concentrations exerts a strong suppressive effect on the dynamics of the individual microtubules; it has no significant effect on the extent of net microtubule assembly indicating that griseofulvin may be exerting its inhibitory effect on cell proliferation by suppressing the dynamic behavior of microtubules (*48*). Griseofulvin in combination with nocodazole enhances the effect of nocodazole against the colon cancer cells *in vivo* (*26*). Since griseofulvin is known to have less toxicity in human, it can be used in combination with other anticancer drugs in adjuvant therapy.

Sulfonamides

Sulfonamides are known to interact with different cellular targets and produce diverse pharmacological effects. In addition to their antibacterial, antiviral, antidiabetic, and antithyroid activities, several sulfonamides are currently undergoing clinical trials as anticancer agents (clinicaltrials.gov Identifier: NCT00297089). Antitumor sulfonamides have been mainly divided into two groups based on their structure-activity relationship. One group is represented by *N*-(3-chloro-7-indolyl)-1,4-benzenedisulfonamide (E7070) and its analogues and the other group by *N*-[2-[(4-hydroxyphenyl)amino]-3-pyridinyl]-4-methoxybenzene sulfonamide (E7010) (Fig. 1). Indole sulfonamides inhibit the binding of colchicine to tubulin; however, unlike colchicine, sulfonamides do not increase the GTPase activity of tubulin indicating that these agents bind to tubulin by different mechanisms (*50*). Docking studies also indicated that E7010 and the indole sulfonamides derived from E7010, may have distinct binding modes at the colchicine site on tubulin (*51*). The indole sulfonamides like E-68384 and E-68378 (Fig. 1) are shown to inhibit microtubule polymerization *in vitro* (*17*). Further, some of the indole sulfonamides are found to suppress the dynamicity of individual microtubules by reducing the growing and shortening rates and by inhibiting the catastrophe and rescue frequencies, while increasing the pause state (*17*). The binding of free sulfonamide molecules or sulfonamide-tubulin complexes either at the microtubule ends or their incorporation into the microtubule lattice may stabilize microtubule lattice and prevent further addition of tubulin dimers at the ends. The indole sulfonamides inhibit the proliferation of HeLa cells by blocking the cells at mitosis. At half maximal inhibitory concentration for cell proliferation ($\sim$IC$_{50}$), the indole sulfonamides depolymerize spindle microtubules and perturb the chromosome organization without significantly affecting the interphase microtubules. The spindle microtubules are highly dynamic and more labile than the interphase microtubules and this may be the basis of the differential effects of indole sulfonamides on the microtubules of mitotic and interphase cells. How-

ever, high concentrations (3 $\times$ IC$_{50}$) of these agents are found to depolymerize microtubules of both interphase and mitotic cells. Abnormal spindle organization, with condensed ball shaped chromosomes is induced by these drugs, which prevents the mitotic progression of these cells. Sulfonamides induce the formation of multinucleated polyploid cells, which may be due to the occurrence of nuclear division without being followed by cytokinesis (*52*). The basis of the antimitotic activity of sulfonamides is somewhat similar to that of known antimicrotubule agents; it involves suppression of microtubule dynamics (*17*).

CONCLUSION

In spite of their potent anticancer action, many of the antimicrotubule drugs have limited clinical use because of their strong toxic effects. Another limiting factor lies in the inherent or acquired resistance of the tumors towards these drugs. Tumors may develop resistance against a particular drug due to the overexpression of drug efflux pumps, mainly P-glycoprotein and multidrug resistance associated protein 1, which pump out the drug from the cells and reduce the intracellular concentration of the drug (*53*). Another *modus operandi* adopted by oncogenic cells involves mutations in the genes encoding α and β subunits of tubulin, which reduce the binding of a drug to tubulin (*54*). In addition, differential expression of tubulin isotypes may also contribute to the development of drug resistance. Discovery of new antimicrotubule drugs with novel mechanism of action may be helpful to overcome these problems. Another way to overcome these shortcomings might lie in the combination therapy, which reduces the doses of individual drugs, thereby lessening their toxicities (*13, 20, 28–31*). Griseofulvin, benomyl, and sulfonamides are weak antimicrotubule agents and these agents are considered to have less harmful side effects compared with the highly potent antimicrotubule agents such as vincas and taxanes. Therefore, these agents can be used in combination with low doses of other microtubule targeted drugs to have a synergistic inhibitory effect on the proliferation of cancer cells in the absence of significant toxicity.

ACKNOWLEDGEMENTS

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