Recent Development and SAR Analysis of Colchicine Binding Site Inhibitors

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Abstract: Microtubules are cytoskeletal components that play important roles in a number of cellular processes. Colchicine binding site inhibitors (CSIs) is one major class of tubulin polymerization inhibitors, inhibiting microtubule polymerization and blocking cell proliferation at metaphase during mitosis. Many CSIs were discovered or designed and synthesized as anticancer agents in the past several years and great progress had been made. Here, we discuss the insights gained so far relevant to the mechanism of CSIs and their common pharmacophore. The recent development of CSIs with their biological activity and structure-activity relationship (SAR) are also reviewed.

Key Words: Microtubules, colchicine binding site, inhibitor, SAR, anticancer.

INTRODUCTION

Microtubules, the key components of cytoskeleton of eukaryotic cells, are cylinder-shaped protein polymers composed of α, β-tubulin heterodimers arranged head to tail. They form stable interphase microtubule networks and are highly dynamic mitotic spindles. Besides, being critical for cell division, it is involved in various cellular processes, such as cell shape maintenance, cell signaling, migration and transportation [1-9]. Microtubule dynamics is specifically important for the proper attachment and movement of chromosomes during various stages of the mitotic phase [3-5]. Its suppression blocks the cell division machinery at mitosis, leading to cell death. Therefore, the dynamics of microtubule represents a potential target for developing anticancer drugs.

Known microtubule-targeted agents can be broadly divided into three groups according to their binding site, vinca alkaloids, colchicine alkaloids and taxanes. Among them, the first two groups are tubulin polymerization inhibitors, which inhibit microtubule polymerization and block cell proliferation at metaphase during mitosis [10]. And the taxanes are polymerization promoters, which stabilize microtubules and disturb microtubules depolymerization [11]. In the past years, the binding sites of taxol, colchicine, and vinblastine in tubulin were well characterized [5]. It was reported that taxol and vinblastine bind to the β-tubulin, whereas colchicines is located in β-tubulin at the interface with α-tubulin. While the vinca alkaloids and the taxanes have well-established roles in the treatment of human cancers [12-14], no CSIs are currently approved for cancer therapy. Over the years, a large number of natural and synthetic small molecules of varied structures have been identified as CSIs, some are undergoing intensive investigation as vascular targeting agents for cancer therapy, thus, suggesting a high plasticity of this binding site. This review is primarily focused on recent development (2005-2008) of structurally different CSIs and their interactions with tubulin.

COLCHICINE AND ITS ACTIVITY MECHANISM

Colchicine (**1**) was extracted from the poisonous meadow saffron *Colchicum autumnale L.* It is the oldest known natural product that binds to tubulin and inhibits its assembly into microtubules [15]. It possesses many biological activities such as anti-inflammatory, anti-mitotic, anti-fibrotic, and anti-cancer [16]. It is used in the treatment of pseudogout, familial Mediterranean fever, cirrhosis of the liver and bile, and amyloidosis [17, 18]. Besides, colchicine is found to be used as a selective neurotoxin in animal models to study Alzheimer's dementia [19, 20]. Although its high toxicity has limited its application on cancer therapy, it plays a central role in elucidating the physical properties and biological functions of microtubules in cells [21].

Colchicine blocks cell division by disrupting microtubules. It binds to soluble tubulin leading to the formation of a tubulin-colchicine complex (TC-complex). This complex then undergoes co-polymerization into microtubule end with the majority of the tubulins being unaffected. The microtubule ends have the ability to polymerize further, but TC-complexes induce a conformation, which prevents the microtubules growth by sterically blocking further addition of the tubulin dimers at the ends [16]. It is known as the "end conserving mechanism", which suggests the TC-complex doesn't completely prevent the tubulin addition but only slows new tubulin addition [22]. This process retards and eventually causes the microtubule spindle to disassemble because of the structural instability during the metaphase of mitosis. Thus, the number of TC-complexes incorporated into the microtubules determines the stability of the microtubule ends. Colchicine depolymerizes microtubules at high concentration and arrests microtubule growth at low concentration [23]. The cells blocked at mitosis undergo abnormal mitotic cycle, named "colchicine-mitosis" or "c-mitosis". C-mitosis is characterized by partial or complete absence of spindle appa-

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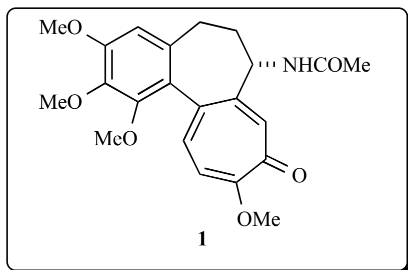
ratus followed by the breakdown of nuclear envelope, condensed chromosomes and undivided centromeres [24]. Other studies showed that colchicine could inhibit the function of several ion channels and its depolymerizing effect was attributed to this [25]. Besides, colchicine could alter the membrane potential of the mitochondria, leading to the release of proapoptotic factors like caspases, cytochrome-c and apoptosis-inducing factors, and eventually leading to the apoptotic cell death [26].



THE LOCATION OF COLCHICINE AND A COMMON PHARMACOPHORE OF COLCHICINE SITE LIGANDS

1. Structure of Tubulin

To analyze the mechanism of drug-tubulin interaction, we need a detailed description of the receptor, tubulin. From 1995, a three-dimensional model of tubulin at 6.5Å, 4 Å and 3.7 Å resolutions, by using electron crystallography of zinc-induced tubulin sheet, were presented continuously [27]. And it was further refined using standard X-ray crystallography methodology [28]. In this structure, α- and β-tubulin possessed identical principal structure, each monomer had compact structure being composed of a core of two β sheets surrounded by a helix. The monomer could be divided into three functional domains. First one was an amino-terminal domain, a nucleotide-binding region, having six parallel β-strands (S1–S6) alternating with helices (H1–H6). The loops (T1–T6) connected each strand with the start of the next helix in binding to the nucleotide. The nucleotide binding was completed by interaction with the *N*-terminal end of the core helix H7. This helix also connected the first region with the second one, the intermediate taxol-binding domain, which was formed by three helices (H8-H10) and a mixed β sheet (S7-S10). The third one was the carboxy-terminal domain comprising the binding site for motor proteins. It was formed by two antiparallel helices (H11-H12) that were crossing over the previous two domains.

2. Location of Colchicine

Experimental data showed that colchicine bound to β-tubulin at its interface with α-tubulin [29], resulting in inhibition of tubulin polymerization. This binding mode was then confirmed by the determination of a 3.58 Å X-ray structure of α, β-tubulin complexed with *N*-deacetyl-*N*-(2-mercaptoacetyl)colchicine (DAMA-colchicine), a close structural analog of colchicine [30] (PDB code 1SA0). In this crystal structure, the rings A and C of DAMA-colchicine interacted with the β-tubulin, while the side chain of ring B interacted with the α-tubulin. Later, Nguyen *et al.* [31] described the colchicine site in detail. It was reported that the colchicine site was located mostly in the β-tubulin and was bordered by H7, which contained Cys β239, and H8. In additional, α-tubulin formed crucial interactions at the colchicine site, notably the loop connecting S5 and H5. The latter contained Thr α177 and Val α179, both appeared to form hydrogen bonds with CSIs. The dimensions of the colchicine site are ~10 Å× ~10 Å× 4-5Å.

3. A Common Pharmacophore for CSIs

Over the years, a large number of natural and synthetic small molecules have been identified as CSIs. Their structure differences give rise to the question about the essential structural features required for the activity of a drug. The identification of a common pharmacophore among these structures provides an answer to this.

A pharmacophore is a three-dimensional substructure of a molecule that carries the essential features responsible for a drug's biological activity. Nguyen and coworkers [31] selected fifteen CSIs on the basis of occupation at same chemical space, consistent topology and binding modes (Fig. **1**). Tubulin-DAMA-colchicine crystal structure was chosen as a template. Molecular dynamics simulations and docking studies were deployed to construct binding models for these structurally different CSIs. They divided these drugs into two groups. The first one included those sharing structural similarity with colchicine and bearing the following three important features: a diaryl system, a trimethoxyphenyl (TMP) moiety and a constrained conformation. The second group did not possess any of the characteristics of the former group. Thus, it would be more structurally diversified than the first group. The study on these CSIs indicated the frequency of hydrogen bonds between CSIs and tubulin. The following was the conclusion: (1) All of the fifteen CSIs formed hydrogen bonds with the thiol group of Cys β239. (2) Among the fifteen CSIs, eleven CSIs formed hydrogen bonds with the nitrogen atom of Val α179. (3) Hydrogen bonds with the nitrogen atoms of Ala β248, Asp β249 and Leu β250 were found in eight CSIs. (4) One hydrogen bond was formed with the oxygen atom of Thr *α*179 in four CSIs. Then a common pharmacophore model was constructed by using the binding models of these compounds. The consistent structural features and recurring tubulin-ligand interactions were the basis for the pharmacophoric points. Thus, the different structural classes of CSIs were proposed attributing to a seven-point pharmacophore consisting of three hydrogen bond acceptors (A1, A2, and A3), one hydrogen bond donor (D1), two hydrophobic centers (H1 and H2) and one planar group (R1) (Fig. **2**). These points could be distributed in two planes with an angle of about 45°. (Fig. **2**) also presented the calculated distances to imply the interactions between the pharmacophore groups and tubulin. Group H1 (generally consists of the carbon atom of the methoxy group) was located between Val α181 and Met β257. Group H2 (generally are the aromatic ring) performed a hydrophobic contact with Leu β255, Ala β316, Val β318 and Ile β378. Besides, it showed six potential hydrogen bonds: A1 with the amide hydrogen atom of Val α181 (3.3-4.6 Å); A2 with the thiol hydrogen atom of Cys β239 (3.2-4.2 Å); A3 with the amide hydrogen atoms of Ala β248, Asp β249 and Leu β250 (3.9-6.4 Å); D1 with the carbonyl oxygen atom of Thr α179 (3.0-4.9 Å). The authors also concluded that groups A2, H2 and

Fig. (1). Selected CSIs for identification of a common pharmacophore.

R1 were common to all of the selected CSIs, while H1 occurs in fourteen drugs. This suggested that A2, H1, H2 and R1 might be essential features for activity. The hydrogen bond between A2 and Cys β239 was presented in all structures, and the hydrogen bond between A2 and Val α181 was formed in most structures, which also indicated the importance of these two hydrogen bonds.

All of the above results extend our understanding of the binding interactions at the colchicine site and provide an explanation for many SAR data of CSIs. It would encourage and guide researchers to find more active compounds through definite modifications of the known ligands.

COLCHICINE BINDING SITE INHIBITORS

1. Colchicines

The total synthesis of colchicine (**1**) was first achieved in 1959 and now various synthetic strategies have been reported in literature [32]. Colchicine is consisted of three rings, a trimethoxy benzene ring (ring A), a methoxy tropone ring (ring C) and a seven-membered ring (ring B) with an

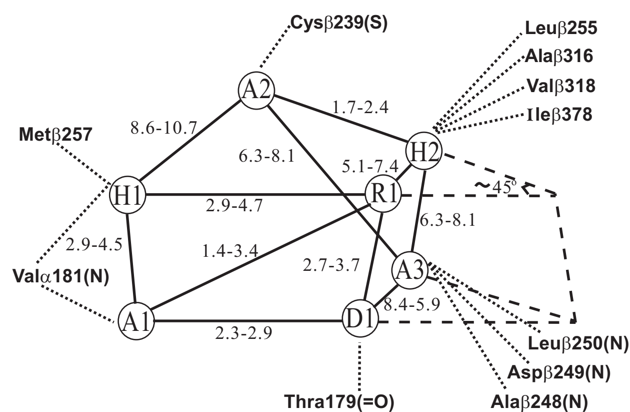


Fig. (2). Common pharmacophore of CSIs. The distances between the pharmacophoric points are given in Angstroms.

acetamido group located at its C7 position. Although it is one of the oldest antimitotic drugs, its therapeutic window is too narrow to be of clinical value. In the past decades, hundreds of colchicine analogues have been isolated or prepared in order to search for more active compounds with low toxicity. Among them, some promising ones are currently under clinical trials as antitumor agents. The SAR of this series is concluded in (Fig. **3**).

The trimethoxyphenyl group of colchicine serves as a stabilizer for the TC-complex. It consists an essential pharmacophore for tubulin affinity in conjunction with ring C and is essential for antitubulin activity [33]. It was found that the 3-methoxy group was important for binding ability. Replacing it with bulky groups resulted in great reduction of the affinity [34]. And 1-methoxy group might be important in setting the correct conformation of the molecule [35].

As mentioned above, the tropone ring of colchicine is also crucial for colchicine-tubulin interaction [36-39]. This part of colchicine can easily undergo photochemical decomposition and transform into a fused four and five-membered ring having carbonyl and methoxy substituents [36]. This change leads to reduced binding ability. It was also reported that rearrangement of the bond system led to inactive compound [34]. The same result was found when exchange the relative position of the methoxy and carbonyl group to get isocolchicine (**2**) [37-39]. The 10-methoxy group can be replaced with halogen, alkyl, alkoxy or amino groups without affecting tubulin binding affinity, while steric bulk substituents decrease the activity [40, 41]. In addition, changing the seven-membered tropolone into an aromatic phenol doesn't impact affinity with tubulin. For example, allocolcichine (**3**), a natural product, is also a tubulin inhibitor [42-44].

It is believed that the seven-membered ring B and its C7 side chain are not crucial for tubulin binding ability. But they may affect the conformation of colchicine analogues and their tubulin binding properties such as on-rate, off-rate, activation energy reversibility and quantum yield of the drug-tubulin complex [35]. Thus, modification on ring B is mainly aimed to modulate the kinetic properties of the compounds [45]. It is known that introducing a double bond into the ring B improved binding ability with tubulin [46]. Conversing the configuration of C7 leads to the unnatural 7*R*-colchicine (**4**) without any tubulin binding activity [47]. And replacing the substituent at 7 position is tolerated and results in a number of active compounds [48, 49].

Korde *et al.* [50] reported some ^{99m}Tc-labeling of colchicine derivatives showed good tumor cell uptake *in vitro*. The tumor-specificity of these agents provided further insight toward their possible utilities for imaging multi-drug resistant states.

A rational design for compounds that included independent tubulin-binding and androgen receptor (AR)-binding moieties lead to the compound CCN (**5**), which bound cyanonilutamide to colchicine through a rigid alkyne linker [51]. It could bind to tubulin and inhibit tubulin assembly with greater potency (1.1±0.1μM) than colchicine (2.2±0.4μM). Besides, it still retained AR-binding activity. It was also found that CCN was more potent in killing androgen-independent prostate cancer cells than the combination of colchicine and cyanonilutamide.

Most of the researches proved the isocolchicine analogues being no activity on tubulin inhibition. Nevertheless, 7-NH-dansyl-isocolchicine (**6**) was found exhibiting certain activity recently. Its improved tubulin affinity was related to the binding of ring A and B with tubulin, while the ring C remained inactive [52]. Additionally, an investigation of connecting a sugar moiety to the colchicine scaffold led to colchicine neoglycoside (**7**) with physiologic effect different from that of the colchicine alkaloids but similar with that of taxane which acted by stabilizing tubulin formation [53].

Recent years, Büttner *et al.* [54, 55] synthesized allocolchicinoid derivatives and discussed the effects of the modification on ring B. It was showed that expansion of ring B decreased the antitubulin activity, probably due to the decrease of the torsional angle between the planes of rings A and C. All derivatives with a substituent at C5 were inactive. Annulated heterocyclic ring systems, such as tetrazole (**8**), to the ring B obtained inhibitors possessing equivalent potency to that of colchicine. Ring size and substitution pattern of the annulated heterocycles seemed to have a no remarkable impact on activity.

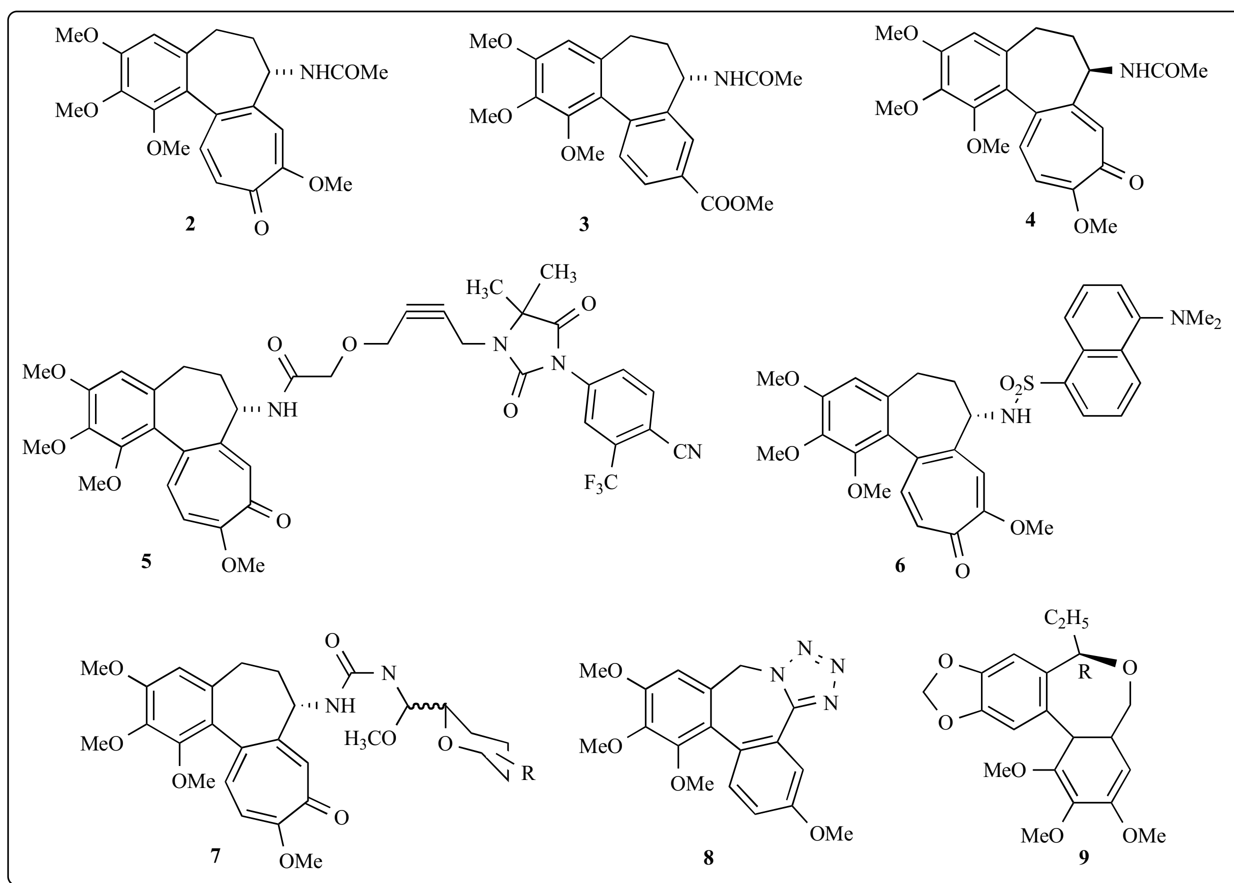
Joncour *et al.* [56] described an asymmetric synthesis of biaryl hybrids of alloclochicine and steganacin, a podophyllotoxin analog, containing a seven- or eight-membered heterocyclic medium ring. Compound (**9**) displayed the most potent antitubulin activity, as it was approximately two-fold active compared with that of colchicine and showed interesting cytotoxicities (0.70~3.5 μM).

2. Podophyllotoxins

Podophyllotoxin (**10**) was isolated from dried roots of *Podophyllum peltatum* and related species [57]. It is a potent antimitotic agent inhibiting microtubule by binding to the colchicine site at submicromolar concentrations. Although its clinical trial failed partly due to its toxicity, the excellent cytotoxicity of podophyllotoxin leads to its investigation as an anticancer agent, and many natural or synthetic analogues have been studied. The SAR study of podophyllotoxin derivatives is more complicated for another potential mechanism contributed to their cytotoxicity, topoisomerase II (TOP II) inhibition. However, inspecting the extensively published data of podophyllotoxin analogues, a good understanding of the SAR could be obtained [58, 59] (Fig. **4**).

The dioxolane moiety (ring A) is essential for the cytotoxic activity [60, 61]. Opening of the ring A and giving rise to one or two hydroxy groups decrease cytotoxicity. Based on the common pharmacophore, it may be attributed to its violation of favorable hydrophobic interactions [58].

Aromatization or opening of the ring C leads to compounds with low or no activity, probably due to the fact that ring E is no longer positioned properly. The C4-hydroxyl is not fundamental. Removing it doesn't impact its cytotoxic property [62]. Additionally, moving the hydroxyl to C5 position retains its antitumor activity [63]. Magedov *et al.* [64] have replaced carbon atom at 4 position with nitrogen atom leading to the dihydropyridopyrazole analogues of podophyl-



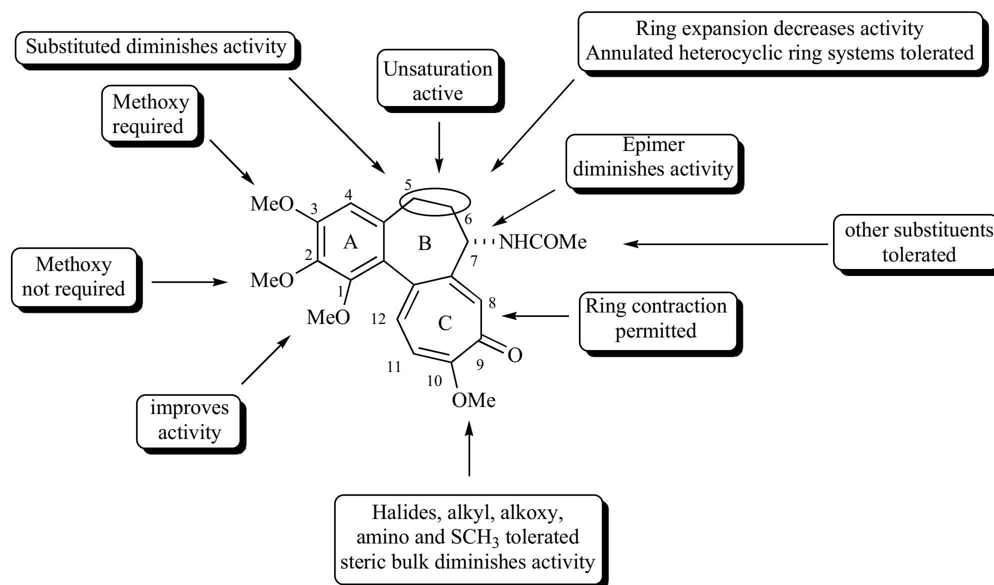


Fig. (3). SAR of colchicine analogues.

lotoxin. This novel heterocyclic scaffold represents the most significant structural departure from that of the natural lead compound and retains considerable portion of its cytotoxicity. However, inversion of the C4-hydroxyl leads to epipodophyllotoxin which could be glycosylated to get etoposide, possessing TOP II inhibition instead of antitubulin activity [65].

The stereochemistry of the C1 position is important in keeping the correct position of ring E. The enantiomer is inactive. Removing the methyl of the 4'-methoxyl has little impact for its ability to tubulin inhibition, while replacing it with bulky groups decreases its activity [66]. In contrast, the methyl of the 4'-methoxyl is important to the TOP II inhibition. It has been found that 4'-methylation removes the TOP

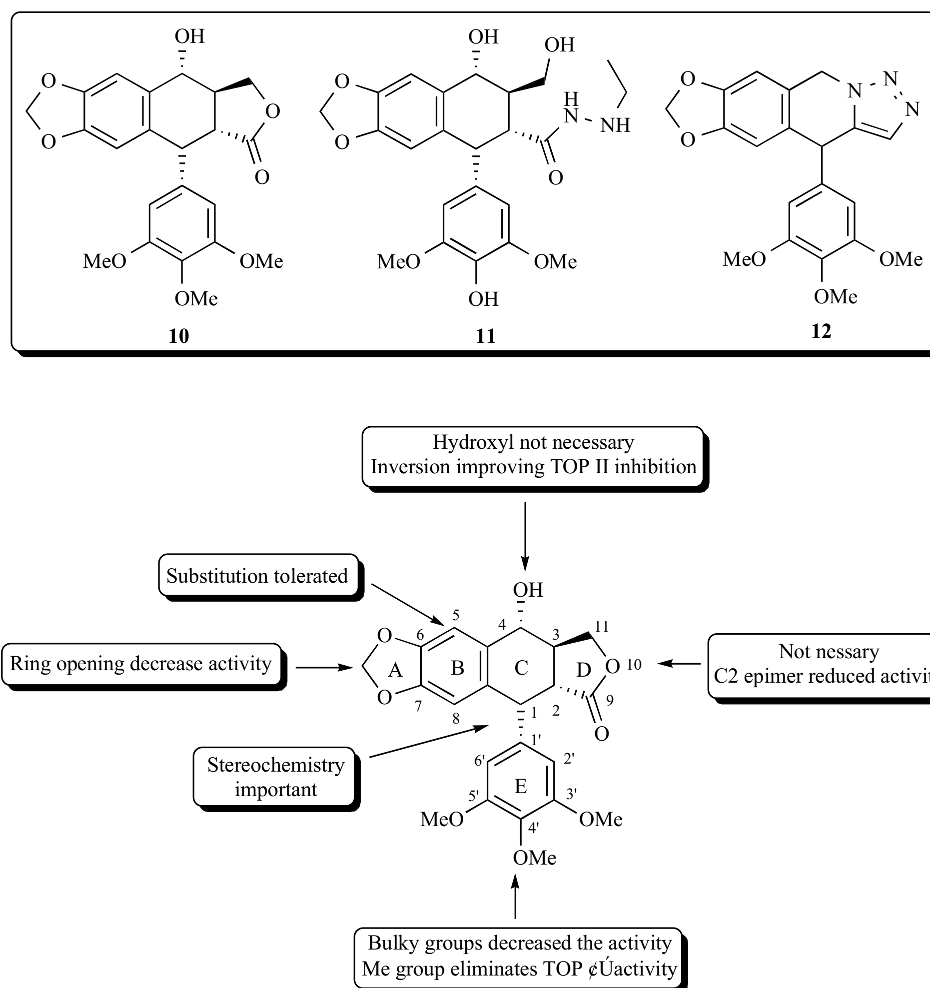


Fig. (4). SAR of podophyllotoxin analogues.

II activity, which may lead to the rational design of selective compounds [67].

The lactone moiety itself is not necessary [59]. Indeed, opening of the lactone with hydrazine yielded the podophyllic acid hydrazides (**11**) used clinically as an anticancer agent. But the configuration of the *trans* fused lactone is crucial. When it undergoes epimerization to the more thermodynamically stable C2 epimer, cytotoxicity and antitubulin activity are significantly reduced [58, 68-70]. Moreover, the fundamental quasi-axial position of the trimethoxyphenyl group to the tetracyclic core can be retained only when lactone is in a *trans*-conjunction. Considering of the thermodynamic instability of lactone, Imperio and coworkers [71] replaced this ring with a triazole moiety and obtained compound (**12**) which retained antitubulin activity. Although it was less potent than podophyllotoxin, the present structures might provide a good platform for the fast generation of metabolically stable compounds.

3. Combretastatins

Combretastatin A-4 (CA-4, **13**) is a natural product isolated by Pettit and coworkers from the bark of the South African bush willow tree *Combretum caffrum* in 1982 [72]. It is the most potent compound in combretastatins family [73], as well as one of the most potent inhibitors of colchicine site [74]. CA-4 is not a substrate of the multi-drug resistance (MDR) pump, and exhibits superior activity against MDR positive cancer cell lines [75, 76]. Moreover, it also has been reported to have angiogenesis inhibition which interferes the process essential for tumor growth [77]. For its simple structure and high activity, CA-4 becomes the most representative ligand of the colchicine site and is important in the design of structural diverse CSIs [78, 79]. Modification of CA-4 can be divided into three parts (Fig. **5**).

3.1. Modification on Ring A

Various studies have shown that the 3,4,5-trimethoxy on ring A of CA-4 is indispensable for potent cytotoxicity and antitubulin activity. However, following the further studies during these years, this conclusion has been made up continuously.

Pettit *et al*. [80] introduced different halogens in the 3 position of ring A. The fluoro, chloro, and bromo halocombretastatins were almost equivalent to CA-4 as inhibitors of tubulin polymerization. A 3,4,5-trifluorinated derivative of CA-4 (**14**), first reported by Hadfield and coworkers [81], showed excellent inhibition on tubulin polymerization (IC_{50}=2.9μM). The good biochemical and biological activity

of compound (**14**) established it as a new lead compound among trifluorinated combretastatins. But further study showed that although compound (**14**) inhibited tubulin polymerization at a concentration comparable to that of 3'-amino-CA-4, its cytotoxicity was somewhat diminished [82].

Fortin *et al*. [83] used *N*-phenyl-*N'*-(2-chloroethyl)urea pharmacophore to mimick the trimethoxy phenyl moiety and obtained CA-4-chloroethylurea hybrid derivatives (**15**), which was cytotoxic on tumor cells through its nucleophilic covalent binding to the colchicine site.

Besides, when ring A was replaced by heterocycles, such as naphthalene, the cytotoxic activity was largely decreased [84].

3.2. Modification on Ring B

Structural modifications on the ring B suggest that the 4'-methoxy group is crucial for cytotoxicity, while the 3'-hydroxy group is optional [85-87].

A series of analogues with nitro or serinamide substituents at the C2'-, C5'-, or C6'-position of CA-4 were synthesized and evaluated for their tubulin polymerization inhibition and cytotoxic effects against several tumor cells [88]. Among them, the most active compounds 2'-aminostilbenoid (**16**) and 2'-amino-3'-hydroxystilbenoid (**17**) were potential for further modification and development as vascular disrupting agents for the treatment of solid tumor cancers.

In order to overcome the low solubility of CA-4, a number of prodrugs bearing hydroxy group on ring B have been synthesized [89]. Replacing 3'-hydroxyl by a bioisosteric boronic acid was an alternative approach to improve the solubility of the compounds. The resulting compound (**18**) showed nanomolar cytotoxicity and similar affinity to tubulin as that of CA-4 [90]. A series of compounds bearing CA-4 and the nitrogen mustard cores were also synthesized [91]. All the compounds were cytotoxic and inhibited tubulin polymerization. Joining CA-4 with chlorambucil *via* an ester linkage led to compound (**19**), which was significantly more potent than the original two lead compounds and inhibited tubulin with IC_{50} of 0.64nM. Whereas, when CA-4 was joined with nitrogen mustard *via* an ether linkage or without any linkage, loss of potency was observed.

Besides, plenty of literatures reported the replacement of ring B with various of heterocycles, such as indole [92, 93], thiophene [94], benzo[*b*]thiophene [95], naphthalene [84], tetrahydrothienopyridine [96], benzo[b]furan [97]. Their antitubulin activities could be maintained in most cases.

3.3. Modification of the Linker

It is believed that *cis*-olefin configuration at the bridge is the prerequisite for potent cytotoxicity. It keeps the two aromatic rings in the correct conformation for potency, which appeared to be 66° according to the crystal structures [98]. To retain the *cis*-olefin configuration of CA-4, alternative bridge groups were introduced instead of the double bond, such as ether, ketone, sulfonamide, sulfonate, amine and amide [78, 84, 99, 100]. Recently, Hsieh *et al*. [101] reviewed various CA-4 derivatives and analogues. They classified them into one-atom, two-atom and three-atom bridge-head analogues, and concluded their effects on the pharmacological profiles. Modification on the linker attracted more and more attention during recent decades.

Borrel *et al.* [102] introduced carboxamide and carbamate onto the olefin site adjacent to the ring A in order to improve the water solubility and stabilize the *cis*-configuration of the double bond. Their tubulin polymerization inhibition and cytotoxic effects against several tumor cells were evaluated. The carboxamide series exhibited potential inhibitions on both tests. In contrast, most carbamates were either inactive or displayed only moderate cytotoxicities.

It was demonstrated that it was possible to introduce one or two fluorine atoms on the stilbene double bond of combretastatins without altering their biological properties [103]. The introduction of a fluorine atom, both in R_1 and R_2 (**20**) or only in R_2 (**21**), resulted in a slight reduction of tubulin aggregation, with IC_{50} of 7.3 and 8.8μM, respectively. On the contrary, when the fluorine atom was introduced only on R_1 (**22**), the improvement of tubulin inhibition with IC_{50} of 2.5μM was observed compared with CA-4 (IC_{50}=4.9μM). Besides, *cis*-double bond of CA-4 could also be replaced by a 1,2-diketone unit without significant loss of cytotoxicity and inhibition of tubulin assembly potency [104].

A series of aryl- and aroyl-substituted chalcone CA-4 analogues were prepared and assessed for their tubulin polymerization inhibition. Compound (**23**) inhibited tubulin polymerization at low concentrations (IC_{50}=2.5μM), but did not exhibit antimitotic or cytotoxic effects, which might be promising to be developed as selective vascular disrupting agents [105].

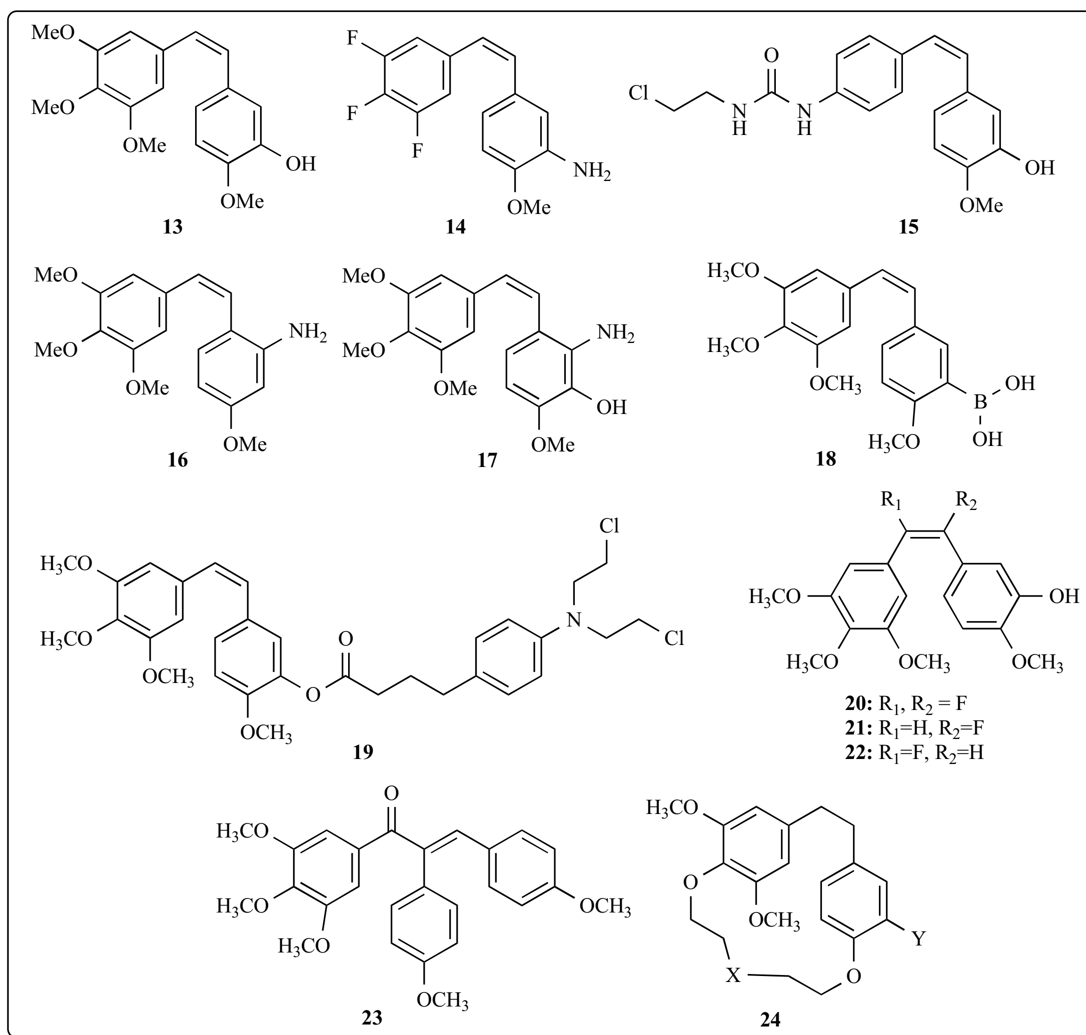
Heterocyclic bridges including indole, pyrimidine, benzopyrazine, pyridopyrazine [106], indazole [107], triazole [92, 108], imidazole [109], imidazol-2-one [110], pyrazoline [111], furazan [112], furan [113], isoxazole, isoxazoline, oxadiazole [114, 115], cyclopentenone [116], maleimide [117], thiadiazole [118] *et al.* were also used to maintain the spatial arrangement of both aromatic systems, resulting in some highly potent and soluble compounds whenever their substitution pattern was close to that of CA-4. Among them, the five-membered rings considered to be the optimal.

Mateo *et al.* [119] designed some new analogues of combretastatins (**24**) in which a macrocycle was formed by linking the *para* positions of both aromatic rings through the corresponding oxygen or nitrogen atom. This restriction generated new *cis*-locked analogues, irrespective of the bridge structure. However, all of them were less potent than CA-4.

4. Arylindoles

An increasing number of synthetic indoles as potent tubulin polymerization inhibitors were reported in the past few years and antitubulin agents bearing an indole as their core nucleus were reviewed recently [120]. For the majority of this series of compounds, experimental data were consistent with the inhibition of tubulin polymerization by binding to the colchicine site.

6-Methoxy-3-(3',4',5'-trimethoxybenzoyl)-*1H*-indole (BPR0L075, **25**) was first designed as a heterocyclic analogue of CA-4 [121, 122]. It appeared to be a potent inhibitor



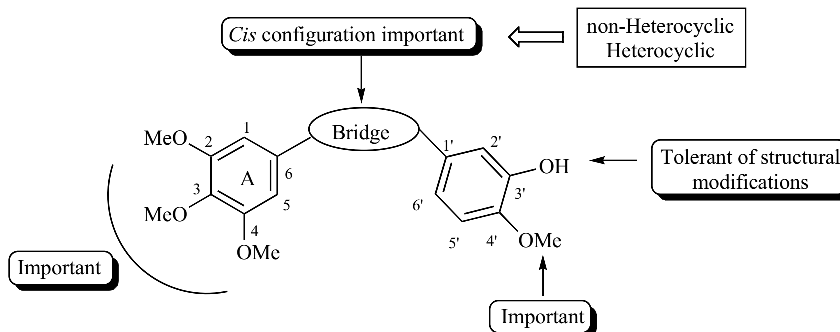


Fig. (5). SAR of CA-4 analogues.

of tubulin polymerization (IC_{50}=3.76μM) and bound tubulin at the colchicine site. It exhibited strong cytotoxic activity (in nanomolar range) against a panel of human cancer cell lines, including multi-drug resistant cells. This compound was also found to exert significant antitumor activity in several human xenografted tumors and was selected for further preclinical development as an antimitotic anticancer drug [123, 124]. Later, introducing a hydroxy group at the 7 position of the indole nucleus and evaluating the influence of this modification on cytotoxic and antitubulin activities were carried out [125]. The resultant compound (**26**) displayed marked morphological activity at nanomolar concentrations on endothelial cells, indicative of potential antivascular activity.

1-Aroylindoline, 1-aroyl-1,2,3,4-tetrahydroquinoline, and 1-aroylindole derivatives were synthesized and evaluated for their anticancer activity [126]. The 4-amino (**27**) and 4-hydroxy-1-aroylindoles (**28**) exhibited antitubulin activity with IC_{50} of 0.9 and 0.6 μM, respectively, superior or compa-

rable to that of colchicine and CA-4. They also showed antiproliferative activity with IC_{50} of 0.3~5.4 nM against a set of human cancer cell lines. The SAR data revealed that the introduction of amino or hydroxyl group at the 4 position of 1-aroylindole series significantly improved activity. This group also described the synthesis and SAR of 4- and 5-aroylindoles as potent antitubulin agents [127]. Among them, compound (**29**) exhibited antiproliferative activity, with IC_{50} values ranging from 10 to 15 nM against a diverse set of human cancer cell lines from different organs, including the MDR positive resistant line (KB-vin10). It also demonstrated more potent antitubulin activity (IC_{50}=1.1μM) than colchicine (IC_{50}=2.9μM) and comparable to that of CA-4 (IC_{50}=1.3μM).

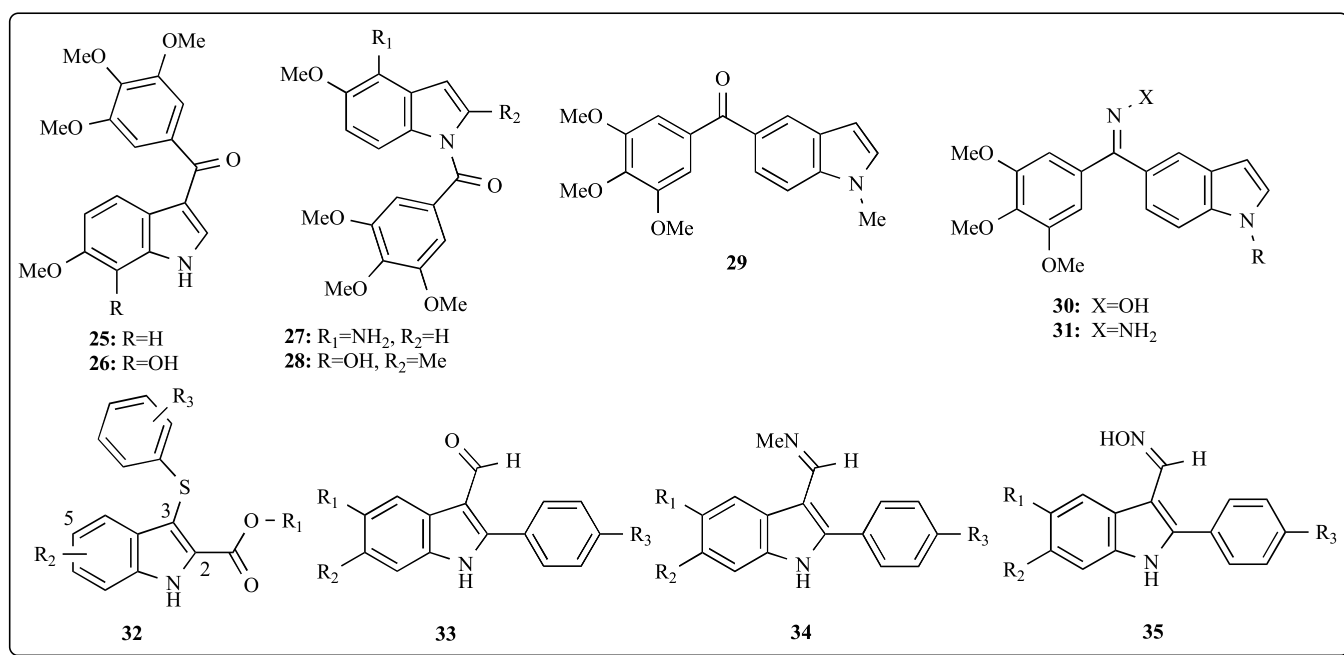
Álvarez *et al*. [128] replaced the ketone with oxime and hydrazone and synthesized a series of compounds with different modifications on the nitrogen atom of the bridge. Several compounds showed potent tubulin polymerization inhibitions as well as cytotoxic activities against cancer cell lines. Diaryloximes (**30**) exhibited more potent tubulin polymerization inhibition than diarylhydrazones (**31**). But their cytotoxicities against several cancer cell lines were lower than those of the lead compound, diaryketones. Other substituents at the imine nitrogen greatly reduced the tubulin inhibitory and cytotoxic activities.

Arylthioindoles (ATIs, general structure **32**) is a new class of potent tubulin assembly inhibitors that bind to the colchicines site [129]. Initial SAR analysis clarified structural requirements for this class of inhibitors [130]. Essential structural features for an active agent included a small-size ester function at 2 position of the indole, the 3-arylthio group, the sulfur atom bridge and a substituent at 5 position of the indole. But recently, the same group designed new ATI derivatives without the ester moiety at 2 position of the indole and found most of the compounds binding to the colchicine site of tubulin in the same orientation as the previously studied ATIs [131]. These new ATI derivatives were also potent inhibitors of tubulin polymerization in comparison with colchicine and CA-4. A halogen atom at 5 position decreased their free energy of binding to tubulin, with concomitant reduction in cytotoxicity, while methyl and methoxy groups at 5 position resulted in more cytotoxic compounds.

2-Phenylindole-3-carbaldehydes (**33**) was an interesting class of compounds with high antiproliferative activity against two breast cancer cell lines. It was showed that tubulin was the primary target of these agents. They inhibited the tubulin polymerization by binding to the colchicine site. However, these aldehydes didn't inhibit the growth of transplanted murine tumors *in vivo*. One of the possible reasons might be the instability of the aldehyde function toward metabolic reactions. Insufficient bioavailability could be another reason. Further modification by conversion to the methylamines (**34**) did not affect the activity in comparison to the free aldehyde, whereas the formation of oximes (**35**) reduced the antiproliferative activity by one order of magnitude [132].

5. Sulfonamides

Sulfonamides is a class of compounds possessing a range of biological activities. This attracted researchers to study them as antimitotic activity. The sulfonamide (**36**) was thus found through *in vitro* antitumor screening [133]. The SAR study of this series indicated that replacement of the methyl group of (**36**) by a methoxy group increased both *in vitro* and *in vivo* activities. The efficiency of substituents at 4 position of the anilino group of (**36**) was hydroxy>hydrogen> methoxy. E7010 (**37**), a 4-hydroxy derivative of (**36**), displayed good activity against a range of human tumors *in vitro*, including MDR expressing resistant cell lines, and was subsequently shown to inhibit tubulin polymerization by binding to the colchicine site [134, 135]. This molecule was orally active with good dose-dependent bioavailability and is



currently undergoing both phase I and phase II clinical trials for nonsmall-cell lung, breast and colorectal cancers [136].

A pentafluorophenylsulfonamide, T138067 (**38**), was reported binding irreversibly to the Cys β239 of tubulin, which was found in the colchicine binding site [137]. It was thought that thiol group of Cys β239 interacted with the 4-fluoro atom of the pentafluorophenyl ring. And this molecule had undergone a phase II trial.

Chang *et al*. [138] reported a novel series of 7-substituted indoline-1-benzenesulfonamides which showed excellent activity as inhibitors of tubulin polymerization through binding to the colchicine site of microtubules. The SAR information of this series revealed that the 7-amide bond contributed to a significant extent for maximal activity rather than the carbamate, carbonate, urea, alkyl, and sulfonamide linkers. Besides, the amide bridge with an electron-withdrawing property group, such as 7-aroyl or 7-heteroaryl substitutions could effectively improved activity. Compound J30 (**39**) showed potential activities against various resistant and nonresistant cancer cell lines *in vitro*. Further studies indicated that the J30-mediated apoptotic signaling pathway depended on caspases and mitochondria. Oral administration of J30 significantly inhibited tumor growth in nonobese diabetic/ severe combined immunodeficiency (NOD/SCID) mice bearing human oral, gastric and drug-resistant xenografts. The results suggested that J30 had potential as a chemotherapeutic agent for treatment of various malignancies [139].

Numerous substituted tricyclic sulfonamides, with a thiadiazepine skeleton as constraint analogues of E7010, were prepared and evaluated, leading to the identification of potent and original cytotoxic antimitotic agents. The SAR showed that the phenylethyl chain substituted on the pyridine core of benzopyridothiadiazepine tricyclic moiety was more beneficial than on the seven-membered ring. Compound (**40**), one of the most active compound in this series, inhibited L1210 leukemia cell proliferation in the submicromolar range and inhibited tubulin polymerization in the micromolar range [140].

6. 2-Methoxyestradiols

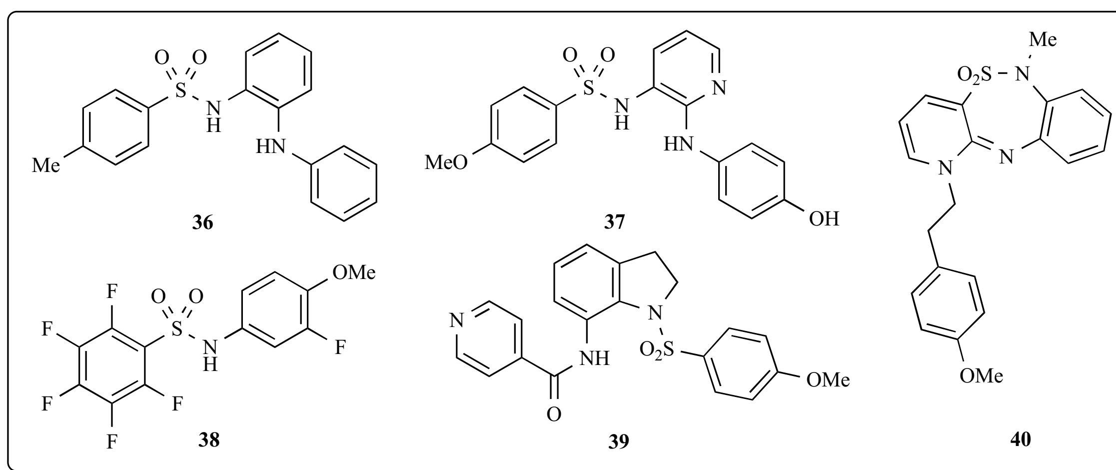
2-Methoxyestradiol (2ME2, **41**) is a natural microtubule inhibitor that exhibits antiproliferative activity against a wide range of tumor and endothelial cell types *in vitro*, as well as significant antiangiogenic and antitumor activities *in vivo*. Although not fully characterized, 2ME2 shows to bind to the colchicine site of tubulin and disrupt microtubule polymerization in cells, resulting in G2/M cell cycle arrest and the induction of apoptosis [141, 142]. Unlike many antitubulin agents, 2ME2 is not a substrate for multi-drug resistance pumps [5, 143]. It has minimal toxicity at its pharmacologically active doses [144, 145] and is well tolerated in preclinical and clinical studies [146]. In marked contrast to most of other antitubulin drugs, neurotoxicity or myelosuppression was not arised by treatment with 2ME2 for more than five years [147-150].

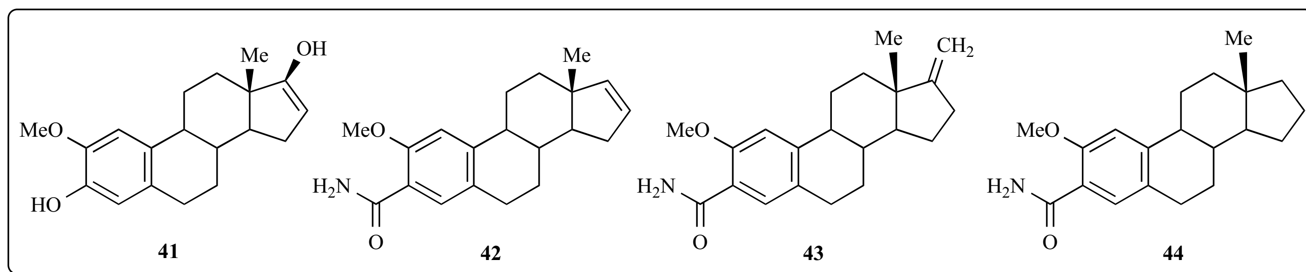
Clinical studies in humans and *in vivo* studies in rodents have shown that orally administered 2ME2 is metabolized mainly by the processes similar to that of estradiol and other steroid hormones, with conjugation at 3 and 17 positions as well as oxidation at 17 position to form 2-methoxyestrone. A strategy to improve potency and metabolism of 2ME2 was modifying the two hydroxy groups. Thus analogues of 2ME2 with substituents at the metabolically active 3 and 17 positions were synthesized [151]. Several compounds with single or combined substitutions at 3 and/or 17 positions showed similar or improved antiproliferative activities and further reduced estrogenicity as compared to 2ME2. In this series, three lead compounds, ENMD-1198 (**42**), ENMD-1200 (**43**), and ENMD-1237 (**44**), were selected for further studies. Pharmacokinetic studies showed that oral administration of these compounds resulted in increased plasma levels compared with that of 2ME2. And ENMD-1198 was selected as the lead molecule in this series and is currently in a phase I clinical trial in patients with refractory solid tumors [152].

7. Flavinoids

A binding model of chalcone (**45**) with colchicine site was proposed recently [153]. Based on the molecular docking study, several chalcone derivatives were found to be tubulin inhibitors and displayed cytotoxic activities. Among them, compound (**46**) was the most promising compound and inhibited tubulin polymerization with IC_{50} of 15.7 μM.

2-Phenylquinolin-4-one derivatives (2-PQs, general structure **47**) are natural or synthetic small molecules known for their high antimitotic activities. They are structurally derived from the flavone nucleus by isosteric substitution of





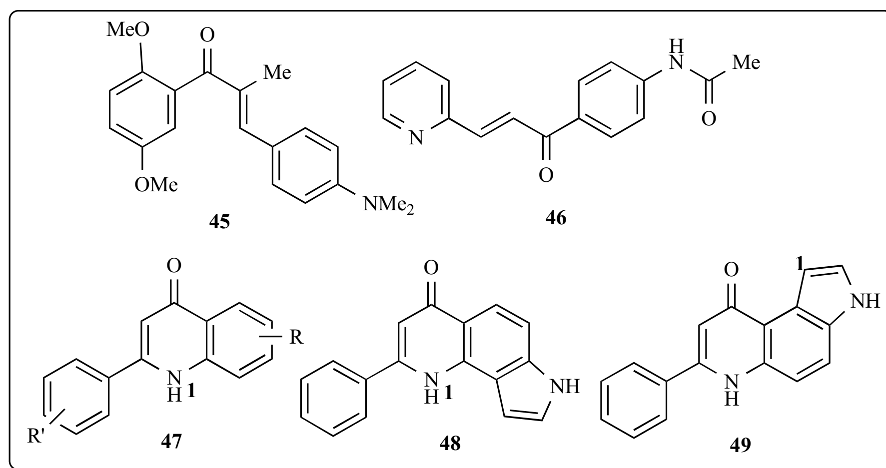
the pyran oxygen with a nitrogen atom. In comparison with flavone derivatives, 2-PQs showed higher antiproliferative activity through a more specific mechanism involving inhibition of tubulin polimerization by interaction with the colchicine binding site [154-157]. Recently, as a further development of 2-PQs, two series of phenylpyrroloquinolinone (PPyQ) derivatives, 2-PPyQs (for example, **48**) and 7-PPyQs (for example, **49**) were synthesized and studied by Ferlin *et al.* [158, 159]. The SAR revealed that the 7-PPyQs (IC_{50}=0.4~0.8μM) were more active and wide-spectrum than 2-PPyQs (IC_{50}=0.7~10μM). Then, a novel series of 3-alkyl-substituted 7-PPyQs were synthesized with the aim to optimize the cytotoxic activity of 7-PPyQs [160]. It showed that introducing a lipophilic group at the *N*-3 position of pyrrole ring, generated potent compounds, with IC_{50} values up to 100-fold lower than the previous compounds. Moreover, the introduction of longer side chains was more active than bearing a methyl group, while substituted with a bulky group (cyclopropylmethy) being the most cytotoxic.

8. Others

In recent years, several other new structural classes of CSIs have been reported.

Zune *et al.* [161] reported the potent antitumor activity *in vitro* of 10-[(3-hydroxy-4-methoxy-benzylidene)]-9(10*H*)-anthracenone (**50**), a potent colchicine site binder with IC_{50} of 0.67 μM against tubulin polymerization and 20 nM against K562 cell growth. In continuation of structural modification studies concerning the anthracenone core obtained 9-benzylidene-naphtho[2,3-*b*]thiophen-4-ones (**51**). The most active compound in this series strongly displaced radiolabeled colchicine from its binding site in tubulin in competition experiments, showing IC_{50} values virtually 3- to 4-fold lower than that of colchicine. Later, this group synthesized another series of sulfonate derivatives (**52**) [162] and identified several representatives of this series to be highly potent antiproliferative agents and inhibitors of tubulin polymerization. The SAR indicated that a methoxy group substituted at the phenyl ring played an essential role to exhibit potent antitubulin and antiproliferative activities, while electronic or lipophilic factors account for the loss of activity. They believed this structural class of compounds was attractive for further structural modifications and the findings might provide useful information for the design of novel antitumor agents.

In the view to identify novel microtubule polymerization inhibitors, Lisowski *et al.* [163] described an unknown heterocyclic family: thienopyrrolizinones or tripentones. The first hit in that series, NSC 676693 (**53**), detected by the National Cancer Institute (NCI), exhibited an cytotoxic activity *in vitro* in submicromolar concentrations against all tested cell lines. The SAR indicated that the 3-phenyl group bearing small substituents at its *meta* and *para* position was essential for cytotoxicity. However, linking aryl groups at 2 position seemed unfavorable. Thus, a new lead (**54**) substituted at 3 position with a 4-methoxy-3-hydroxyphenyl group was discovered. Its cytotoxicities over a panel of tumoral cell lines were in nanomolar range [164]. According the results of preliminary flow cytometric studies, tripentones were first supposed to interact with the mitotic spindle. Tubulin polymerization inhibitory test was then performed and showed an IC_{50} of 2.9 μM, similar to that of deoxypodophyllotoxin (2.4μM). However first *in vivo* evaluations by the NCI were disappointed and indicated the insufficient bioavailibility



was due to a lack of solubility in physiological conditions[165]. Aiming to deal with solubility and specify the structural requirements, Rochais *et al*. [166] extended the study toward synthesis of novel tripentones based on other heterocyclic rings such as pyrrole, and found NSC 733670 (**55**) was the most active derivative. But it remained less active than the first hit in the thiophene series (**53**). Guo *et al.* [167] focused on the modification of 2, 3, and 8 positions on tripentone scaffold and obtained derivatives (**56-59**). The SAR indicated the *O*-substituted oximino-pyrrolizines (**57-59**) exhibited more potent antitumor activities than the corresponding oximes (**56**) in most cases. Morpholine substituted at the amino alkoxy moiety of 8 position dramatically decreased the activity, while the pyrrolinyl showed stronger activity than other amino analogues. Besides, oxidation of the sulfur atom to sulfoxide or sulfone was found to be detrimental to the antitumor activity.

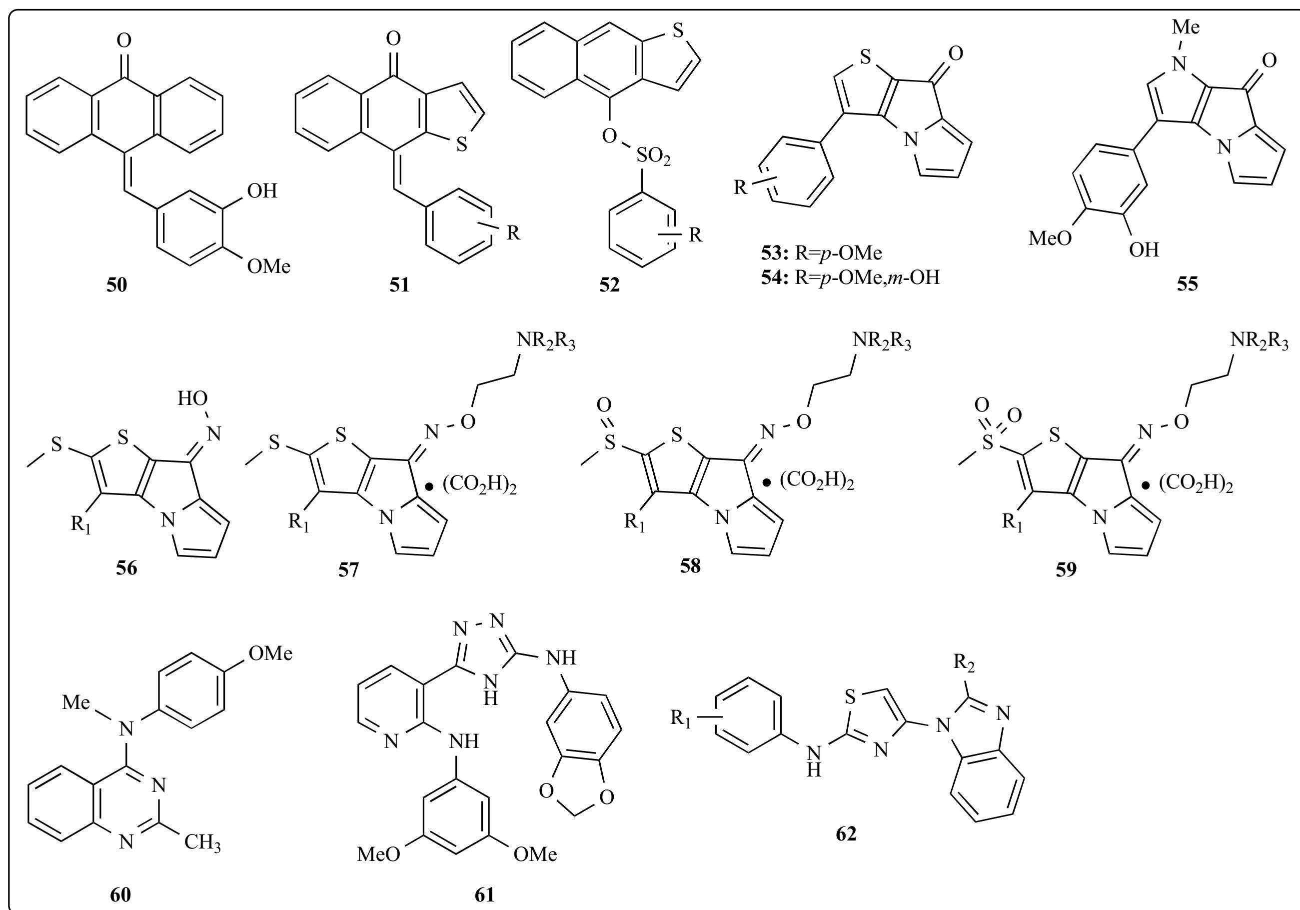
A novel series of 4-arylaminoquinazolines were identified from a cell-based screening assay as potent apoptosis inducers [168]. Through SAR study, MPC-6827 (**60**) was discovered as a proapoptotic molecule and mitotic inhibitor with IC_{50} value at low nanomolar concentrations against multiple tumor cell lines. These studies also indicated that MPC-6827 inhibited tubulin polymerization effectively, competed with colchicine binding, and disrupted the formation of microtubules in a variety of tumor cell lines, which supported the conclusion that tubulin was its target. In addition, it was not a substrate for ABC MDR transporters *in vitro*, and displayed a broad spectrum of antitumor activity.

A new class of ligands of the colchicine site was found among substituted triazoles [169, 170]. In this class, compounds containing dioxolane fragment (for example, **61**) exhibited the highest activity. It was hypothesized that the binding of compound (**61**) to tubulin was similar to that of podophyllotoxin.

Mahboobi *et al.* [171] reported a novel series of highly active antimitotic, tubulin interfering agents with a [4-(imidazol-1-yl)thiazol-2-yl]phenylamine type structure (**62**). It also inhibited microtubule polymerization by interfering with the colchicine binding site of β-tubulin.

CONCLUSION AND PERSPECT

From the discussions above, we believe that the colchicine binding site on tubulin is a principal target for anticancer agents. Discovery and development of small molecular CSIs are currently hotspots for academic and industrial groups. Although the first observed CSIs is colchicine, it is not limited to such defined structure since many compounds with various structures can bind in this region. Though no such inhibitors are clinical applicable on cancer therapy, further studies on the structure of tubulin, experimentally supported model of the colchicine site and identification of the common pharmacophore of CSIs will undoubtly promote



the rational design and discovery of the novel, potent, and drug like inhibitors.

ABBREVIATIONS

AR	=	Androgen receptor
ATIs	=	Arylthioindoles
CA-4	=	Combretastatin A-4
CSIs	=	Colchicine binding site inhibitors
DAMAcolchicine	=	N-deacetyl-N-(2-mercaptoacetyl)colchicine
MDR	=	Multi-drug resistance
2ME2	=	2-Methoxyestradiol
NCI	=	National Cancer Institute
NOD/SCID	=	Nonobese diabetic/severe combined immunodeficiency
PPyQ	=	Phenylpyrroloquinolinone
2-PQs	=	2-Phenylquinolin-4-one derivatives
SAR	=	Structure-activity relationship
SCID	=	Severe combined immunodeficiency
TC-complex	=	Tubulin-colchicine complex
TMP	=	Trimethoxyphenyl
TOP II	=	Topoisomerase II

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