Tubulin inhibitors targeting the colchicine binding site: a perspective of privileged structures

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Abstract

The vital roles of microtubule in mitosis and cell division make it an attractive target for antitumor therapy. Colchicine binding site of tubulin is one of the most important pockets that have been focused on to design tubulin-destabilizing agents. Over the past few years, a large number of colchicine binding site inhibitors (CBSIs) have been developed inspired by natural products (NPs) or synthetic origins, and many moieties frequently used in these CBSIs are structurally in common. In this review, we will classify the CBSIs into classical CBSIs and non-classical CBSIs according to their spatial conformations and binding modes with tubulin, and highlight the privileged structures from these CBSIs in the development of tubulin inhibitors targeting the colchicine binding site.

Keywords: microtubule • privileged structures • tubulin inhibitors • colchicine binding site inhibitors • colchicine domain • prodrug.

Microtubules are formed by the association of α- and β-tubulin heterodimers, and serve as important components of the cytoskeleton in eukaryotic cells. They are extremely important in the process of mitosis and cell division, which make them an important target for anti-cancer drugs [1]. Microtubules are not simple equilibrium

polymers, instead they show complex polymerization dynamics which are crucial to their cellular functions. Microtubule targeting agents (MTAs) that stabilize or destabilize microtubule can interfere with these microtubule dynamics, which lead to mitotic block and cell apoptosis [2].

Microtubule targeting agents

Unlike the targets involved in signal transduction and transcriptional regulation, MTAs have not been shown to have a cancer-specific function without affecting normal cells [3]. Even though microtubule is not a disease-causing target, this intervention is promising in cancer therapy because microtubule is essential for tumor cell proliferation regardless the expected adverse effects on normal cells. However, due to the highly rapid proliferation of tumor cells, their dynamic microtubule system is more vulnerable than normal cells, which makes tumor cells more sensitive to MTAs.

MTAs can potently alter microtubule dynamics at low concentrations, and at relatively higher concentrations (10-100 times higher), they can block mitosis and induce cell apoptosis by destabilizing or stabilizing microtubules, based on which they can be divided into two classes including microtubule destabilizing agents and microtubule stabilizing agents [2]. At least three binding sites in tubulin have been identified which can be interfered by MTAs including taxane, vinca alkaloid, and colchicine binding sites. Inhibitors targeting taxane binding sites can stabilize microtubule while those targeting the vinca alkaloid and colchicine binding sites can destabilize microtubule [4]. All these microtubule-binding agents can alter microtubule dynamics, thus their main potential mechanism of action seems to be the specific inhibition of the dynamics of mitotic spindle microtubules. However, the colchicine site binders that destabilize microtubule can also exhibit anti-angiogenesis and vascular disruption activities which are not found in other site binders [5].

These three binding sites in tubulin all have natural ligands with high affinity to the respective binding pocket, which provides many opportunities for the development of anti-cancer agents with potential activities or druggability. However, most of the NPs targeting taxane and vinca alkaloid binding sites, such as paclitaxel and vinblastine, have extremely complex structures. Although total synthesis or semi-synthesis methods have been developed, the structural complexity and low aqueous solubility as well as limited availability of source materials hampered their clinical applications. Besides, multidrug resistance (MDR) and dose-limiting toxicity caused by the established taxane and vinca alkaloid binding sites inhibitors in cancer therapy have also limited their applications. As for the colchicine binding site, natural CBSIs, such as colchicine, combretastatin A-4 and podophyllotoxin, have relatively simpler structures which can be easily modified. Thus, the emerging CBSIs that have the potential to overcome these limitations represent a promising type of anti-tubulin agents, especially those accessible synthetic CBSIs by high throughput screening or rational drug design.

Over the past two decades, many research efforts have been concentrated on developing CBSIs inspired by natural sources or synthetic origins. Drug design

methods including scaffold hopping, bioisosterism, conformation restricting, prodrug strategy or computer-aided drug design have been utilized to overcome the drawbacks encountered during the development of CBSIs. To better understand the common structures of these CBSIs and provide insights into the development of CBSIs in future, herein we review the development of CBSIs from the perspective of privileged structures shared by the CBSIs. The frequently used prodrug strategies to improve the aqueous solubility and bioavailability of CBSIs will be uncovered in this review.

Colchicine domain

Colchicine domain is the generalized naming of colchicine binding site, which comprises a main site (colchicine binding site) and additional neighboring pockets. Massaroti et al. [4] have divided the colchicine domain into three zones including zones 1, 2, and 3. The zone 1 is located at the α subunit interface and surrounded by residues Valα181, Serα178, Metβ259, and Asnβ258. The zone 2 is an accessory hydrophobic pocket located in the β subunit and formed by residues Lysβ352, Asnβ350, Ileβ378, Valβ318, Alaβ317, Alaβ316, Leuβ255, Lysβ254, Leuβ252, Alaβ250, Leuβ248, Leuβ242, and Cysβ241. And the zone 3, which is buried deeper in the β subunit, is formed by residues Thrβ239, Valβ238, Tyrβ202, Gluβ200, Pheβ169, Asnβ167, Glnβ136, and Ileβ4 (Figure 1A).

Owing to the chemical instability of tubulin, the structural information of ligands binding to the colchicine-binding site was not clear until Ravelli et al. first reported the structure of α,β-tubulin complexed with *N*-deacetyl-*N*-(2-mercaptoacetyl) colchicine (DAMA−colchicine, PDB code 1SA0) [4]. This pioneering work illuminated the location of colchicine in the pocket where it prevents curved tubulin from adopting a straight conformation, which inhibits assembly. As shown in Figure 2, the methoxy group in ring A of colchicine forms a hydrogen-bond interaction with Cysβ241 while ring C interacts with the α subunit by a hydrogen bond formed between the carbonyl group of ring C and the backbone of Valα181.

To date, several compounds with diverse structures have been crystallized in the colchicine binding site of tubulin [4, 6, 7], which showed many new and interesting binding modes and provided rationales for the design or discovery of new ligands targeting this domain. Based on the analysis of María-Jesús Pérez-Pérez et al. [8], colchicine domain ligands were classified into classical and non-classical CBSIs according to their spatial orientations. The superposition of these ligands with X-ray crystal structures indicated that classical CBSIs with more globular or butterfly like shape occupy zones 1 and 2, mimicking the colchicine-binding mode. However, non-classical CBSIs with more planar structures tend to locate deeper into the β-subunit making use of zones 2 and 3 (Figure 1B, 1C).

Privileged structures of CBSIs

A vast number of CBSIs with diverse structures have been developed inspired by natural sources and synthetic origins in the past two decades. Many fragments namely privileged structures shared by these CBSIs are frequently found in the construction of molecules with potent tubulin polymerization inhibition and anti-proliferative

activities. Many of these privileged structures could be interchanged with retained or better biological activities, thus some experiential rules may be applied to develop novel CBSIs. To better understand the tremendous research results achieved in last two decades and provide rationales for the development of novel CBSIs in the future, herein we summarize these privileged structures of both classical CBSIs and non-classical CBSIs, and the privileged prodrug forms are also highlighted.

Classical CBSIs

Typically, this class of CBSIs characterized with a " aromatic ring–bridge–aromatic ring" scaffold mostly has globular or butterfly like shape forming a specific spatial conformation so that they can be accommodated into the binding pocket. As depicted in Figure 1B, these ligands locate ring A in zone 2, ring B in zone 1 and the bridge in the space between α and β tubulins. In this section, privileged structures of ring A, ring B and bridge will be discussed, respectively.

Ring A of classical CBSIs

The trimethoxyphenyl moiety that derived from natural occurring CBSIs such as colchicine, combretastatins, podophyllotoxin is one of the most important fragments regarded as ring A of classical CBSIs. It plays a crucial role in interacting with tubulin, nevertheless, many other structures have also been discovered to replace the trimethoxyphenyl with retained or better biological activities. Due to the relatively ample space of zone 2, ring A of classical CBSIs can tolerate variations. However, the hydrophobic character of this cavity and a critical Cysβ241 residue determine whether these units are suitable. Thus, these moieties are generally hydrophobic or can form specific interactions such as hydrogen bond or covalent bond with residues in zone 2.

(1) Trimethoxyphenyl

The most remarkable privileged structures as ring A is trimethoxyphenyl. Luduena and Roach evaluated the binding affinities of several compounds bearing 3,4,5-trimethoxyphenyl fragment (trimethoxy benzaldehyde, trimethoxybenzylalcohol) with tubulin and found that these fragments can inhibit the binding of colchicine to tubulin at micromole concentrations, suggesting that this moiety can solely act as weak CBSI occupying zone 2 without ring B and bridge [9]. To date, hundreds of CBSIs comprising the trimethoxyphenyl moiety have been developed and some of them are investigated in clinical trials (Figure 3A).

Colchicine (**1**), the first discovered tubulin destabilizing agent extracted from the poisonous *meadow saffron Colchicum autumnale L*, has been the powerful antimitotic agent to study the tubulin target. Even though the dose-limiting toxicity of colchicine restrains its application as an anti-cancer agent, it exerts critical roles in this field since it innovated the subsequent CBSIs developments. The trimethoxyphenyl moiety of ring A of colchicine plays a crucial role in tubulin binding, it binds to the hydrophobic pocket by a hydrogen bond with Cysβ241 residue (Figure 2). Insertion of a bulky group in ring A or replacement of trimethoxyphenyl caused loss of activity [10]. The tropolone ring of ring C in colchicine skeleton is also a key structural moiety for its binding to tubulin, which can also be substituted by other similar

structures. ZD6126 (**2**) with hexatomic ring replacing tropolone of colchicine was developed by AstraZeneca, it exhibits potential anti-angiogenesis and antineoplastic activities, however, the clinical study in phase II was terminated due to the apparent cardiotoxicity at pharmacological doses [11, 12].

Despite the remarkable biological activity of colchicine in microtubule depolymerization, its undesirable action such as toxicity, multidrug resistance (MDR) has stimulated the exploration of new CBSIs with improved anti-cancer activity and low systemic toxicity. However, the development of CBSIs has been standstill until another impressive natural CBSI bearing trimethoxyphenyl moiety was discovered. Combretastatin A-4 (**3**, CA-4), a natural *cis*-stilbene derivative isolated from the bark of the African willow tree *Combretum caffrum*, is a typical tubulin inhibitor binding to the colchicine binding site and possesses a simpler and easier synthesized structure compared to colchicine [13]. Combretastatin A-4 has strong cytotoxicity against a variety of tumor cells with a broader therapeutic window, thus it has initiated the discoveries of a large number of combretastatin analogues for overcoming its isomerization to the less active *trans*-form. Some prodrug forms or analogues of CA-4 have already been evaluated in clinical trials, such as CA-4P (**4**), Oxi4503 (**5**), AVE8062 (**6**), Phenstatin (**7**), CC-5079 (**8**), BCN-105P (**9**), CDK-516 (**12**) **(**Figure 3A**)**.

The trimethoxyphenyl, *cis*-olefin and *para*-methoxyphenyl moieties on the ring B are the key components of combretastatin structure, and trimethoxyphenyl group exerts the most dominant effects on modulating the pharmacological properties [14]. Molecular modelling methods showed that this motif in CA-4 resembles the binding mode of colchicine though tubulin–CA-4 complex has not been reported so far [15]. The modifications of CA-4 analogues have been focused on the ring B and the bridge while the trimethoxyphenyl moiety is generally fixed, these contents will be detailed in the corresponding sections.

Podophyllotoxin (**10**), extracted from dried roots of *Podophyllum peltatum*, was also found to inhibit microtubule assembly, it competitively inhibits the binding of colchicine to the colchicine binding site [16]. The antimitotic properties of podophyllotoxin are similar to those of colchicine possibly because of the overlap of their binding sites. However, these molecules do not show complete overlap in the binding site. The trimethoxyphenyl moiety of colchicine and podophyllotoxin have different binding modes in the pocket of tubulin [17]. The trimethoxyphenyl of podophyllotoxin is critical to the microtubule depolymerization activity while the 4'-demethylation of trimethoxyphenyl leads to the loss of anti-tubulin activity, which can be exemplified by etoposide (**13**) and teniposide (**14**). Both have a different mechanism of interfering with topoisomerase II and causing DNA damage [18], because the oxidative metabolism of 4'-phenol gives 3', 4'-catechol derivatives which can be further oxidized to 3', 4'-ortho-quinone. These two structures cause DNA damage through forming free radicals or covalent bonds between the quinone and the DNA [19] (Figure 3B). This case also strengthens the significance role of trimethoxyphenyl moiety as a privileged structure in maintaining tubulin binding ability.

(2) Nitrogenous heterocycles

Nitrogen heterocyclic moieties are often present in privileged structures that are mostly found in drugs. Replacing methine (CH) of benzene by nitrogen atom generates nitrogen containing heterocycles, such as pyridine or pyrimidine which are important structures in medicinal chemistry. The introduction of nitrogen atom greatly improves the basicity of the system due to its basic character, furthermore, the protonation of nitrogen turns it into a strong hydrogen bond thus it provides additional hydrogen bonding interaction. Another important property of nitrogen-containing heterocycles is polarity which can be used as a mean of reducing the lipophilic character and improving water solubility and oral absorption. These heterocycles have been applied in the construction of CBSIs [20]. Herein, we mainly focus on the substitution of trimethoxyphenyl with these heterocyclic structures which can interact to the zone 2 of colchicine binding pocket.

In 2008, Nilantha Sirisoma firstly reported some aminoquinazolines endowed with potent cytotoxic effects, compounds **15** and MPC-6827 (**16**) showed the most potent cytotoxicity against multiple tumor cell lines with IC_{50} values of 2-10 nM [21]. Afterwards, they proposed and validated tubulin as the main target of these compounds [22]. Early clinical trials of MPC-6827 was carried out for patients with advanced cancer [23], however, this promising candidate has been discontinued by Myrexis Corporation in order to reallocate resources towards advancing lead candidates from earlier stage programs.

Aleem Gangjee et al. also discovered some similar structures, compound **17** exhibited excellent anti-proliferative activity in searching for receptor tyrosine kinase (RTK) inhibitors [24]. However, mechanism studies validated that **17** was not a RTK inhibitor but tubulin binding agent. Studies of the structure-activity relationships (SARs) suggested that N-methyl and 4-methoxy groups appear important for the potent activity [25]. Recently, compound **18** with a furo[2,3-*d*]pyrimidines structure exhibited potent activity with GI_{50} values less than 10 nM in a panel of cancer cell lines, the biological effects of **18** was identified as a novel, potent microtubule depolymerizing agent [26].

Studies independently researched by Xiao-Feng Wang et al.[27] afforded a similar compound **19** [28, 29], it showed extremely high cytotoxicity against a panel of human tumor cell lines with GI_{50} values ranging from 1.5 to 1.7 nM. Extensive SARs studies on the tetrahydroquinoline moiety have found that six or seven membered ring was desirable for activity. Compound **20** showed a comparable activity to **19**, both of them strongly inhibited colchicine binding to tubulin indicating that they can interact with the colchicine binding pocket of tubulin as CBSIs [30].

All these cases show that aminoquinazoline fragment is a privileged structure utilized in the design of CBSIs, and N-methyl, 4-methoxy groups are essential for potent activity. However, recent work carried out by Mouad Alami et al. affording **21** and **22** showed that N-methyl and aminoquinazoline could be respectively replaced by olefin and 2-methyl quinoline with more potent activity [31, 32]. Molecular modelling experiments illustrated that 2-methyl quinoline interacts with zone 2 of colchicine domain by a hydrogen bond between N-1 and Cysβ241 residue, resembling a similar

positioning with trimethoxyphenyl of IsoCA-4 [32]. These interesting results testify this new 2-methyl quinoline privileged structure could replace the traditional trimethoxyphenyl group, which may provide a new direction for discovering structurally novel CBSIs with potent activities.

(3) Dimethylbenzopyran

Dimethylbenzopyran motif is frequently found in secondary metabolites isolated from natural sources, many NPs containing this fragment exhibit a wide range of promising biological activities [33]. Millepachine (**23**) (Figure 5), first isolated from the *Millettia pachycarpa* by Lijuan Chen's group [34], was found to have potent cytotoxicity against a variety of human cancer cells. Modifications of millepachine have led to the discovery of many derivatives with more potent activity, such as **24** [35], **25** [36], **26** [37], **27** [38]. These structures were found to be tubulin polymerization inhibitors by binding at the colchicine site. Among these compounds, **27** showed higher anti-tubulin polymerization activity than colchicine, the bioavailability of the hydrochloride salt of **27** was up to 47%, and it significantly inhibited tumor growths in four xenograft models including resistance-tumor bearing mice models without causing significant loss of body weight [38]. All these results indicate that **27** can be developed as a promising new orally active anti-cancer agent, and it is worth mentioning that **26** and **27** possess a structural similarity with the chalcones type compound **85** .

(4) Covalent privileged structures.

Irreversible inhibitors also represent an important class of CBSIs, they irreversibly bind into the colchicine binding pocket by covalent bonds formed with some specific residues. These binding modes may circumvent drug resistance caused by mutations of tubulin residues and trigger microtubule disruption.

Urea moiety is one of the most common structures of covalent CBSIs, and it can be found in the structures of N-Phenyl-N'-(2-chloroethyl)urea (CEU) (**28**) and its analogues (**29-31**) (Figure 6). These anti-tubulin agents can acylate Gluβ198 that is a residue located in a pocket adjacent to the colchicine binding site [39, 40]. Urea moiety of CEU could be a bioisostere of the trimethoxyphenyl, which has been validated by the hybrid compound **32** with potent anti-proliferative activity at micromolar level [41]. Gaudreault's group reported the cyclisation of the CEU moiety into the corresponding phenylimidazolidin-2-ones (IMZs) **33** [42], leading to a new class of anti-tubulin agents. *Cis*-olefin moiety can also be replaced with sulfonate or sulfamide groups as the bridge, thus the obtained compounds **34** [43] and **35** [44] exhibited potent biological activities. However, these IMZs with steric hindrance can prevent the IMZ moiety from binding to the adjacent pocket behind colchicine binding site, thus they may take a different positioning from that of **32**. Molecular modeling experiments showed that the IMZ moiety might mimic the tropolone or the vanillin moieties of colchicine and CA-4, respectively to stretch into zone 1 of the pocket[45], which is validated by the most active compound **33** that bears a trimethoxyphenyl moiety.

Another important structure used as covalent CBSIs is pentafluorphenyl, a

clinical evaluated compound T138067 (**36**) and its analogue T113242 (**37**) can covalently alkylate tubulin Cysβ241 residue and induce microtubule depolymerization [46]. Other new anti-tubulin agent PRR 112378 (**38**) with a novel structure has been isolated by Combeau et al.[47] from the fresh water plant *Ottelia alismoides*. This highly cytotoxic compound with an IC_{50} of 0.02 nM against KB cell is an efficient inhibitor of tubulin polymerization (IC_{50} 1.2 μM), it may covalently bond with the thiol of Cys241 residue by a 1,6-michal addition reaction. A simplified analogue **39** with potent anti-proliferative activity has also been reported by Tsai-Yuan Chang et al.[48].

Ring B of classical CBSIs.

Generally, ring B of typical CBSIs can accommodate into zone 1 of colchicine domain and usually forms a hydrogen bond with the residues around. This moiety of classical CBSIs lacks variation due to the relatively narrow hydrophobic cavity of zone 1. Two proper groups that can exactly accommodate into zone 1 are 4-methoylphenyl and indoles, which will be briefly discussed in this section.

(1) 4-methoylphenyl

This motif derived from natural CBSIs such as CA-4, colchicine (a similar tropolone motif) was often templated in the development of CBSIs. Structurally, the presence of a para-methoxy group is required for the biological activity; steric effect in ring B plays a determinative effect on activity; substitutions with an ethoxy or propoxy group or methylthio group cause a loss in activity [49]. The meta position tolerates variations which could be electron-donating (e.g., amino, **40**) (Figure 7A) or electron-withdrawing groups (e.g., fluorine, **41**). Hydroxyl and amino groups are most used substitutions introduced at this position, such as CA-4 (**3**) and AC7700 (**40**). Other variations including boronic acid (**42**) and azide group (**43**) have been reported with maintained activities [50, 51].

2-Methoxyestradiol (2-ME, **44**) (Figure 7B) is an endogenous estrogen metabolite with potent inhibition on tumor vasculature and tumor cell growth by binding to the colchicine binding site of tubulin [52]. The 4-methoylphenyl motif of 2-ME exerts a crucial role in its activity. Due to the formation of sulfate conjugates of 3- and 17-hydroxyl groups, its rapid metabolization and poor pharmacokinetic (PK) profiles have launched the synthesis and biological evaluation of novel 2-ME analogues. Potter's group has been engaged in developing novel 2-ME analogues with metabolically stable and improved anti-tubulin properties, the modifications were mainly made on the sheltering of two hydroxyl of 2-ME. The introduction of sulfamate into 3-hydroxyl resulted compounds **45** and **46** with 15-33 folds of improvement in anti-proliferative activities against MCF-7 cell lines compared with 2-ME [53]. The 18-OH position of 2-ME tolerates modifications, compounds **47**, **48**, **49**, **50**, and **51** all exhibited potent cytotoxicities in nanomolar ranges [54-57]. Replacing the 3-hydroxyl with amide group is another approach to improve the metabolic stability, resulting the discovery of ENMD-1198 (**52**) [58], which is found to be a new CBSI and is being evaluated in ongoing clinical trials. A simplified structure mimicking the spatial conformation of 2-ME has been reported,

tetrahydroisoquinoline derivative **53** [59] exhibited potent cytotoxicity with improved physicochemical properties.

(2) Indoles

Indole, an important nucleus of many biologically active natural and synthetic products, represents one of the most important privileged structures in drug discovery. It occurs in a wide range of therapeutically important drugs with various biological activities. Indole is one of the most successfully studied heterocyclic rings that can replace the 4-methoylphenyl motif in CA-4 or colchicine, most of these CBSIs incorporating indole moiety are derived from synthetic sources. Indole molecules as inhibitors of tubulin polymerization are continuing to attract the interest of chemists and biologists [60, 61], herein we will focus on the indole privileged structures as the ring B of CBSIs.

Indole as ring B of CBSIs was initiated by Liou et al. [62] with the discovery of compound **54** and **55 (**Figure 8**)**. SARs studies revealed that a methoxy group located at the C-6 position of 3-aroylindole (**54**) or C-5 position of 1-aroylindole (**55**) resulted in the best activity. In continuation of their works on 1-aroylindoles, Liou et al. developed 4-hydroxy (**56a**) and 4-amino-1-aroylindoles (**56b**) as potent tubulin polymerization inhibitors [63], suggesting that the introduction of hydroxyl or amino group at C-4 position of 1-aroylindoles was an effective way to increase the cytotoxicity. The effectiveness of this strategy was also proven by Ty et al. taken in 3-aroylindole resulting in the discovery of **57a** and **57b** [64]. It is evident that the indole moieties in these compounds all shared similar structures to 4-methoylphenyl. 2-aroylindoles were also identified by Mahboobi et al. as antimitotic agents, compound **58** exhibited the most potent activity with IC_{50} value of 0.06 μM against HeLa cells [65]. A series of 2-, 3-, 4-, 5- and 6-aroylsindoles were prepared by Liou et al., compound **59** was the most active with IC_{50} values ranging from 10 to 15 nM in six different cancer cell lines, suggesting that substitution on C-5 position of indole ring may be the best option for this class of CBSIs [66]. Interestingly, when allocolchicinoids analogue **60** was incorporated with indole moiety based on colchicine skeleton, it showed higher anti-proliferative activity with IC_{50} value of 1nM against BJAB cell line [67]. All these cases indicate that indole moiety could be an alternative of 4-methoylphenyl as the ring B of CBSIs.

The Bridge of classical CBSIs

The potent antiangiogenic and antitumor profiles of CA-4 open up a new era of CBSIs since its discovery. However, isomerization of the active *cis*-olefinic conformation into the corresponding inactive *trans*-analogs impedes its clinical development, thus hundreds of CA-4 analogues have been developed by modification on *cis*-double bond. This linkage between two aromatic rings tolerates modifications due to the ample space where olefin structure is located in the pocket. These moieties all share a common character that the unique spatial conformation can orient ring A and ring B into the cavities of zone 2 and zone 1, respectively. Such moieties including *cis*-olefin, one atom bridge, chalcones, heterocycles and sulfonamides are discussed in this section.

(1) *Cis*-olefin

Cis-olefin occurred in CA-4 remains the most classical and original bridge, it initiates the modifications on this *cis*-double bond to avoid the isomerization of *cis*-olefin to *trans*-olefin. Hydrogenation of the double bond leads to erianin (**61**) (Figure 9) that retains activity but with less potent. Studies extending the bridge via methylene, ethylene, propylene, and butylene revealed that two carbon linkers were the most active and other linkers were less active both in cytotoxicity and tubulin assays [68].

(2) One atom bridge

IsoCA-4 (**62**) with two aromatic rings on one alkenyl carbon represents another class of CBSIs resembling the structure of CA-4, which showed comparable activity with that of CA-4 [69]. Another compound comprising indole nucleus in this class is **63** [70], which has tubulin polymerization inhibition value in the submicromolar range and cytotoxicity in the subnanomolar range. Isoerianin (**64**), the hydrogenation form of isoCA-4, exhibited excellent anti-proliferative activity against HCT-116 cells with IC_{50} value of 28 nM [71]. Substitution of the double bond with carbonyl leads to phenstatin (**65**) with comparable activity of CA-4 [72]. Replacing the isovanillic ring with an indole (**54**) [62] or an aniline derivative (**66**) [73] also led to new compounds with potent activities.

Amino group is an important privileged linkage, the amino is often substituted with a methyl and this *N*-methyl group plays a vital role in maintaining activity probably because the introduction of methyl leads to a triangle pyramidal shape which could orient ring A and ring B into the pocket of zone 2 and zone 1, respectively. As Soussi et al. described [74], compound **67** exhibited potent activity with IC_{50} value of 7 nM against HCT 116 cells. *N*-methyl in other aforementioned structures (**15-18**) also validated this unit as a privileged structure in the construction of CBSIs.

(3) Chalcones

The length of the bridge can be extended with maintained biological properties, which is exemplified by two series of compounds including vinylogous analogues of CA-4 and chalcones. Vinylogous analogues bearing two olefinic bonds have four geometric isomers while only one of which is active (**68**) [75] (Figure 10). The development of **68** as a tubulin inhibitor was hampered due to its synthetic inaccessibility, while chalcones that can be prepared by the simple procedure are adaptable for further exploitation.

Chalcone with a α,β-unsaturated ketone structure represents a key structural motif in biologically active molecules both from synthetic and natural sources. Research regarding the anti-tubulin activity of chalcones was initiated by Ducki et al., **69** and **70** showed remarkable anti-proliferative activities with respective IC_{50} values of 4.3 nM and 0.21 nM against K562 cell lines [76]. The introduction of methyl on α position of carbonyl led to a dramatically improved cytotoxicity due to the more preferential s-trans conformation adopted by **70**, while s-*cis* conformation by **69**. Other substitutions on α position of carbonyl have been reported by Lawrence et al. [77] and Ducki et al. [78] with a conclusion that methyl introduced on α position of

carbonyl remains the best substitution for activity improvement. The vital effect of methyl on activity was confirmed by the work of Jun Yan et al. , compound **72** was 70 folds more potent than **71** with IC_{50} value of 3 nM against A549 cell lines [79]. Since tubulin was identified as the potential target for chalcone-type compounds, extensive researches have been carried out to design and synthesize new anti-tubulin cytotoxic chalconoids, which have been reviewed by Mirzaei et al. [80] recently. Thus, α,β-unsaturated ketone especially a methyl substituted moiety is a privileged structure to replace *cis*-olefin as the bridge of CBSIs.

The spatial relationship between the two aromatic rings of CA-4 and colchicine is an important structural feature that determines its ability to bind to tubulin. The enone structure of chalcones is a semi-rigid backbone with three rotatable bonds. As mentioned above, substitution on α position can act as a conformational locker forcing the enone to adopt an s-trans conformation, which presumably increases the affinity of chalcone to tubulin. Thus, enone rigidification may be an approach for activity improvement. The first attempt by Lawrence et al. promoted the synthesis of **73** and **74**, both showed potent cytotoxicity against K562 cell lines with IC_{50} values ranging from 40 to 50 nM [81]. In 2015, Xingshu Li's group systematically studied these rigidified chalcones with the finding that five-membered ring fused into ring A may be the best option, compound **75** showed potent activities with IC_{50} values ranging from 172 to 570 nM against several cancer cell lines [82]. In their efforts to continue the research of rigidified chalcones as anti-tubulin agents, compound **76** with an indole nucleus was found to possess potent cytotoxicity against several cancer cell lines and exhibited greater than 217-fold selectivity over human normal cells [83]. Other conformation locking strategy of fusing enone structure into ring B was reported by Romagnoli's group, compounds **78** [84] and **79** [85] templated from **77** [86] were all endowed with promising activities.

(4) Heterocycles

Heterocycles as one of the most studied privileged bridges are effective *cis*-locked structures to fix the *cis* double bond in CA-4. The ample space where olefin structure located in the pocket allows expansion of double bond to a larger fragment, such as heterocycles. This heterocycle bridged strategy has enriched the library of CA-4 analogues since the first attempt was made by Shirai et al. through replacing the olefinic moiety with dioxolane scaffolds (**80**), however, it was devoid of anti-tubulin activities [87]. To date, a large number of this class of analogues with retained anti-tubulin activities have been reported and detailed reviews can be found in the summaries of Yan Lu et al. [88] and Pérez-Pérez et al. [8]. The privileged heterocyclic rings include heteroaromatic rings such as pyrazole (**81**), imidazole (**82**), 1,3-oxazole (**83**), [89] triazols (**84**) [90], tetrazoles (**85**) [91], furazan (**86**) [92], 2-aminothiazoles (**87**) [93], N-methyl imidazole (**88**) [94] and other nonaromatic heterocycles such as β-lactams (**89** [95]**, 90** [96] and **91** [97]) (Figure 11). All these hetero-aromatic compounds share five-membered heterocycle rings as the bridge, suggesting that five-membered rings seem to be the best option for this class CBSIs. The exceptions of four-membered rings are β-lactams, compounds **89**-**91** with β-lactams as the bridge showed impressive cytotoxicity, C-3 aryl substituted **90** and

91 with better activities indicate that this position tolerates modifications probably because another ample space exists in the corresponding zone of colchicine binding site.

(5) Rings fused into ring B

Fusing *cis*-olefin into ring B represents another tactic to lock the *cis* double bond. The works of fusing carbocyclic rings into ring B were mainly described by Pinney's and Alami's groups. Pinney's group was the first to attempt this modification leading to the discovery of **92** (Figure 12) with seven membered carbocyclic ring fused into vanillin ring. Compound **92** exhibited highly potent cytotoxicity against several human cancer cell lines with IC_{50} values ranging from 3.2 to 28 pM [98]. Templated with the structure of **92**, other molecules were successively reported by both groups. For examples, **93** [99], **94** [100], **95** [101], **96** [102] all exhibited potent activities. Recently, Xingshu Li's group described the fusing of carbocyclic rings into indole nucleus, compound **97** with seven membered ring was the most active with IC_{50} values ranging from 22 to 56 nM [103]. Heterocycles fused into ring B have also been achieved by Galli et al. [104], compound **98** showed similar cytotoxic and anti-tubulin activities compared with CA-4 in neuroblastoma cells but a better PK profile. All these cases suggest that rings especially seven membered rings fused into ring B are privileged motifs mimicking the structure of colchicine.

(6) Sulfonamides

Sulfonamides have long been privileged structures in drug discovery. Drugs with sulfonamide motif have many biological activities, such as antibacterial, diuretic, antidiabetic, antithyroid, antihypertensive and antiviral activities. Sulfonamide structures can be alternative of *cis*-olefin to act as the bridge as exemplified by clinically investigated compounds **36**, **37**, **99** [105], and **100** [106] (Figure 13). Another important application of sulfonamide in CBSIs is ABT-751 and its analogues, these compounds bearing planar or rigid structures tend to be located deeper into the β-subunit. Therefore, they are grouped into the non-classical class and will be discussed in the relevant section. Other bridges comprising sulfonamide were described by Reddy's group, the discoveries of **101** [107] and **102** [108] with remarkable antitumor activity led to the most potent compound **103** [109], which was proved to be an anti-tubulin agent with highest anti-proliferative activity having IC_{50} value of 3 nM against K562 cell lines.

Non-classical CBSIs

Unlike the classical CBSIs that share a common structure (ring A, ring B and a bridge), these non-classical CBSIs are characterized with diverse structures. Their space conformations are generally planar or rigid skeletons that is capable to stretch into the deeper zone 2 of the β-subunit (Figure 1C). Naturally derived CBSIs including plinabulin and nocodazole or synthetic origins, such as ABT-751 and its analogues, anthracenones, will be reviewed in this section.

Diketopiperazine

Diketopiperazines are cyclodipeptides obtained by the condensation of two α-amino acids, and they are often found alone or embedded in larger, more complex architectures in a variety of NPs. These privileged structures have several characteristics that make them attractive scaffolds for drug discovery. The conformational constrained heterocyclic scaffolds with stability to proteolysis mimics a preferential peptide conformation that has the ability to bind to a wide range of targets [110].

Phenylahistin (**104**) (Figure 14**)** with a 2,5-diketopiperazine skeleton was firstly isolated by Kanoh et al. from *Aspergillus ustus* as a racemic mixture, the enantiomer **(−)-104** exhibited potent cytotoxicity with inhibition of tubulin polymerization [111]. Compound **105**, the dehydrogenation form of **104**, was reported as an antimitotic agent being 1,000 times more active than **(−)-104**. The tert-butyl derivative **106** was developed by Hayashi's group with 2.8 folds increase in cytotoxic potency compared with **105** [112]. SARs studies on the tert-butyl and the phenyl groups of **106** afforded compounds **107** with a 2,5-difluorophenyl and **108** with a benzophenone, both were found to have more potent activities with 5.7 and 10 folds increase in cytotoxicity against HT-29 cell lines, respectively [113]. Modifications of benzophenone and tert-butyl of **108** led to the discovery of **109** [114] and **110** [115], **109** exhibited increase in potency of 2.8 folds, however, **110** with pyridine replaced of tert-butyl imidazole slightly decreased in potency of 5 folds, as compared with **108**.

Due to the intermolecular hydrogen bonds and π-π stacking interactions from lines or networks of 2,5-diketopiperazine, this class of CBSIs shares a limitation of poor solubility. Aiming at solving this drawback, Yonghong Liu's group introduced protective groups to replace one or two of the amide hydrogen atoms, thus the formation of hydrogen bonds and π-π stacking interactions were interrupted. Compounds **111** [116] and **112** [117] were endowed with improved solubility, however, their activities were decreased with IC_{50} values ranging from 0.36 to 4.5 μM against several cell lines.

Benzimidazoles

Benzimidazoles derivatives (BZs) characterized by carbamate substituted at the position 2, such as albendazole (ABZ), fenbendazole (FBZ), mebendazole (MBZ), oxibendazole (OBZ), parbendazole (PBZ), and luxabendazole (LBZ) (Figure 15) are commonly used for antinematodal treatment. They are also very effective anti-tumor agents, it was believed that BZs can exert their cytotoxic effects by disrupting the functions of the microtubule system [118]. Thus, BZs may represent a new class of structurally novel antimitotic agents as tubulin inhibitors. Some of them are currently being investigated in clinical trials, for example, nocodazole (**114**) is a rapidly-reversible inhibitor of microtubule polymerization with potential antitumor activity, and is often used as a lead compound for the discovery of novel CBSIs. Another compound in clinical study is MN-029 (**115**), this L-alanine prodrug of MN-022 (**116**) is rapidly metabolized to its active form **116** *in vivo*. Other than carbamate substitution on the benzimidazole ring, benzimidazole-2-urea derivatives were reported by Wenna Wang et al., compound **117** showed promising cytotoxic activity with IC_{50} values ranging from 6 nM to 1.77 μM [119].

Unlike CA-4 or colchicine, these BZs do not interact with the α-subunit but are located into the deeper site of β-tubulin, this site overlaps very little with that of colchicine. Meanwhile, the benzimidazole-carbamate structure can accommodate into zone 3 of colchicine domain to form hydrogen bonds with residues Asnβ165 and Gluβ198 [6]. This unique binding mode of BZs with tubulin provides new insights into the discovery of CBSIs with benzimidazole-2-carbamate moiety.

ABT-751 and its analogues

ABT-751 (**118**) and its analogues sharing a characteristic structure of benzsulfamide have long been studied as structurally unique CBSIs since ABT-751 was identified as a potent anti-proliferative agent [120], and was subsequently found to be an anti-tubulin agent by targeting the colchicine binding site [121]. ABT-751 interacts with all three zones in its X-ray structure reported by Audrey Dorle ́ans et al.[122], which is distinct from all reported binding modes with tubulin. The 4-methoylphenyl is accommodated into zone 1 with pyridine ring in zone 2, and the phenol extends into the deeper site of β-tubulin, namely zone 3 with phenolic hydroxyl formed hydrogen bonds with Tyrβ202 residue. ABT-751 was the only known ligand occupying all the three zones in colchicine domain until Yan-Na Liu et al. [123] reported that the *m*-ethoxyaniline group of **119** with a novel rigid skeleton extends into zone 3 of colchicine binding pocket with hydrogen bonded with Tyrβ202, while ethyoxyl substituent at the 6-position occupied only partial hydrophobic cavity in zone 1 with the adjacent acetamide group formed a hydrogen bond with Serα178 at the interface (Figure 16A).

Due to the conjugation effect of pyridine nitrogen, the structure of diphenylamine moiety is almost planar, thus it can be accommodated into the colchicine binding site. Numerous ABT-751 analogues with the similar spatial conformation have been acquired since the discovery of ABT-751. Takashi Owa et al. [124] first identified E7070 (**120**) (Figure 16B) as a potent anti-cancer agent with a conformation-locking strategy, this strategy of fusing the secondary amine into ring B was testified as an effective tactic to maintain the planar conformation. Subsequently, Jing-Ping Liou's group [125, 126] synthesized the compound **121, 122, 123** by a similar way to prove that fusing the secondary amine into ring B with the opposite orientation was more effective. The IC_{50} values of **121-123** ranged from 8 to 40 nM against a panel of cancer cell lines. The secondary amine fused into ring A has also been reported by Nicolas Lebegue's group, while sulfamide was replaced with ethyl, the resulting compound **124** [127] exhibited potent cytotoxicity against L1210 cells with IC_{50} value of 110 nM. However, lack of chemical and metabolic stability of **124** led to relatively lower *in vivo* antitumor activity, which provoked their continuing work to overcome these drawbacks. A more stable quinazolinone motif was introduced to replace the previous three membered ring, the afforded compound **125** showed more potent activities both *in vitro* and *in vivo* [128]. Another analogue HMN-214 **(126)** (prodrug of **127**) with *trans*-olefin replaced with sulfamide was originally discovered and developed by Nippon Shinyaku, it showed strong antitumor activity by causing M-phase arrest of tumor cells and inducing apoptosis, and is currently undergoing clinical trials against various tumor types. However, this

compound showed no interaction with tubulin [129].

Anthracenone

Molecules comprising anthracenone skeleton are another representative structurally planar CBSIs. This class of CBSIs was started from Helge Prinz's group, compound **128** (Figure 17) showed remarkable cytotoxicity with IC_{50} of 20 nM against K562 cell lines by targeting colchicine binding site of tubulin [130]. This unique anthracenone motif in **128** motivated their efforts to continue the research on this class of CBSIs, compounds **129** [131], **130** [132], **131** [133], **132** [134], and **133** [135] were discovered to exhibit potent anti-proliferative and anti-tubulin activities. The binding mode had not been elucidated until **134** was suitably docked into colchicine domain with a hydrogen bond formed between methanone group and Valα181 as well as two cation-π interactions formed between the phenyl rings and Lysβ352 and Lysβ254 [136]. All these cases showed that anthracenone is a privileged structure in the skeletons of non-classical CBSIs.

Prodrugs of CBSIs

CBSIs either from natural sources or synthetic origins usually suffer from poor aqueous solubility and PK profiles that hinder the progression of these CBSIs in preclinical studies or clinical evaluations. Many strategies to improve aqueous solubility of CBSIs have been centered on making use of prodrug strategy or searching for new structures with improved solubility. Herein, the frequently used prodrug forms will be discussed including phosphate, amino acid introduced at hydroxyl or amino groups and other forms. Other delivery systems such as polymeric micelles, liposomes, dendrimers to improve the poor aqueous solubility and PK properties will not be covered here.

Phosphate

The most classical strategy is the derivatization as the phosphate prodrugs which have good chemical stability and also are rapidly converted to the active drugs *in vivo* by phosphatases. This approach is often applied to the prodrugs bearing a phenolic hydroxyl group as exemplified by CA-4P (**4**) (Figure 18). CA-4P has excellent water solubility, good stability and potent cytotoxicity, which is rapidly dephosphorylated to CA-4 *in vivo* within a few minutes. CA-4P is currently being investigated in phase 3 clinical trials for the treatment of anaplastic thyroid cancer and in phase 2 clinical trials for non-small cell lung cancer and platinum-resistant ovarian cancer [137]. Other than phenolic hydroxyl group, the NH in indole ring of CBSIs is also a good modifiable position to produce phosphate prodrug. For example, water solubility of compound **72-P** was increased to 125 mg/mL in sodium phosphate buffer, and the $t_{1/2}$ of **72-P** (157.5 min) was greatly improved in comparison with CA-4 ($t_{1/2}$ < 60 min), which may lead to a higher bioavailability *in vivo* [79].

Amino acid

Like phosphate prodrugs, amino acid prodrugs can also increase aqueous solubility. This modification strategy is usually applied in prodrugs with an amino group. As the aqueous solubility improvement is expected, amino acid conjugation

strategy may also increase the cell uptake of prodrugs in aid of peptide transporters. An example of this approach is **135**, the amino acid prodrug of CA-4, which is being investigated in clinical trials for advanced-stage soft tissue sarcoma, solid tumors and advanced solid tumors [138].

Others

Other approaches to improve aqueous solubility include hydrochloride (**6, 136**) [139] and carboxylate (**137**). Compound **137** is the prodrug of plinabulin, and it was developed by Hayashi's group [140] via a synthetic methodology [141]. This novel modification method to improve the aqueous solubility of plinabulin provides a new modification site for this class of CBSIs.

Conclusion & future perspective

NPs with structurally diverse frameworks have always been the inspiration for the development of small druggable molecules [142]. NPs inspired CBSIs discovery is a powerful and economical strategy to develop the novel CBSIs, which avoids the time-consuming *de novo* design for scaffold production. To overcome the limitations encountered in the development process of these natural CBSIs, such as colchicine, CA-4 and phenylahistin, synthetic alternatives that possess better cytotoxic and PK profiles have been discovered using these NPs as the templates.

In addition, structural biology has greatly accelerated the development of CBSIs since the identification of complexes of α,β-tubulin with a wide range of structurally diverse ligands. Knowing their binding modes with tubulin, those established CBSIs can be modularly analyzed and their pharmacophore models may be constructed, which facilitates medicinal chemists to discover more structurally novel CBSIs.

As mentioned throughout this perspective, privileged structures are often overlapped within different classes. These privileged structures that share common characters possess similar interactions with tubulin within the colchicine binding pocket. With these common characters available, more alternatives resembling the established privileged structures can be discovered and further validated. Thus, off-targets or failing works could be avoided with this privileged structure based strategy taken into account. We anticipate this review will provide such insights into the development of novel CBSIs in the future.

Executive summary

Background

- Microtubules are formed by the association of α- and β-tubulin heterodimers and serve as important components of the cytoskeleton in eukaryotic cells.
- The vital roles of microtubule in mitosis and cell division make it an attractive target for antitumor therapy.

Microtubule targeting agents

- Tubulin inhibitors can be divided into three classes according to their binding sites in microtubule, including taxane, vinca alkaloid, and colchicine binding sites.
- Colchicine binding site inhibitors have been attractive over the past two decades, hundreds of structurally diverse CBSIs have been developed.

Colchicine domain

- Colchicine domain is the generalized naming of colchicine binding site, it comprises a main site (colchicine binding site) and additional neighboring pockets.
- Inhibitors targeting colchicine domain are classified into two classes including classical CBSIs and non-classical CBSIs based on their spatial structures.

Privileged structures in CBSIs

- Privileged structures in CBSIs are discussed based on classical CBSIs and non-classical CBSIs, respectively.
- Classical CBSIs are typically characterized with an "aromatic ring – bridge – aromatic ring" which have a globular or butterfly like shape, and privileged structures of these three fragments are highlighted.
- Non-classical CBSIs are usually characterized with more planar or rigid skeletons, and they are structurally different from the common structures of colchicine and CA-4 analogues.

Prodrugs of CBSIs

- Some prodrug forms frequently used to improve aqueous solubility and PK profiles are highlighted.
- The most classical form is the phosphate prodrug due to its good chemical stability and metabolic profiles.

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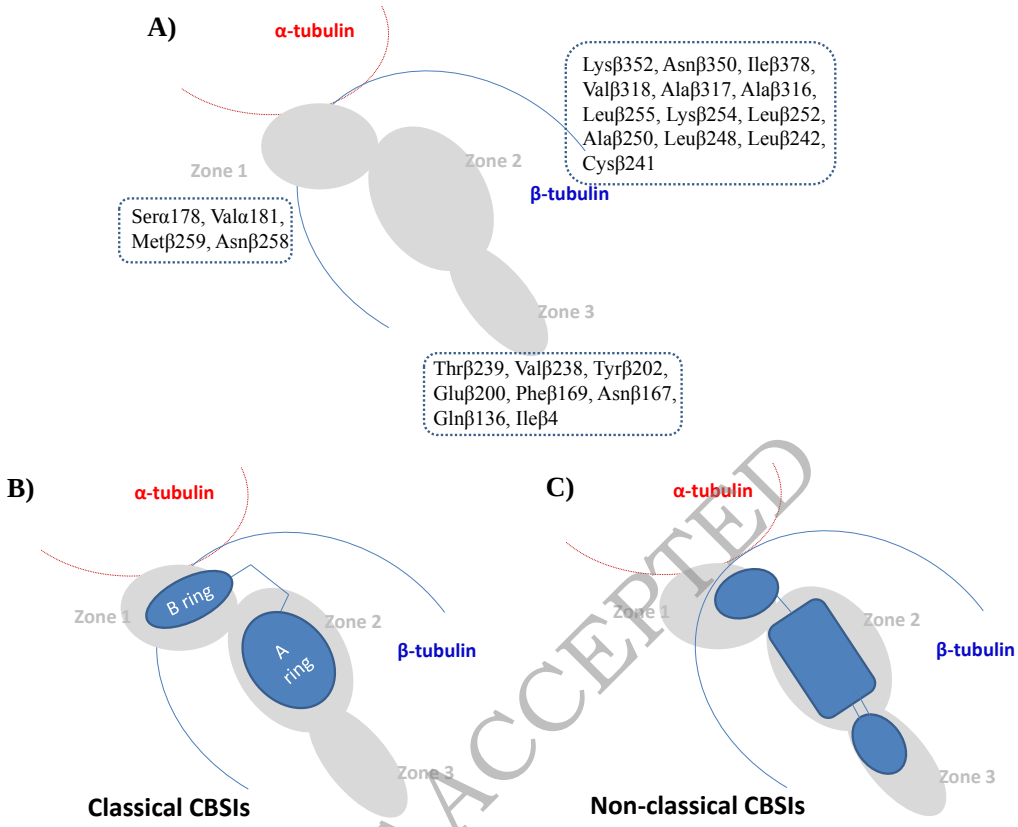
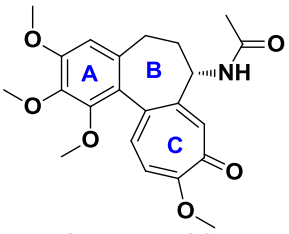


Figure 1. Three zones in the colchicine binding site (A), and classical (B) and non-classical (C) CBSIs binding modes with tubulin in colchicine binding site.



Colchicine (1)

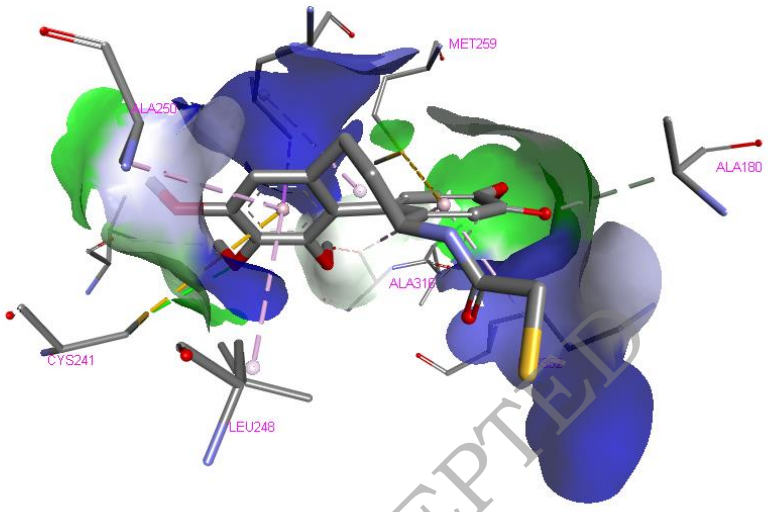


Figure 2. The structure of colchicine and its binding mode with tubulin (PDB code: 1sa0).

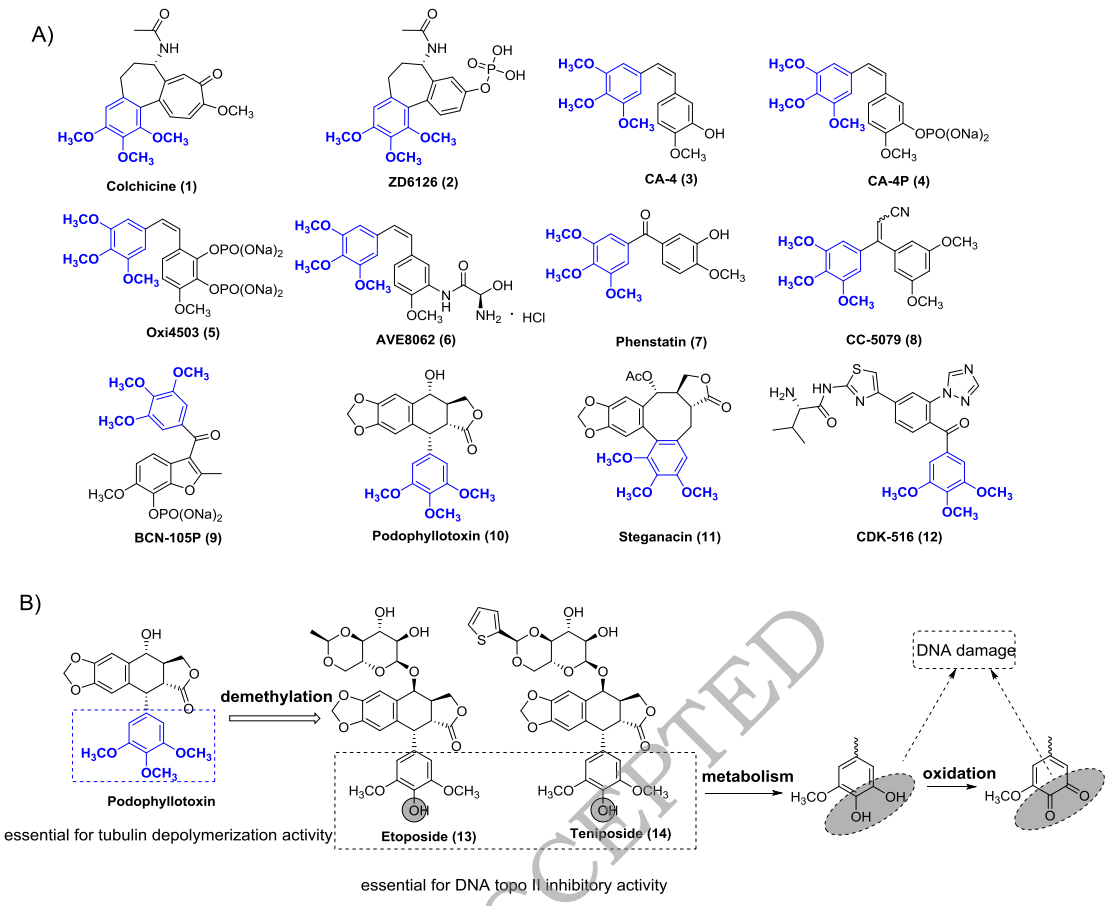


Figure 3. A) Structures bearing trimethoxyphenyl moiety in clinical trials. B) Demethylation of podophyllotoxin leads to loss of tubulin depolymerization activity but DNA topo II activity obtained.

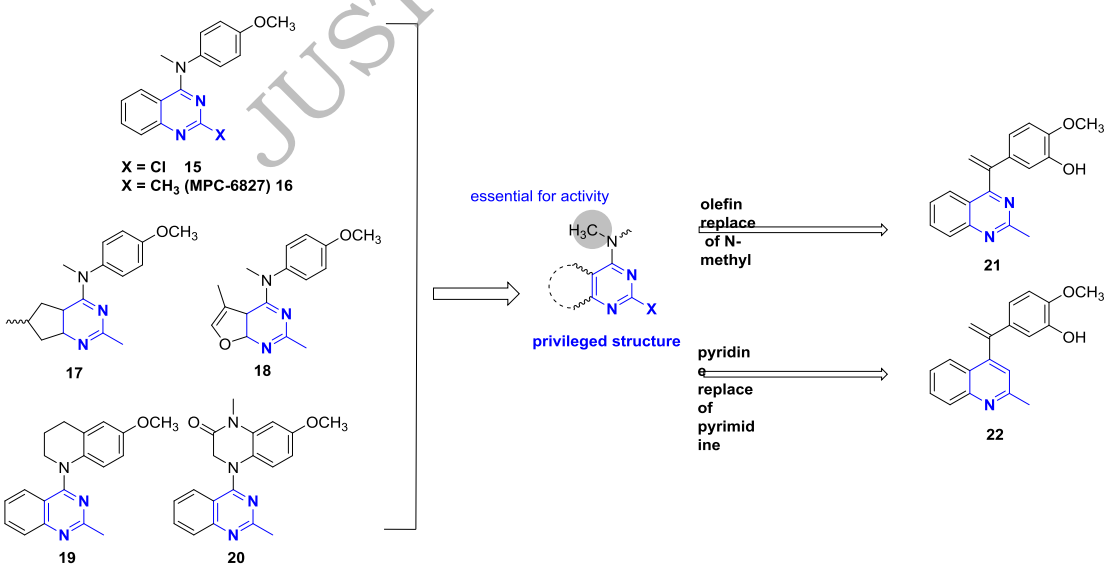


Figure 4. Nitrogenous heterocyclic compounds.

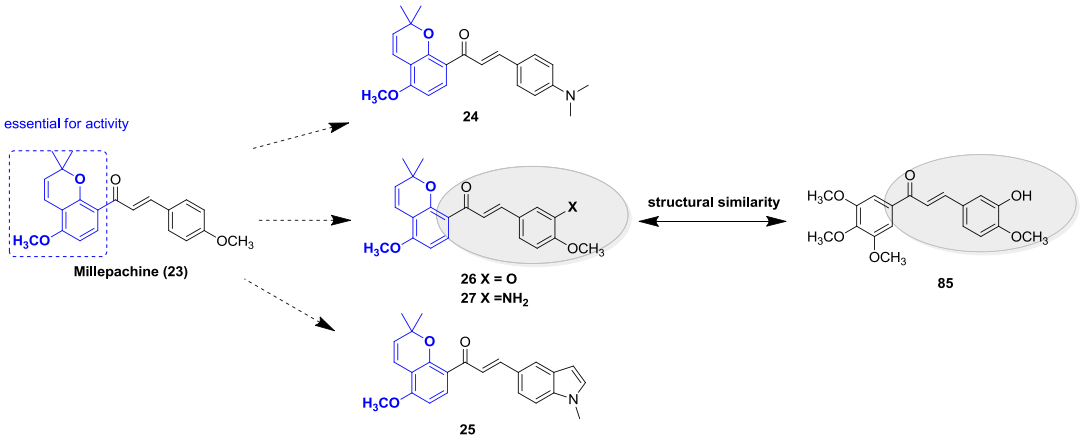


Figure 5. Modifications of millepachine bearing dimethylbenzopyran moiety.

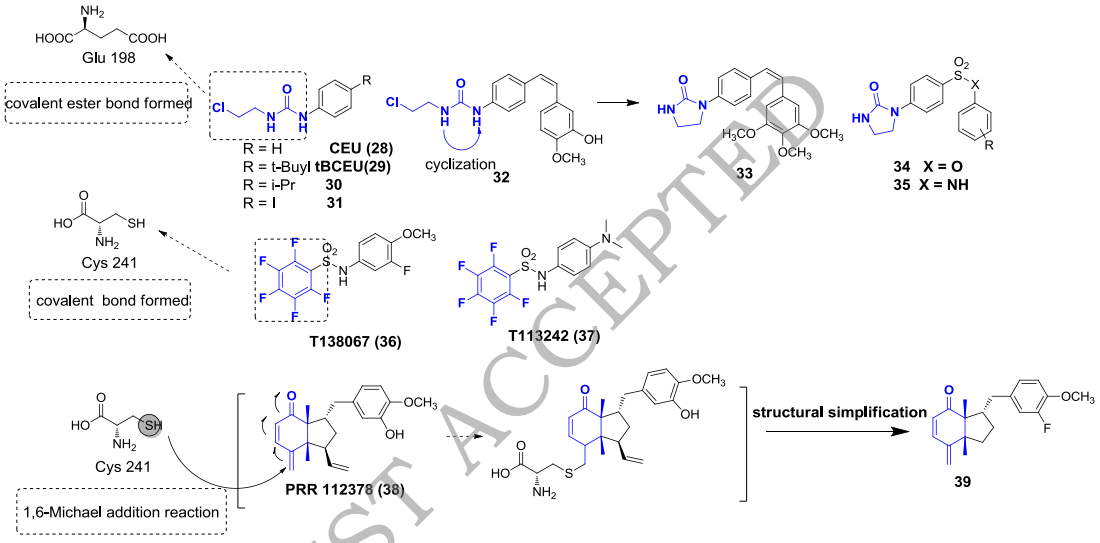


Figure 6. Structures bearing covalent privileged moieties.

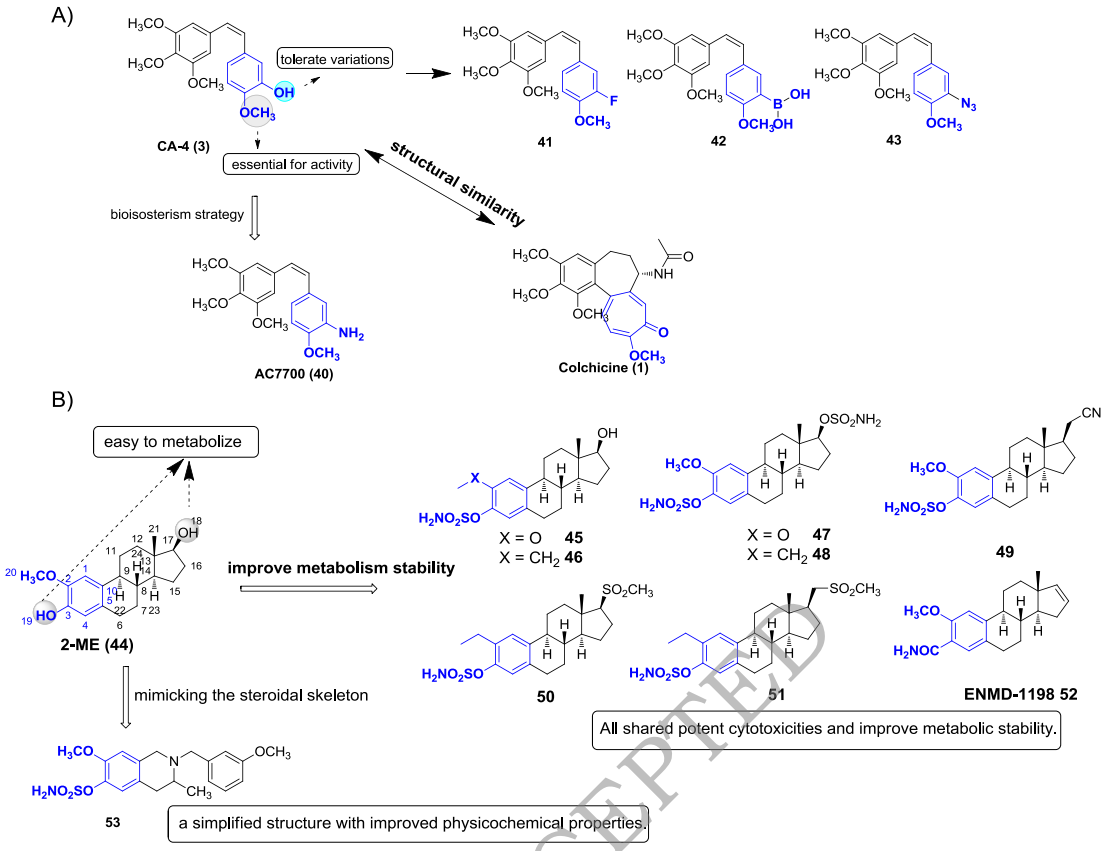


Figure 7. A) Structures bearing 4-methoylphenyl moiety. B) Structures derived from 2-Methoxyestradiol.

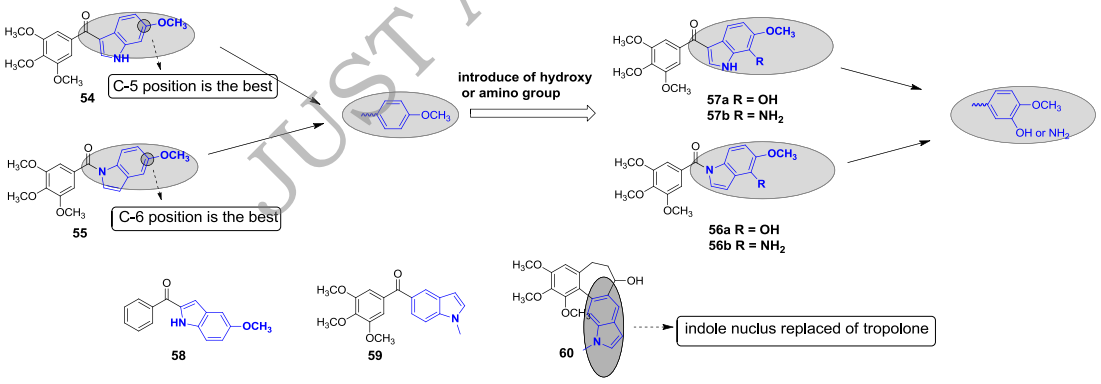


Figure 8. Structures bearing indole moiety.

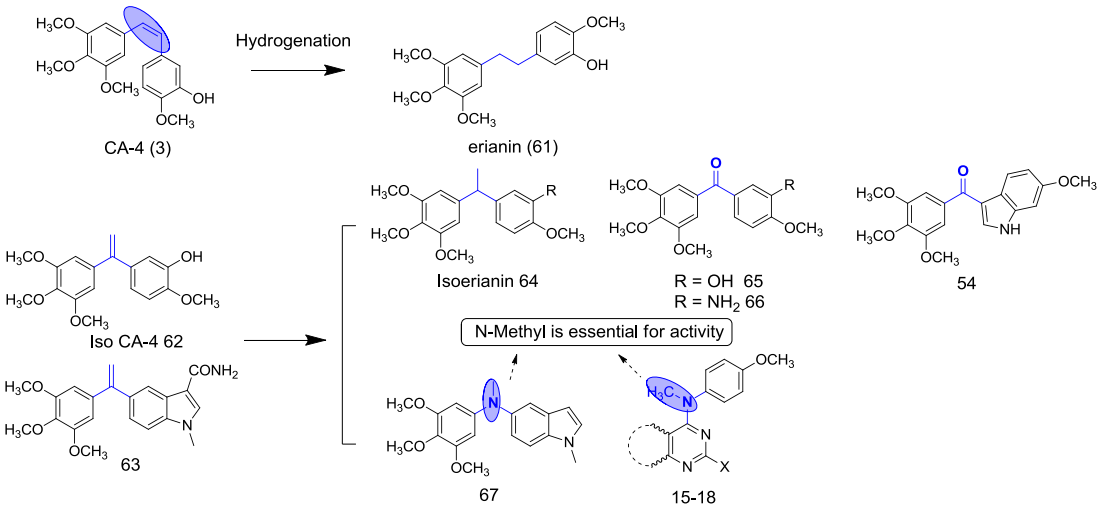


Figure 9. Cis-olefin and one atom as the bridge of classical CBSIs.

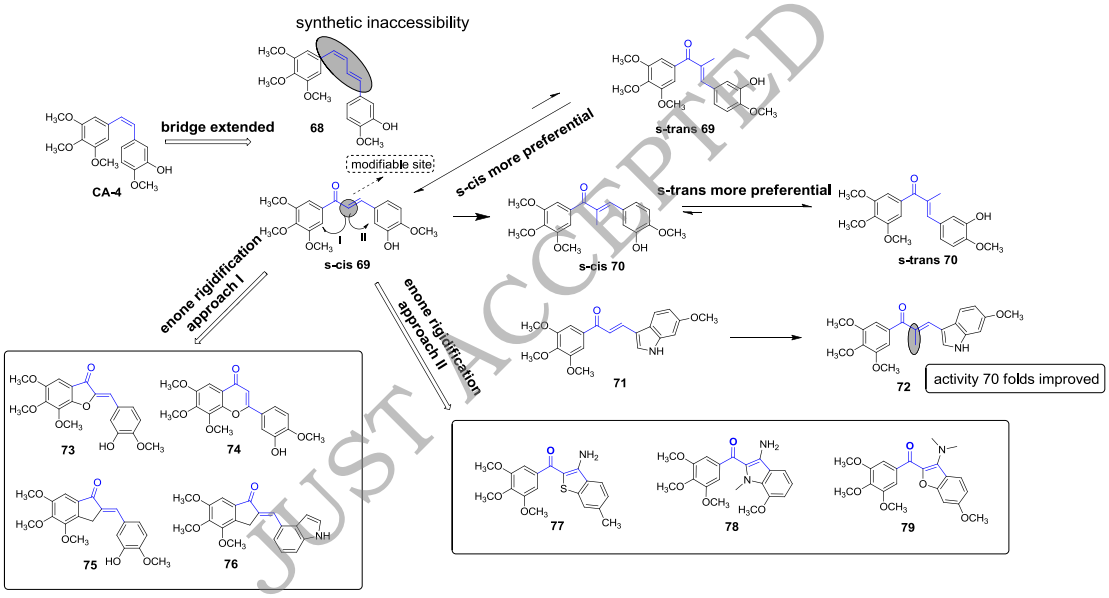


Figure 10. Chalcone structures as the bridge of classical CBSIs.

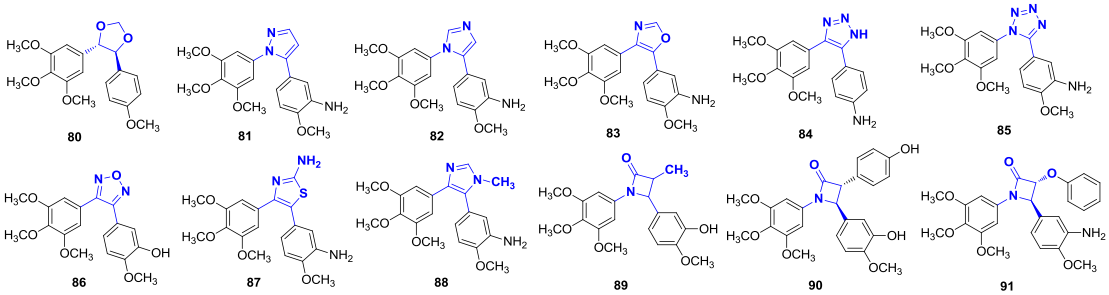


Figure 11. Heterocycles as the bridge of classical CBSIs.

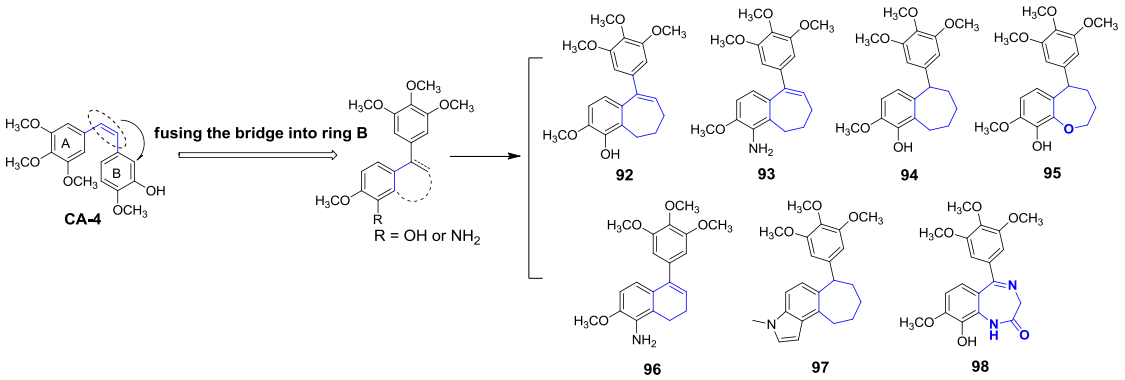


Figure 12. Structures with rings fused into ring B.

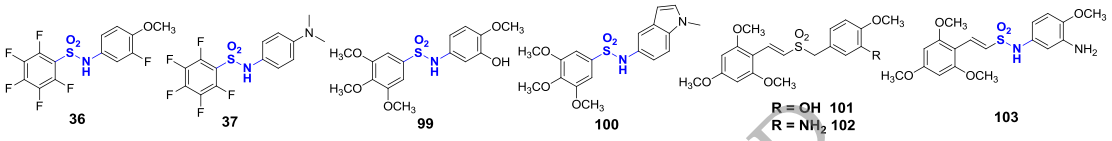


Figure 13. Sulfonamide moiety as the bridge of classical CBSIs.

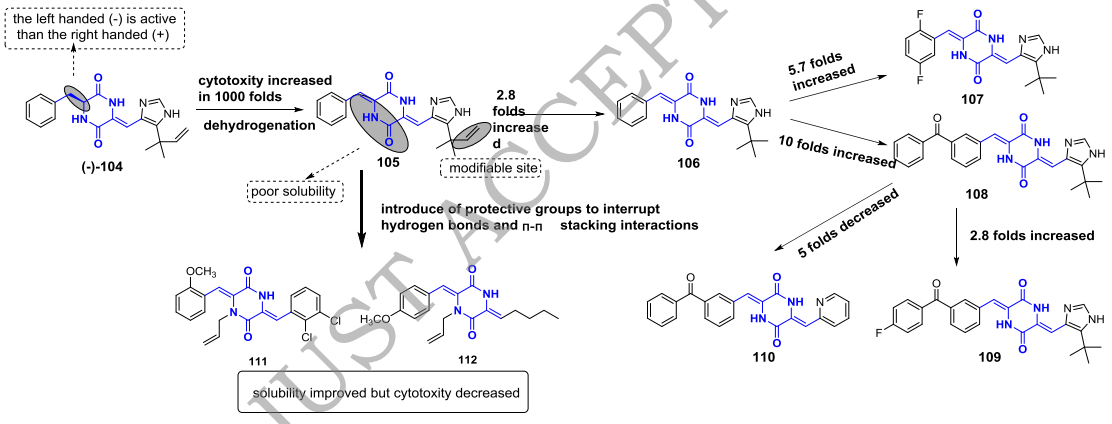


Figure 14. Non-classical CBSIs bearing the diketopiperazine moiety.

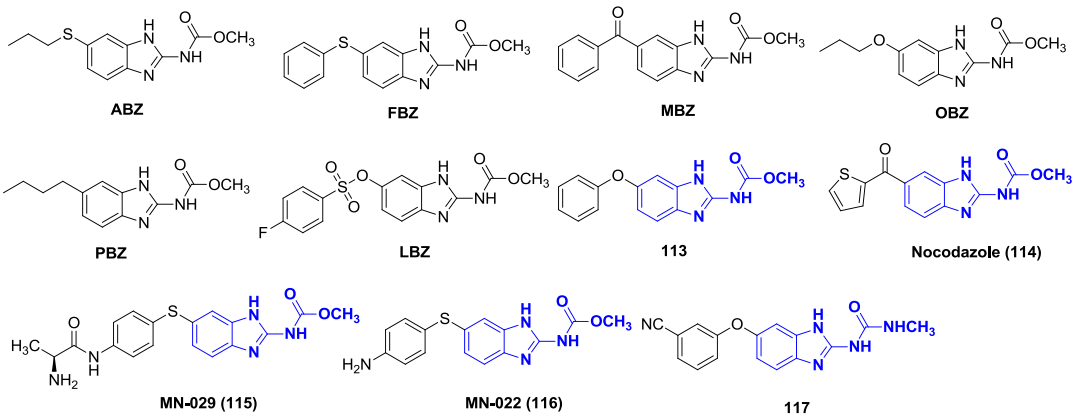


Figure 15. Non-classical CBSIs bearing the benzimidazole moiety

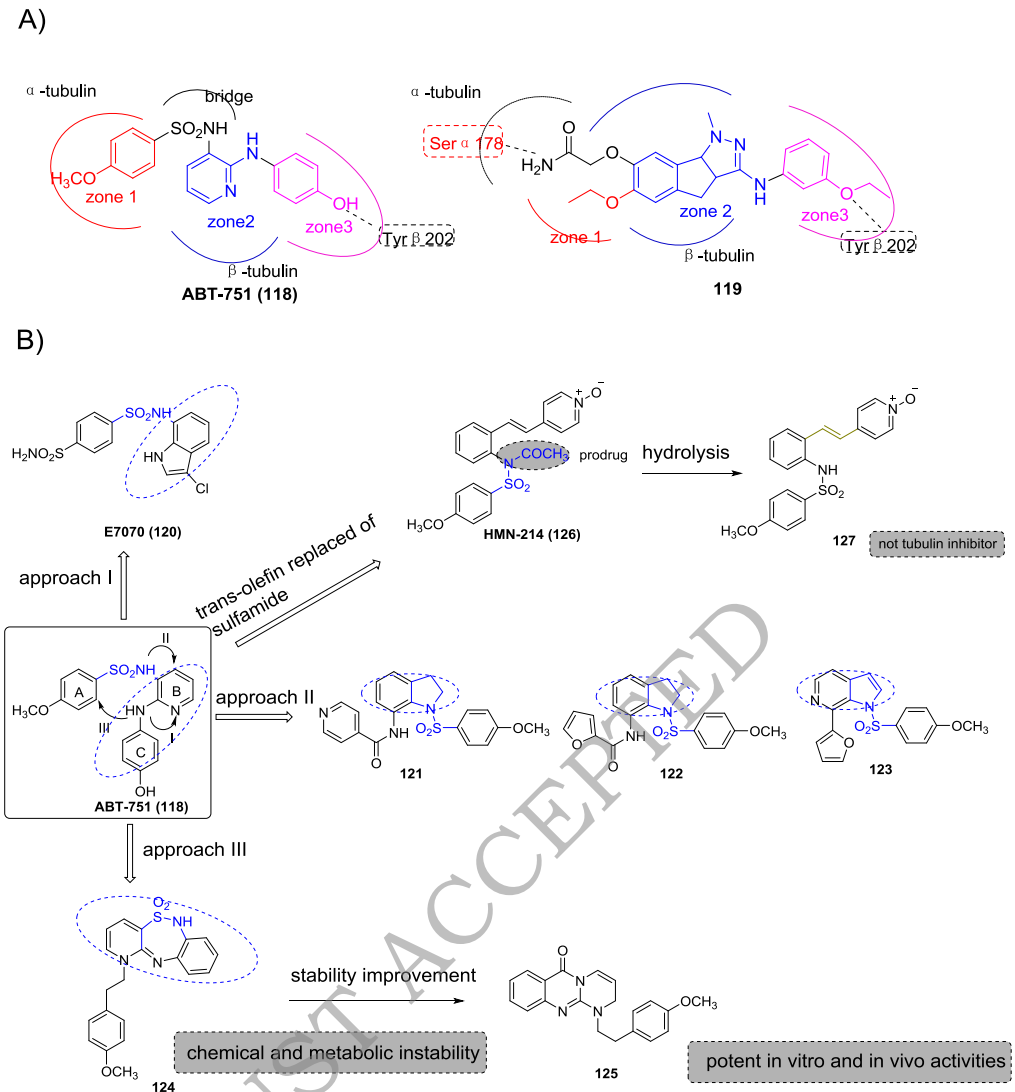


Figure 16. A) Binding modes of ABT-751 and 119 with tubulin. B) The modifications of ABT-751 as the non-classical CBSIs.

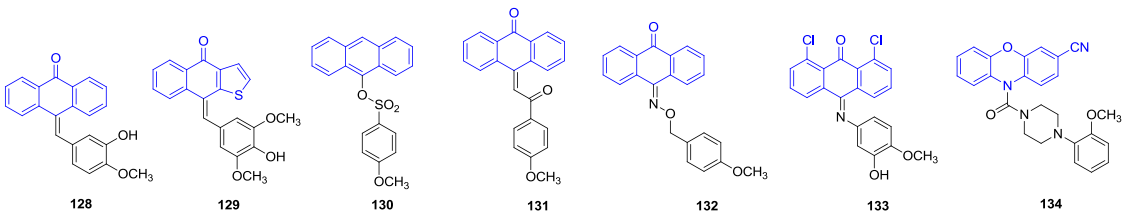


Figure 17. Structures bearing anthracenone moiety.

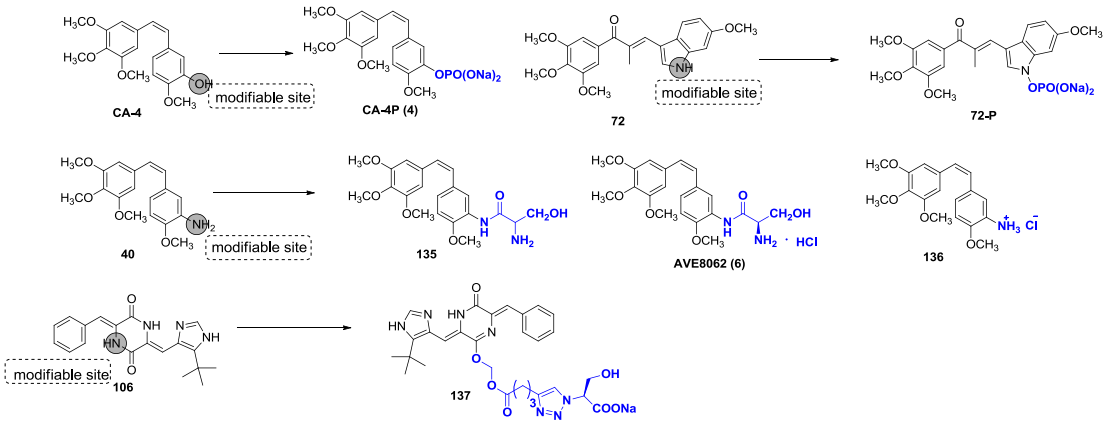


Figure 18. Privileged prodrug forms of CBSIs.