Pyridine Based Antitumour Compounds Acting at the Colchicine Site

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Abstract: Antimitotics binding at the colchicine site of tubulin are important antitumour and vascular disrupting agents. Pyridines and azines are privileged scaffolds in medicinal chemistry and in recent years many colchicine site ligands (CSL) have incorporated them into their structures with the aim of improving their pharmacokinetic and pharmacodynamics properties. CSL have been classified according to their chemical structures and the chemical structures of the pyridine and azine containing antimitotic compounds are described. The design principles behind the structural modifications and the achieved effect on the biological activity upon inclusion of these heterocycles are also discussed. Lessons from the achievements and failures have been extracted and future perspectives delineated.

Keywords: Pyridine, azines, antimitotics, tubulin, colchicine, antitumour, drug design.

1. INTRODUCTION

The discovery of the antitumour activity of the Vinca alkaloids[1] and the subsequent establishment of the inhibition of microtubule polymerization as their molecular mechanism of action[2] marked the beginning of modern anticancer chemotherapy[3] with the use of combination regimens[4]. This finding put the microtubular system at the centre of attention of anticancer chemotherapy, initiating the search for so called antimitotics due to their effect on cell division (Fig. 1). The discovery of the tubulin stabilizing taxanes by scientists at the NCI[5, 6] and the difficulty they present in their development as drugs against solid tumours[7, 8] once again highlighted the therapeutic potential of tubulin interfering agents. Colchicine (**3**), a natural product from the meadow saffron (*Colchicum autumnale*), had already played an important role in the discovery of tubulin[9] and was soon followed by structurally related natural products binding at the same site of tubulin, such as podophyllotoxin (**4**)[10] and the combretastatins,[11] all of them acting as tubulin polymerization inhibitors (TPI). Compared to the taxanes and the Vinca alkaloids, already in clinical use, the ligands binding at the colchicine site have much simpler chemical structures and have therefore been the subject of many synthetic medicinal chemistry programs.[12] Although to date, none of them have yet reached the market.[13]

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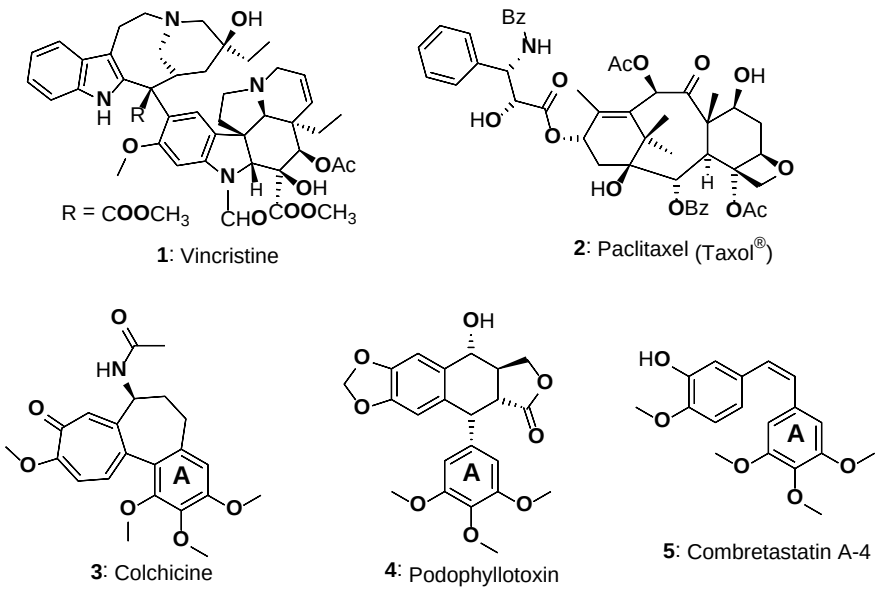
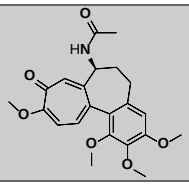
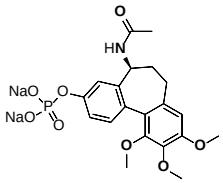
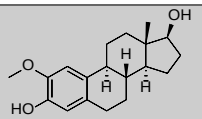
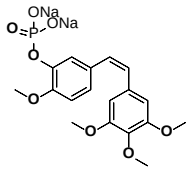
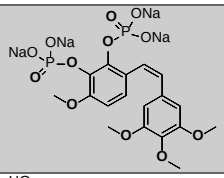
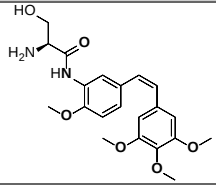
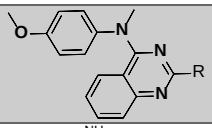
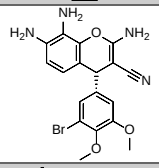
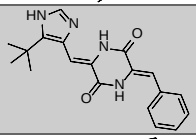
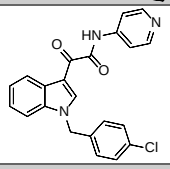
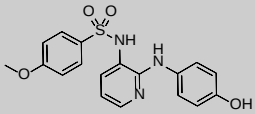
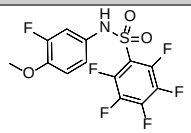
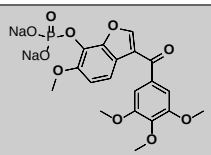
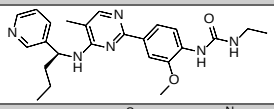
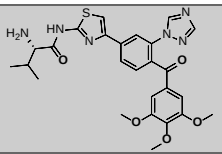
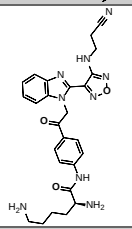
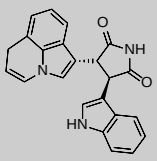


Fig. (1). Structures of tubulin ligands with historical importance

Table **1**. State of the Clinical Trials of CSL

Name	Structure	State of the clinical trials
Colchicine (3)		Withdrawn. Phase I trials failed due to high toxicity.
ZD6126		Withdrawn. Phase II trials for metastatic renal cell carcinoma and colorectal cancer failed due to cardiotoxicity.
2-Methoxyestradiol		Withdrawn. Completed Phase II trials but not effective enough for Phase III
Combretastatin A-4P (Prodrug of 5)		Completed. Phase II for advanced anaplastic thyroid cancer.[14] Completed. Phase II for advanced solid tumours.[15] Completed. Phase II for non-small cell lung cancer. Ongoing. Phase II/III for platinum resistant ovarian cancer. Ongoing. Phase II for pancreatic or gastrointestinal neuroendocrine tumours.
Combretastatin A-1P		Completed. Phase I for hepatic tumour. Completed. Phase I for acute myeloid leukaemia and myelodysplastic syndromes
AVE 8062 (Ombrabulin) (Prodrug of 39)		Completed. Phase III for advanced-stage soft tissue sarcoma after failure of anthracycline and ifosfamide chemotherapies. Completed. Phase I for advanced solid tumours.[16] Completed. Phase II for recurrent ovarian cancer. Completed. Phase II for non-small cell lung cancer.

MPC-6827 (Verubulin)		Completed. Phase I/II for glioblastoma multiforme.[17] Completed. Phase I/II for metastatic melanoma. Completed. Phase I for refractory solid tumours.
EPC-2407 (Crolibulin)		Ongoing. Phase I/II for solid tumours with a focus of anaplastic thyroid cancer.
NPI-2358 (Plinabulin) (17)		Completed. Phase I/II for advanced non-small cell lung cancer. Recruiting. Phase III for non-small cell lung cancer. Completed. Phase I for advanced solid tumours or lymphoma.
Indibulin		Completed. Phase I/II for metastatic breast cancer. Completed. Phase I for advanced solid tumours.
ABT-751 (11)		Ongoing. Phase II for children with recurrent neuroblastoma. Completed. Phase I/II for non-small cell lung cancer.[18] Completed. Phase I/II for advanced lung cancer.[19] It did not show efficacy. Completed. Phase I/II for metastatic prostate cancer. The treatment was not effective. Completed: Phase II for colorectal cancer. Completed: Phase II for renal cancer. Completed. Phase I for refractory solid tumours in young patients.[20] Completed. Phase II for recurrent breast cancer after taxol treatment.
T-138067 (18)		Completed. Phase I for advanced refractory cancer.
BNC105P		Withdrawn. Phase I/II for partially platinum sensitive ovarian cancer patients. Phase I completed and then it was withdrawn. Ongoing. Phase I/II for progressive metastatic clear cell renal cell carcinoma.[21]
CYT-997 (79)		Completed. Phase I/II for multiform glioblastoma. Withdrawn due to difficulties enrolling patients. Phase II for multiple myeloma.
CKD-516		Completed: Phase I for adult solid tumour. Recruiting. Phase I for advanced refractory solid tumours.
BAL101553 (Prodrug of 8)		Ongoing. Phase I/II for solid tumours.
ARQ197 (Tivantinib) (12)		Completed. Phase I for solid tumours.[18] Completed. Phase I/II for metastatic colorectal cancer. Ongoing. Phase II for metastatic non-small cell lung cancer. Completed. Phase II for unresectable hepatocellular carcinoma.[22] Ongoing. Phase III for non-squamous, non-small cell lung cancer.[23] Completed. Phase II for gastric cancer, refractory multiple myeloma

Data in this table have been obtained from www.clinicaltrials.gov and when results of the trials have been published, the references are cited.

The fact that some CSL are not susceptible to some tumour resistance mechanisms against the taxanes and the Vinca alkaloids, such as not being substrates of the multidrug resistance proteins[24] and the discovery of the tumour vascular disrupting activity of some of them[25] has renewed interest in the discovery and development of colchicine site drugs. However, some problems still remain in their development as successful anticancer agents, mainly owing to their low intrinsic water solubility and insufficient potency, which results in the survival of a rim of cancer cells that finally allows tumour to regenerate.[26] In this review, we focus on colchicine site agents carrying pyridine rings or related azines and discuss the pharmacokinetic and pharmacodynamic consequences associated with their presence.

The microtubules of eukaryotic cells form part of the cytoskeleton and play a role in cell shape maintenance, form the mitotic spindle during mitosis and are also implicated in cell movement, intracellular transport and signal transduction.[27] The main constituent of the microtubules are the tubulin αβ heterodimers, GTPases that polymerize along their longitudinal axes to form protofilaments that align themselves side by side to form hollow cylinders. The microtubules are dynamic structures that preferentially grow at one end (plus end) and shorten at the other (minus end). When growth and shrinkage occur at both ends, this is known as a phenomenon called dynamic instability. This event results from the inherent instability of tubulin in the straight conformation required to form the microtubules, a high energy state which is also used by other proteins travelling along the microtubules.[28] The alteration of the dynamic equilibrium of the microtubules more than the actual modification of the polymerization equilibrium between the microtubules and soluble tubulin dimers in cells is considered responsible for the biological effects of antimitotic agents acting on tubulin.[29]

In the absence of structural information at atomic resolution, at least up the 21st century the design of CSL relied heavily on structure – activity relationships[30, 31] which have been generalized to multiple scaffolds by means of several pharmacophore models.[32-35] The first atomic structure of the tubulin dimers was the electronic crystallographic model of taxol-stabilized zinc-induced straight sheets formed by anti-parallel protofilaments.[36, 37] This structure corresponds to the straight tubulin found in assembled microtubules, but the structure of the curved tubulin dimers targeted by CSL was not deciphered until 2004, when the X-ray crystal structures of the complex of two head to tail tubulin dimers in complex with phosphoproteins of the stathmin family was resolved.[38] The same strategy allowed the subsequent determination of the structures of tubulin in complex with several CSL, including classical CSL carrying trimethoxyphenyl rings, such as DAMA-colchicine (**6**) (DeAcetylMercaptoAcetyl-colchicine),[39] podophyllotoxin (**4**),[39] and colchicine (**3**) itself[40, 41] and new chemotypes displaying distinct binding modes, such as the sulfonamides ABT-751 (**11**) and T138067 (**18**),[42] the pyrrolidindione TN16 (**14**),[42] and the two enantiomeric pyridopyridazines NSC 613862 (CI980) (*S*) (**9**) and NSC613863 (*R*) (**10**).[43] Structure based drug design (SBDD) of CSL began with these complexes being resolved at a modest resolution that provided an unprecedented insight into the structural flexibility of the colchicine site of tubulin.[44-46] Recently the inclusion of a third component, the tubulin tyrosine ligase in crystal growth has improved the achieved resolution in the X-ray refinement,[47, 48] thus providing a better starting point for the SBDD.[32, 49, 50]

The X-ray crystal structures of complexes of CSL with tubulin have defined three sequential binding pockets denoted as zones 1 – 3 (Fig. 2); the first two mainly formed by hydrophobic aminoacids whereas in the third one some polar residues are also found.[44, 45] Most of the co - crystallized ligands occupy only two of the adjacent sites, either 1 and 2 or 2 and 3, with ABT751 (**11**) being the only one in contact with all of the three sites although it does not fully occupy the three of them. Zone 1 corresponds to the binding site for the tropolone (B) ring of colchicine (**3**) which binds in the interfacial surface between the α and β tubulin subunits and binding ligands, therefore, making contact with residues from both subunits. Zone 2 corresponds to the binding pocket for the trimethoxyphenyl ring (A) and mostly extends to β tubulin, including cysteine 241β, important for binding, and the gate residue Leu255β which open or closes the access to Zone 3 depending on the nature of the resident ligand. Zone 3 is more deeply allocated in β tubulin and is lined by several polar residues. Ligands binding at Zones 1 and 2 are not usually continuous moieties but consist of two separated moieties (e.g. the A and B rings of combretastatin A-4 (**5**)) connected by a bridge which makes little contact with the protein, as it points toward an open zone in the interfacial region. By contrast, ligands binding at Zones 2 and 3 show less clear separation between the binding substructures.

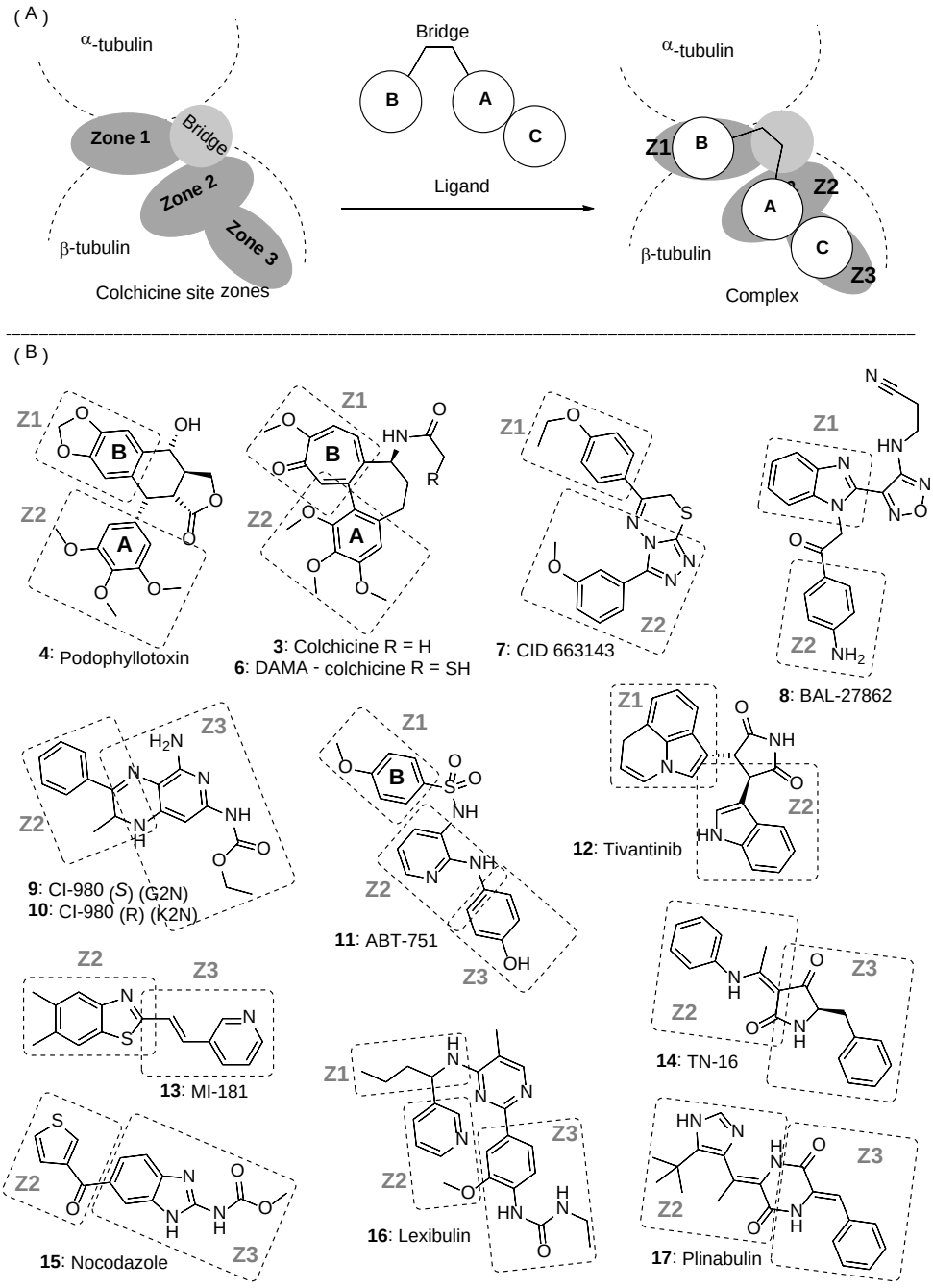


Fig. (2). (A) Schematic representation of the binding of CSL to the site zones.
(B) Disposition adopted by known ligands deduced from X-ray structures.

2. THE PYRIDINE SCAFFOLD

Pyridine can be considered as the result of replacing one methine (CH) of benzene by a nitrogen atom. Further substitution of the methines of pyridine by adding nitrogen atoms generate the azines, many of which are important scaffolds in medicinal chemistry, such as the diazines pyridazine, pyrimidine and pyrazine (Fig. 3).[51-53] The resulting heterocycles are aromatic, but in spite of their similar size dramatic changes in properties and reactivity upon addition of increasing numbers of nitrogen atoms and with respect to the benzenic analogues result, which have important effects on the medicinal chemistry of the compounds. The benzofused pyridines, namely quinoline and isoquinoline and their corresponding aza analogues are also important scaffolds in medicinal chemistry, similar to the pteridine present in folic acid.[54] Additional variations involve azines fused to heterocycles of different sizes, including systems incorporating the azine nitrogens in the fusion. Furthermore, and in contrast to what is

observed for benzenes, all these heterocyclic systems display a great variety of tautomeric forms depending on the substituents introduced on the ring, as evidence the pyridones, thus providing these scaffolds with even more versatility from a medicinal chemistry viewpoint. The totally and partially hydrogenated derivatives behave very much like their acyclic counterparts but are also frequent fragments in medicinal chemistry.[54]

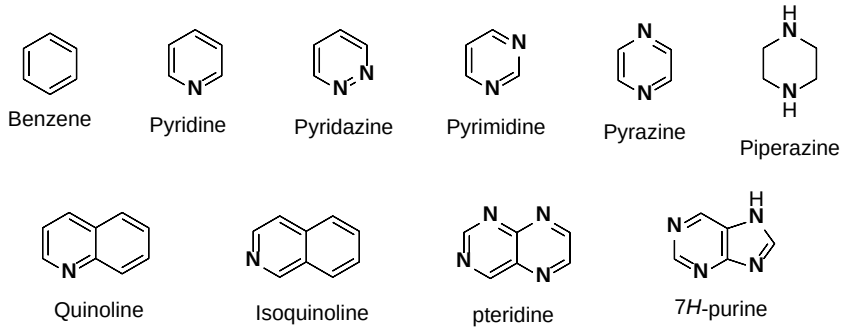


Fig. (3). Structure of benzene and of several azines important in medicinal chemistry.

The initial effect of the introduction of a nitrogen atom into pyridine is its basic character, although unsubstituted pyridine with a pKa of 5.23 is mostly in its neutral form in water. However, introduction of substituents greatly modify the basicity of the system (Fig. 4), and 2- and 4- aminopyridines are expected to be partially and fully protonated in water at physiological pH due to their pKas of 6.9 and 9.2, respectively. The introduction of additional nitrogen into the pyridine ring dramatically reduces their basicity, and the diazines have pKas of 0.4 for pyrazine, 1.1 for pyrimidine and 2.1 for pyridazine, while the triazines show pKa values around 1. On the other hand, the benzofused systems display basicities similar to the non-fused analogues, and pyridine, quinoline and isoquinoline show similar pKa values.[55]

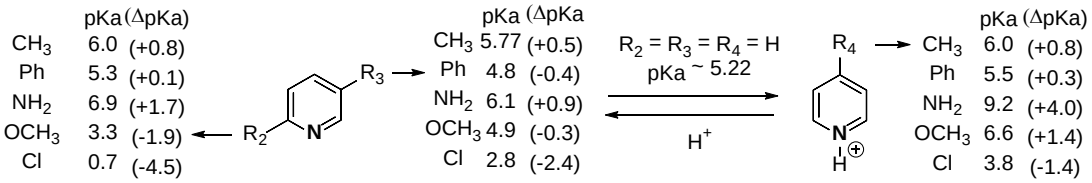


Fig. (4). Protonation equilibrium for pyridine and effects of the substituents on the pKa.

The pyridine nitrogen behaves as a hydrogen bond acceptor of medium strength (pK_{BHX} = 1.86),[56] but this value changes considerably with substituents, with trends similar to those observed in basicity. Pyrimidine and pyrazine are weaker hydrogen bond acceptors while pyridazine and the benzofused systems are of similar strength as pyridine.[57] The protonation of pyridine nitrogen turns it into a strong hydrogen bond donor. Therefore, the replacement of phenyl rings by pyridines or other azines can provide additional hydrogen bonding interactions. However, the energetic consequences are difficult to ascertain, as stronger hydrogen bonding groups also pay higher desolvation penalties.[58] The strong hydrogen bond basicity of pyridines probably makes brain penetration more difficult.[59]

Another important medicinal chemistry property, which experiences substantial variations when going from benzene to pyridine, is polarity. Pyridine is highly polar with a dipole moment of 2.2 D, and the pyridine *N*-oxides are even more polar.[60] The high polarity of azines is often used as a means of reducing the lipophilic character and improving water solubility and oral absorption.[60, 61]

Replacement of electron rich aromatic rings with electron deficient azines is also used as a strategy to improve the biological stability and reduce the metabolic oxidation of drugs. The hydrogen bond basicity of the pyridine nitrogen also contributes to this effect.[57]

3. CLASSIFICATION

Compounds included in this review have been classified according to their structure and accommodation into the colchicine site. In this respect, the presence of more or less extended bridge connecting two aromatic systems, one of them usually a trimethoxyphenyl moiety, is the most general structure of CSL. Nevertheless, many other compounds have been described as binding to the colchicine site of tubulin and several of the crystalline structures of their complexes with tubulin have been published. Accordingly, we have grouped the pyridine based-ligands in four groups: 1) classical, 2) systems lacking the trimethoxyphenyl moiety, 3) quinolones-acridines and 4) polycyclic derivatives. In these groups the pyridine (pyridazine, pyrimidine, pyrazine or other azines) can form part of the basic structure or be introduced as a substituent of a core structure during SAR studies.

3.1. Classical ligands:

We have included in this large group all of the compounds that contain a trimethoxyphenyl system or highly structurally related moiety in an "aromatic ring–bridge–aromatic ring" scaffold. Most of the known CSL belong to this subgroup, as it is the case for colchicines, phenstatins, combretastatins and isocombretastatins... According to the zones of the colchicine site,[44, 45] these ligands locate the trimethoxyphenyl (A ring) in the Zone 2 of the site, the other ring system (B ring) in Zone 1 and the bridge in the space between α and β tubulins. Other compounds included within this group, as those in figures 14 and 15, lack the trimethoxyphenyl moiety, but other rings play the role of binding to Zone 2 in similar pose. Several trimethoxyphenyl derivatives of polycyclic structure are preferably included within group 3.4. Ligands included within this group do not extend to Zone 3 of the colchicine site (Fig. 5).

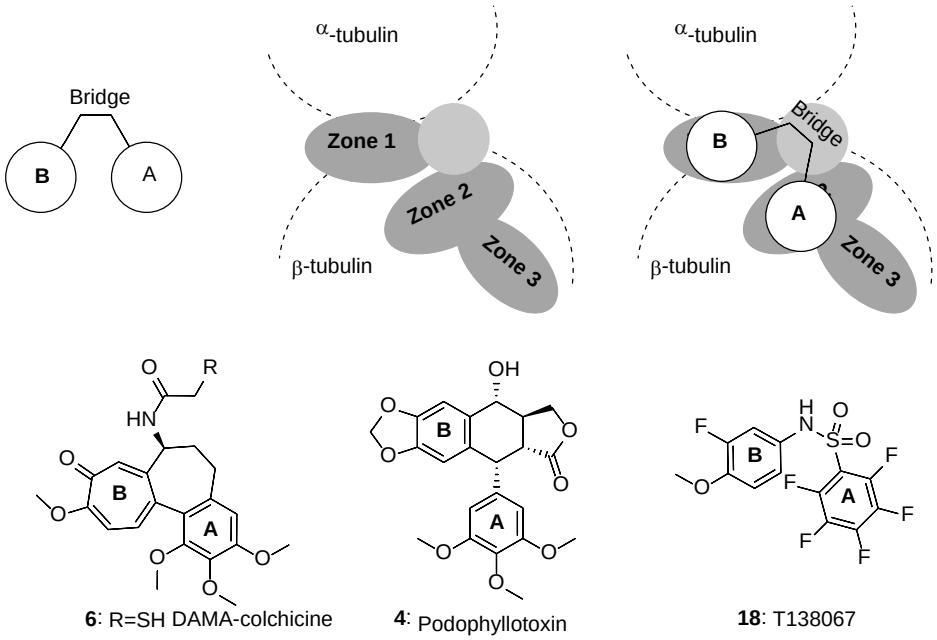


Fig. (5). Classical ligands of the colchicine site, characterized by the presence of a trimethoxyphenyl moiety or related substructure binding to the Zone 2 of the site.

In this group, the pyridine can be present in two of the structural moieties: as a replacement of the ring B system (methoxytropolone of colchicine (**3**), benzodioxole of podophyllotoxin (**4**) or 3-fluoro-4-methoxyphenyl T138067 (**18**)),[62] or as a replacement of the bridge (Fig.6).[63] Another possibility is to introduce the pyridine as a substituent (for example on the bridge), but in this case the contribution to the accommodation in the site is less important.[64]

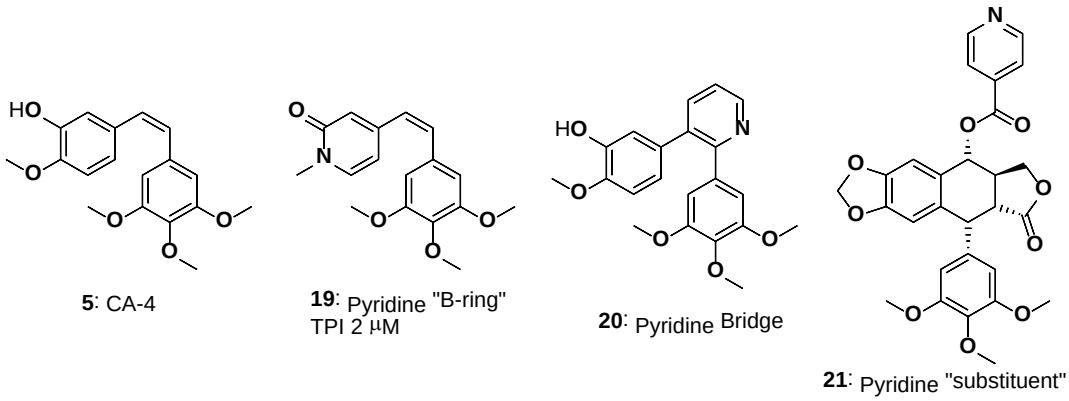


Fig. (6). Classical ligands containing pyridine moieties by replacement of the B ring or the bridge of combretastatin A-4 (**5**) or as substituents in podophyllotoxin (**4**).

3.2. Systems lacking the trimethoxyphenyl moiety

The trimethoxyphenyl moiety is crucial for the binding of a great number of ligands to Zone 2 of the colchicine site. The absence of this substructure, or a highly related one, produces lack of the inhibitory activity on the polymerization of the tubulin in many types of antimitotic agents. But this is not essential, as there are other structural types of ligands that bind to the site accommodating a variety of aromatic systems in Zones 1-3 without the existence of the trimethoxyphenyl or related substructure located in Zone 2.

Among these ligands, those carrying a pyridine present a variety of possibilities and they can be grouped as follows: a) sulfonamides and sulfonates with three aromatic moieties, each one being accommodate in different Zones 1-3, b) Pyrrolidindiones, pyperidindiones and pyperazinones, c) Pyrido[3,4-*b*]pyrazines, and d) 2-(2-(pyrid-3-yl)ethenyl)benzothiazole and other ligands. The last one is a group that has systems connected by a *trans*-double bond, a situation that is detrimental for the inhibitory activity for the classical ligands, such as the large family of combretastatins.[65]

The binding mode is different among these groups. For example, sulfonamides such as ABT-751 (**11**) bind to three zones,[42] a fact that has enhanced the knowledge regarding the colchicine site, including Zone 3 which was not previously considered in the common pharmacophore.[35] On the contrary, pyridopyrazines, such as K2N (**10**) and G2N (**9**) occupy Zones 2 and 3 without binding to Zone 1.[43] The representative binding modes of these types of ligands, whose crystalline structure in complex with tubulin have been described, are shown in Figure (**7**).

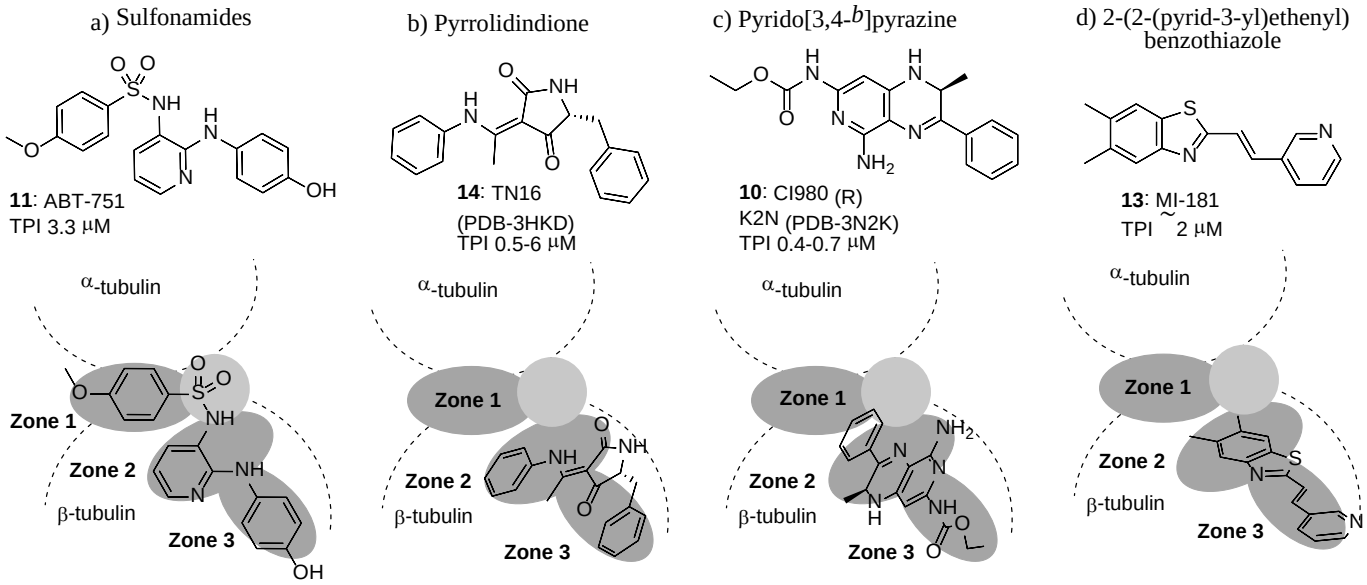


Fig. (7). Types of ligands included in the family characterized by the lack of trimethoxyphenyl moiety or a related substructure.

3.3. Quinolones-acridones

In this group compounds, characterized by the presence of two aromatic systems bonded through a bridge, one of the aromatic systems being bicyclic (quinolones) or tricyclic (acridines and related), have been included (Fig. 8). This family may well be an extension of the classical ligands, but they usually lack the trimethoxyphenyl moiety and can also act as intercalating agents.

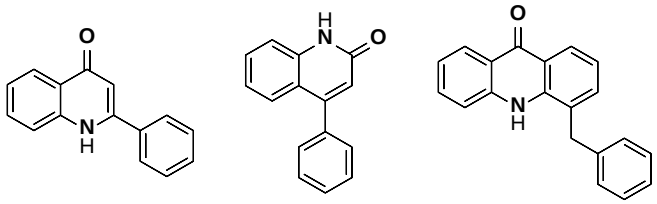


Fig. (8). Generic structures of quinolones and acridones.

3.4. Polycyclic derivatives and other

The fourth group is a miscellaneous one in which, polycyclic systems unrelated to groups 1-3 and the rest of CSL can be included (Fig. 9). For these compounds the binding mode to the colchicine site is not known, except for those with crystalline structures forming complexes with tubulin, and are difficult to classify according to their accommodation to the protein.

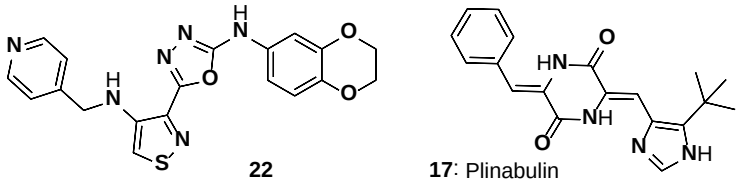


Fig. (9). Structures of representative polycyclic inhibitors of tubulin polymerization.

4. PYRIDINE LIGANDS

In this section, pyridine containing CSL described in recent years are reviewed. Some of them are modifications of known ligands, whereas others have new skeletons characterized by the presence of the pyridine moiety. These compounds are grouped by the structural similarity between them and other readily known, well established, families of CSL.

4.1. Classical ligands

4.1.1. Colchicine derivatives

The use of colchicine is limited because of its toxicity but it has been used as a prototype to prepare new families of potential antitumour drugs with better pharmacological profiles. The acetamido group of thiocolchicine (**23**) was replaced by pyridine carboxylates, and the resulting esters maintained the tubulin inhibitory activity while allowing the aqueous solubility by formation of pyridine hydrochloride salts to be improved (Fig. 10).[66] In contrast, colchicines modified at the C ring as hetero Diels-Alder adducts with pyridinylnitroso reagents have been prepared as prodrugs (**24**) (Fig. 10), with the pyridine rings providing the adequate balance of reactivity in the cycloadditions and stability of the cycloadducts as evidenced by cytotoxic potencies similar

to that of colchicine against PC-3 (prostate cancer) and MCF-7 (breast cancer) cell lines in the absence of in vitro microtubule polymerization inhibition.[67]

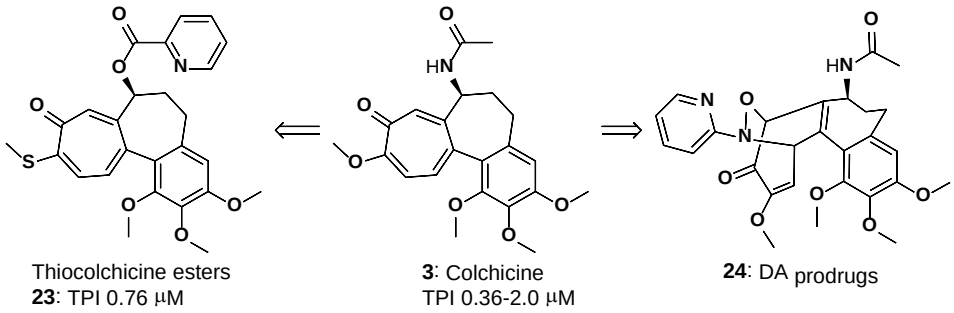


Fig. (10). Colchicine derivatives with pyridine moieties.

4.1.2. Podophyllotoxin derivatives

Podophyllotoxin (**4**) is an aryltetralin lactone isolated from *Podophyllum peltatum* and *Podophyllum emodi* that binds at the colchicine site of tubulin causing microtubule disassembly.[10] Semisynthetic work at Sandoz laboratories fueled by the conviction that glycosylation would improve the pharmacological properties of lignans led to the discovery of the 4'-demethylepipodophyllotoxin derivatives etoposide and teniposide, which were thereafter used as anticancer compounds acting by a totally different mechanism, as they interfere with the scission-reunion step of mammalian topoisomerase II and cause DNA damage.[68] Great efforts, but with limited success, have been devoted to improving the pharmacological properties of podophyllotoxin (**4**), some of them involving the introduction of pyridine rings or related azines (Fig. 11). These changes aimed at improving the pharmacokinetics by increasing the water solubility with polar groups or by reducing the stereochemical instability of the strained, but important for the activity, *trans-trans-cis* stereochemistry of the aryltetralin lactone and also the pharmacodynamics by introducing potential tubulin interacting centers.

The enlargement of the B and E phenyl rings of podophyllotoxin (**4**) as quinoxalines (**29**) or phenazines (**25**) has been of limited success as they result in poorly soluble or much less potent derivatives, possibly due to both not satisfaction of the hydrogen bond acceptors and too much steric bulk, although activities different from tubulin inhibition have arisen as a result.[69, 70] Similar results have been observed after the introduction of pyridine esters on the hydroxyl group of podophyllotoxin (**4**).[64, 71]

One of the earliest aza-podophyllotoxin examples was the conversion of the aryltetralin lactone into an aryltetrahydroisoquinoline cyclic carbamate by substitution of the easily epimerized C2 carbon by a nitrogen atom, thus preventing the inactivation to *cis* lactones of the picropodophyllin series.[72, 73] Despite favorable initial findings, the focus soon turned to dual topoisomerase I and II targeting compounds such as azatoxin, although this route seems to have been abandoned.[74] The replacement of both the C2 and the C4 carbons by nitrogen results in active compounds with increased stereochemical stability and in vivo activity when compared with podophyllotoxin (**4**).[75] The development of synthetic methodologies towards 4-aza-2,3-didehydropodophyllotoxin (**26, 28**) analogues, such as the one-step multicomponent reactions of anilines or heterocyclic amines with tetronic acid or related reagents and aldehydes, has enabled fast access to podophyllotoxin-mimetic compounds.[76-78] The benzo or heteroaryl fused dihydropyridine scaffold preserves the active conformation of podophyllotoxin, (**4**) but with less troublesome stereochemical centers.[78, 79] Structurally related triazolopyrimidines (**30**) selected by virtual screening protocols have also been shown to possess antitubulin activities.[80]

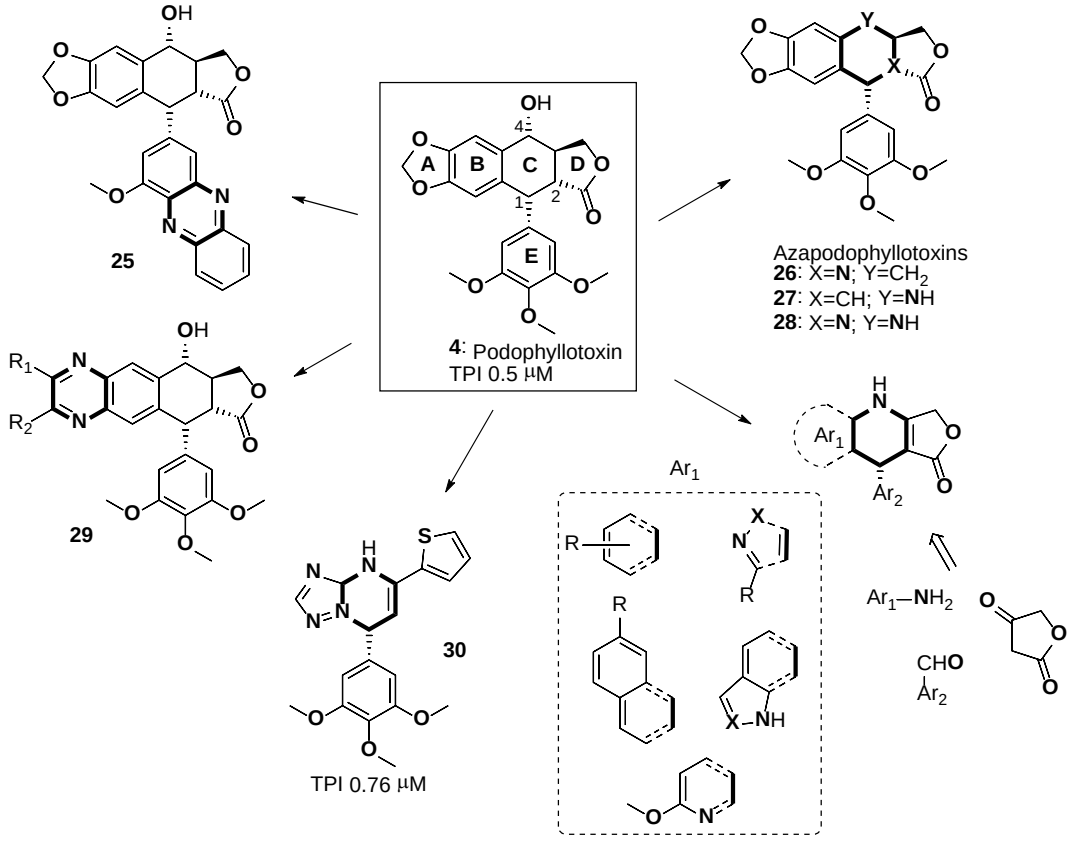


Fig. (11). Podophyllotoxin derivatives with azine modifications. The azines have been highlighted by means of thicker bonds.

4.1.3. *Noscapine derivatives*

Noscapine (**31**) is a phthalideisoquinoline alkaloid isolated from *Papaver somniferum* used as a cough suppressant since mid-1950s. It has very favourable pharmacokinetic properties[81] as it is rapidly absorbed after oral administration with a bioavailability of 30% and a low toxicity profile.[82] It suffers first pass metabolism mainly by breakage of the bond between the tetrahydroisoquinoline and the phthalide.[83] Recently, noscapine (**31**) has been shown to arrest cells at mitosis by inhibiting tubulin polymerization, although with moderately weak activity, and has entered clinical testing[84] and many of its derivatives have been described and have shown improved activity profiles, thus generating meconine (**34**) and (hydro)cotamine (**35,36**).[85, 86] Noscapine (**31**) binds weakly and nonspecifically to tubulin at a site different from the colchicine domain but 7-demethoxy-7-aminonoscapine (**33**) is a more potent cytotoxic agent acting as a tubulin inhibitor competitive with nocodazole, an established colchicine domain binder, but unfortunately suffers from low metabolic stability.(Fig. 12)[87, 88] The formation of carbamates or esters at the 7 position of the benzofuranone results in highly potent cytotoxic compounds, presumably acting as prodrugs of the parent amine or phenol.[89] Noscapine analogues modified at the 9-position also display potent cytotoxic activity but they are weak tubulin polymerization inhibitors (TPI) and compete with colchicine for binding only at very high concentrations.[90]

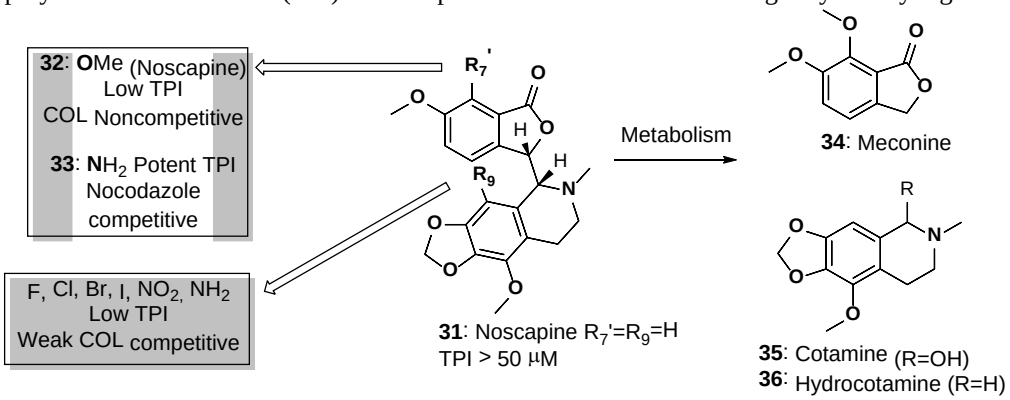


Fig. (12). Modifications on the noscapine structure leading to improved cytotoxic potency and main products of hydrolytic metabolism.

4.1.4. *Combretastatin derivatives*

The combretastatins form a family of natural products originally isolated by Pettit et al. from the bark of the South African bush willow tree *Combretum caffrum*, including stilbenes, dihydrostilbenes, phenanthrenes and the biosynthetically unrelated macrocyclic lactones known as combretastatins D.[91] The most successful of the combretastatins are the *cis* stilbenes known as combretastatins A, some of which have reached clinical trials for the treatment of diverse types of cancers, as for instance the phosphate prodrugs of combretastatin A-4 (**5**) and combretastatin A1. The parent drugs are TPI acting at the colchicine site of tubulin which results in potent cytotoxicity against several cancer cell types and also in vascular disrupting activity.[25] As a result of the success story of these natural combretastatins, many synthetic derivatives have been prepared in an attempt to overcome some of their main drawbacks: their low aqueous solubility which requires the formation of prodrugs, their chemical and metabolic instability due to the easy isomerization of the active *cis* isomers to the thermodynamically favored but inactive *trans* isomers, and insufficient tumour cell killing potency which results in survival of the malignant cells at the tumour rims with eventual regeneration of the tumour mass.[92, 93] The combretastatins are diaryl *cis* stilbenes and the main structural modifications involving the introduction of pyridines or related systems have targeted either aromatic ring and/or the connecting bridge. Along with these modifications, new families of related tubulin inhibitory compounds with bridges of different lengths have emerged, such as the phenstatins (diarylketones), the isocombretastatins (1,1-diarylethenes), the diarylamines and the chalcones.[12, 94]

4.1.4.1 Replacement of the phenyl rings by azines

The 3-hydroxy-4-methoxyphenyl (guaiacol) B ring of combretastatin A-4 (**5**) and related analogues can be successfully changed to a 4-methoxyphenyl ring, a 3-amino-4-methoxyphenyl ring or its amides, naphthyl rings, indole rings linked by several positions and even bigger carbazole rings, thus showing that it is its more easily replaced moiety. Replacement of the B-ring of deoxycombretastatin A-4 by pyridines or other diazines results in a remarkable increase in solubility at the expense of a mild reduction in TPI and cytotoxicity.[62] Further extension to quinolines or quinoxalines have resulted in a preference for the nitrogen on the ring not connected to the bridge and a potency reduction for both the combretastatins'[95] and the phenstatins'[96-98] series, both of them being even slightly less potent than the corresponding naphthalenes.[99-102] Whereas introduction of methoxy groups on the quinoline reduced the potency of the resulting derivatives, 5-amino-6-methoxy-2-(3',4',5'-trimethoxybenzoyl)quinoline (**38**) showed improved TPI when compared with combretastatin A-4 (**5**) and subnanomolar antiproliferative potency.[95] This substitution pattern is reminiscent to that of the active form (AVE-8063 (**39**)) of ombrabulin, an aminocombretastatin prodrug in clinical evaluation.[103] The addition of the quinoline nitrogen not only provides high potency but might also improve the solubility so that no solubilizing prodrugs are further required, thus avoiding additional complications.[104] Replacement of the amino group by larger moieties or the use of different bridges between the two aromatic moieties did not make an improvement on the activity profile of the resulting compounds. (Fig. 13)[105-108]

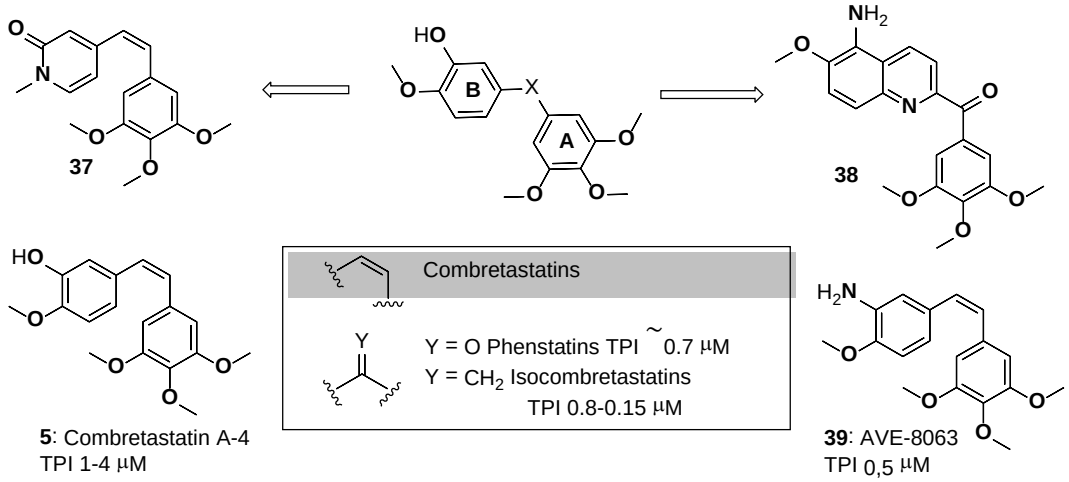


Fig. (13). Combretastatin analogues with phenyl rings replaced by azines.

Remarkably, in a series of 3-(3',4',5'-trimethoxyanilino)benzothiophene-2-carboxylate derivatives with tubulin inhibitory potencies and cytotoxic activities similar to that of combretastatin A-4 (**5**) the introduction of nitrogen atoms on the benzothiophene skeleton yielding thieno[2,3-*b*]pyridines resulted in a substantial potency increase against several tumour cell lines.[109] The thieno[2,3-*b*]pyridine (**43**) required a tailored synthesis from 2-chloropyridine-3-carbonitrile and a thioglycolate, followed by diazotation of the resulting amine and substitution by bromine, previous to the usual Buchwald-type coupling with trimethoxyaniline. Related thieno[2,3-*c*]pyridines (**44**) and tetrahydro analogues (**45**)[110, 111] also maintain the high TPI and antiproliferative potencies. (Fig. 14)

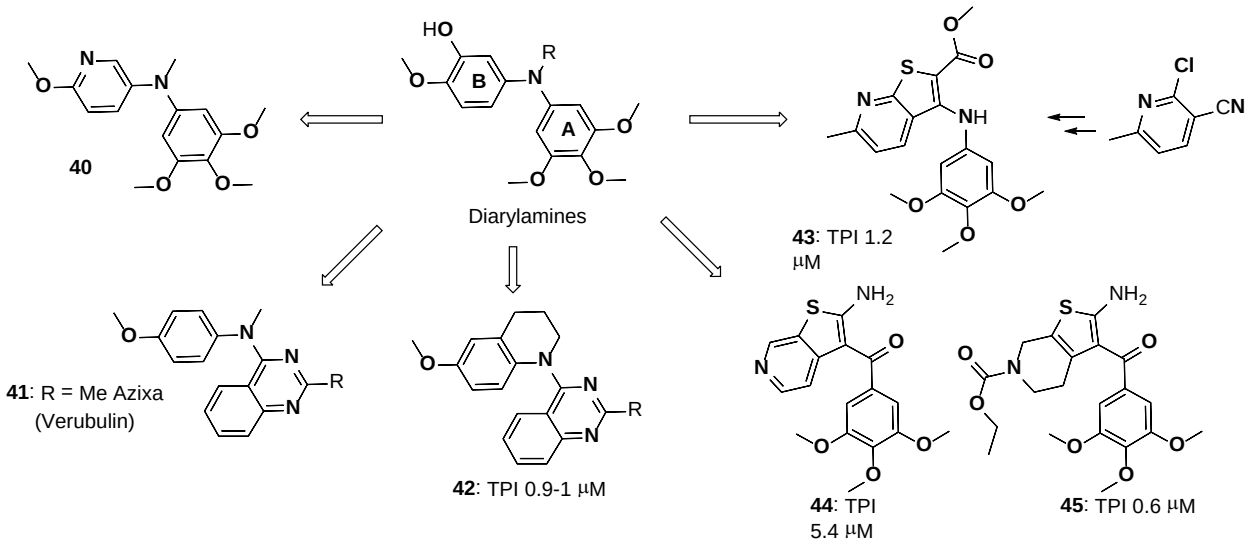


Fig. (14). Diarylamine combretastatin analogues with phenyl rings replaced by azines.

In a structurally related series of *N*-aryl-3,4,5-trimethoxyanilines a quinoline or a methoxypyridine results in significant reductions in potency when compared with *p*-methoxyphenyl rings, naphthalenes, or indoles.[112] This overall different behaviour is also reflected on the amino bridge, where methylation is required in order to achieve cytotoxic activity at a nanomolar range.

Within this series of amino methylated diarylanilines SAR elaboration, after a cell- and caspase-based apoptosis high-throughput screening (HTS) assay, found that replacement of the trimethoxyphenyl ring by quinazolines results in potent cytotoxic compounds,[113, 114] which led to the discovery of Azixa (Verubulin) (**41**), now in phase II clinical trials.[13, 17, 114-116] In a related study, replacement of the trimethoxyphenyl ring by 3,6-disubstituted pyridines results in compounds that are only cytotoxic at micromolar concentrations[117] and even less potent when the bridge is an NH group or an oxygen atom.[118] However, cyclization of the aniline nitrogen to one of the aromatic rings to form a tetrahydroquinoline increases the potency tenfold and one hundred to one thousand fold when quinazolines become the substituting aryl ring on the nitrogen.[119, 120] Further substitution of the quinazoline by different quinolines, isoquinolines or purines do not improve the activity profile but the physicochemical properties can be substantially improved upon by the chemical modification of the quinazoline 2 substituent (**42**).[121]

In a related approach of substitution of the phenyl rings of known CSL by pyridine analogues (Fig. 15) and based on the model microtubule destabilizer chalcone MDL-27048 (**47**)[122] natural products (as for example **46**) were selected from a pharmacophore search[123] or synthesized as cyclopropylcarboxamide analogues, (**48**)[124] but in both instances only weak potencies were observed. Similarly, incorporation of pyridine rings on 1,2,4-oxadiazole and related scaffolds[125-129] results in substantial potency reduction.

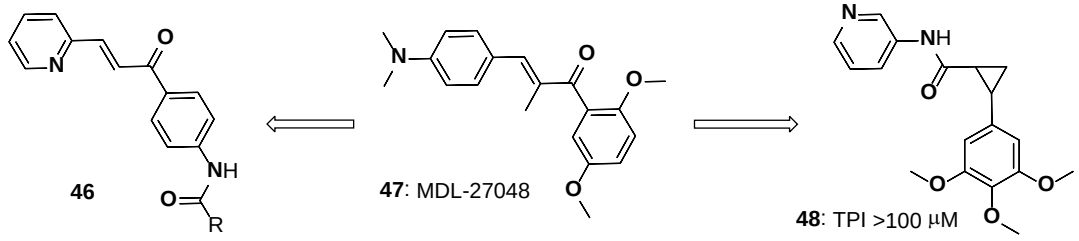


Fig. (15). Chalcone analogues with phenyl rings replaced by pyridines.

4.1.4.2 Pyridine as a replacement of the bridge

Soon after the discovery of the combretastatins the *cis* double bond emerged as a site that could be used for improvements due to its metabolic and chemical isomerization to the biologically inactive *trans* isomers.[130] As a result, a large number of analogues of 1,2- or 1,3-diaryl five- or six- membered carbocyclic or heterocyclic rings (**49**), larger systems such as the benzofused ones, and others such as those with the bridge cyclized onto one of the pending aromatic rings have been prepared and assayed. As a result, compounds with not only high configurational stability but also with enhanced potency and water solubility have been obtained.[58, 91, 92, 131, 132]

The first combretastatin analogues with aromatic azine bridges kept the aryl substituents at contiguous positions of either pyridines[63] or pyrimidines or pyrazines.[101, 133] The resulting pyridines and diazines (**49**) were more potent than the corresponding (inactive) phenyl derivatives [131] but much less so than the original stilbenes or analogues with five membered rings.[63, 132] For these pyridine analogues higher potency was observed when the pyridine nitrogen was closer to the trimethoxyphenyl ring than when it pointed in the opposite direction (Fig. 16). Larger systems such as pyridopyrazines, and quinoxalines caused a drastic reduction in potency, which when combined with similar results obtained for other benzofused systems, such as the indoles, led to the conclusion that there was a limited available space in tubulin for the bridge.[63, 132] Structural information and further structure – activity studies point to a more severe restriction in terms of protrusion out of the plane of ring B, as would be the case for the aromatic systems considered here, rather than an actual steric limitation.[58, 134] In a related strategy, the introduction of pyridones as bridge resulted in a substantial potency reduction. [135]

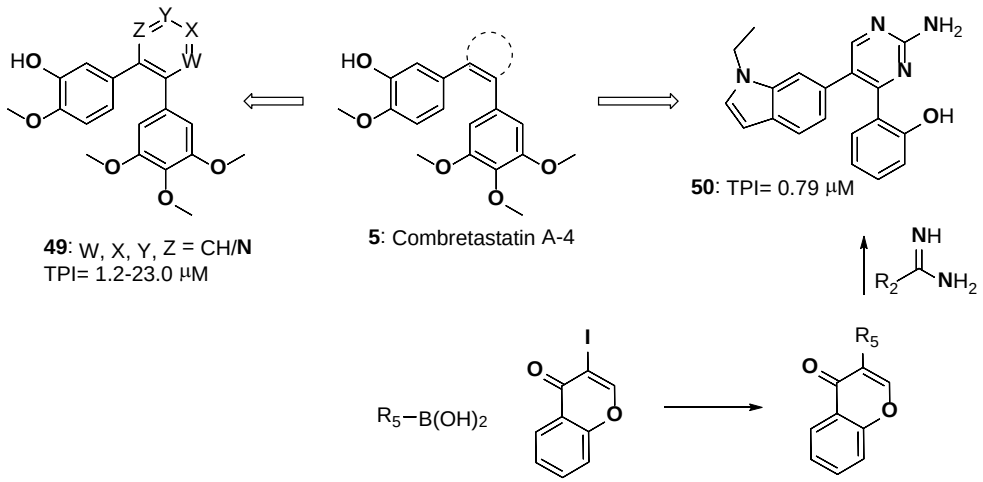


Fig. (16). Combretastatin analogues with azine bridges.

The association of combinatorial synthesis of focused chemical libraries with the pharmacological testing of the synthesized compounds is a powerful technique for the discovery of novel scaffolds deploying biological activities. A combinatorial synthesis of a 2,4,5-substituted pyrimidine library using a sequential three-component, one-pot reaction between a iodochromone, an arylboronic acid, and an amidine followed by its assay for the inhibition of the human hepatocellular carcinoma cell line uncovered 2-methyl-4,5-diarylpyrimidines as a new active scaffold with micromolar potency.[136] Subsequent SAR optimization of the substituents of the positions 2 and 5 of the pyrimidine led to the discovery of 2-amino-4,5-diarylpyrimidines (**50**) as potent inhibitors of tubulin polymerization through binding at the colchicine site and potent cytotoxic compounds. (Fig. 16)[137, 138]

Azines with two aryl substituents on non-contiguous (e.g. 2,6-diarylpyridines) atoms have also been synthesized as configurationally locked colchicine site inhibitors based on chalcones. (Fig. 17) 4,6-diarylthioxopyrimidines analogues (**56**) were prepared by condensation of appropriately substituted chalcones with thiourea in refluxing methanol and the resulting compounds were shown to possess improved water solubility but mild cytotoxic potency.[12, 94, 139, 140] Following a similar rationale, 2,6-diarylpyridines (**52**) were synthesized and shown to be highly potent cytotoxic, vascular disrupting, and tubulin inhibitory compounds with improved bioavailability with respect to combretastatin A-4 (**5**) after oral administration to mice.[141] As indicated above for the contiguous diaryl substitutions on large aromatic systems, attempts to fix the double bond configuration of antimitotic chalcones by construction of imidazo[1,2-*a*]-pyridine/pyrazines (**53, 54**) using a multicomponent Ugi type multi-component reaction and replacement by an amino group[142] or altogether removal of the carbonyl (**55**)[143] to allow a good disposition of the pendant trimethoxyphenyl group disposition of the aromatic rings resulted in a potency reduction compared to

combretastatin A-4 with the extra nitrogen contributing very little to the activity or a complete loss of the tubulin inhibitory activity, respectively.

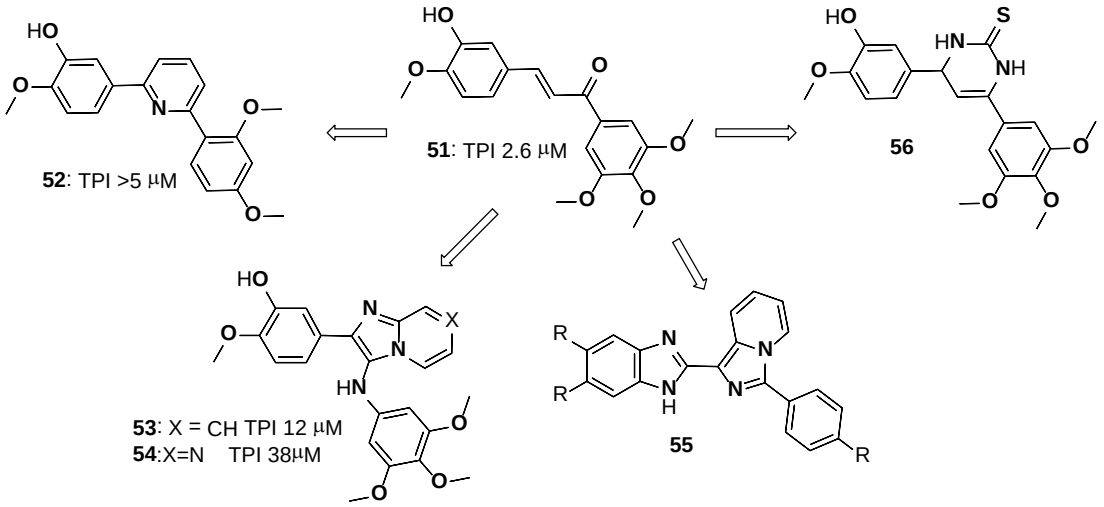


Fig. (17). Chalcone analogues with azine bridges.

The incessant search for new CSL with improved pharmacodynamic, pharmacokinetic, and toxicity profiles has led to the discovery of novel scaffolds which in turn have resorted to the incorporation of azines as a way of optimizing their properties. Thus, due to the biological lability of the ester moiety of 3-(3,4,5-trimethoxyphenylsulfanyl)indole-2-carboxylates (**60**) (a.k.a. arylthioindoles) or related aroylindoles,[144-148] the carboxylates have been replaced by azoles, pyridines and other heterocycles with great success. Several of the resulting compounds are more potent than combretastatin A-4 (**5**) as TPI and cytotoxic compounds against human cancer cell lines including multidrug - resistant (MDR) ones, but also showed improved water solubility, stability against human liver microsomal enzymes, and intestinal permeability in model assays, with pyridines only surpassed by the imidazole and triazole analogues.[129, 149, 150] The 4-substituted methoxybenzoyl-aryl-thiazoles (SMARTs (**57**)),[129] 2-aryl-4-benzoylimidazoles (ABIs (**58**)),[150] and phenyl-amino-thiazoles (PATs (**59**))[149] are potent anticancer agents targeting tubulin by binding to the colchicine which are rapidly metabolized at the trimethoxyphenyl ring or at the ketone by liver microsomal enzymes, thus reducing their bioavailability. Ring fusion of the carbonyl into the azole by formation of the corresponding pyridoazoles improved the metabolic stability and the cytotoxicity. (Fig. 18) [151]

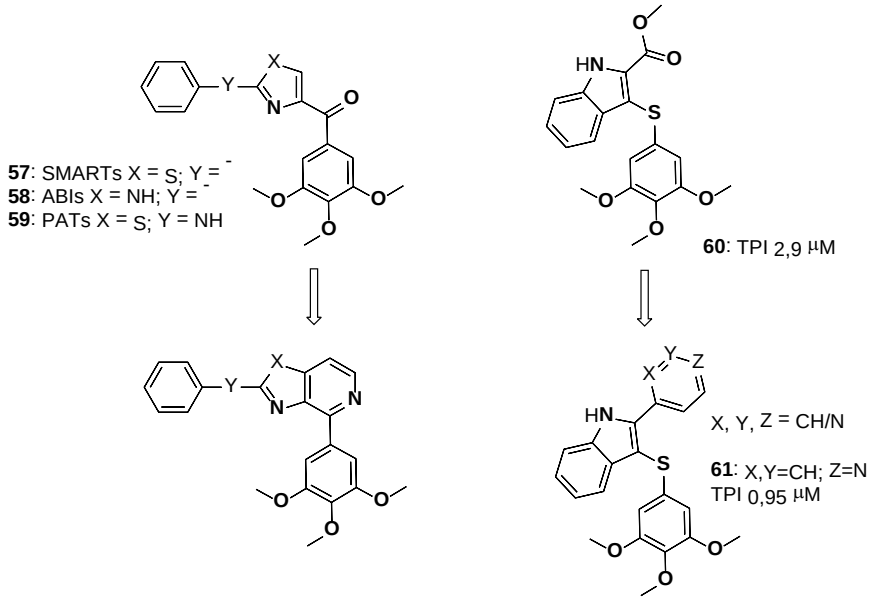


Fig. (18). Azine bridged analogues.

4.2. Systems lacking the trimethoxyphenyl moiety

As the number of ligands binding at the colchicine site of tubulin but lacking the trimethoxyphenyl ring of colchicine (**3**), podophyllotoxin (**4**) and combretastatins started to steadily increase, it became apparent that it could be replaced by several other groups, often incorporating azines or related ring systems.[12, 94] Along with this finding, a new binding zone (Zone C) in the colchicine site of tubulin sitting deeper in the β-tubulin subunit and not being occupied by the ligands carrying the trimethoxyphenyl ring was uncovered for some of these chemical skeletons.[44, 45] When the trimethoxyphenyl ring is present Leu255β closes the access to this pocket, but with less bulky moieties it adopts a different conformation which uncovers a groove lined by several polar groups.[44] This polar cavity has also been occupied by several substituted azines thus increasing the polarity and the aqueous solubility of such ligands and providing improved pharmacokinetics.[32, 43]

4.2.1. Sulfonamides and sulfonates

The sulfonamide group (-SO_2-NH-) is present in many clinically useful drugs with antibacterial, diuretic, hypoglycemic, anti-thyroid, anti-inflammatory, antihypertensive, anti-parasitic and antitumour activities, amongst others.[152-154] These actions are mediated by interactions with many different targets, and anticancer activities have been described for sulfonamides acting on carbonic anhydrases, histone deacetylases, the NF-kB, matrix metalloproteases, tubulin, and many other targets.

Taking into account the privileged structure of sulfonamide drugs that provides orally active drugs with a manifold of biological activities,[155] researchers from the Japanese Eisai Company discovered in the early nineties the sulfonamide ABT-751 as an orally bioavailable non MDR substrate[156] antimitotic drug with antitumour activity against solid tumours,[157] which was later acquired by Abbott Labs and renamed ABT-751 (**11**). The pyridine ring of ABT-751 (**11**) is important for a good oral efficacy in the sulfonamide series[155, 156] but surprisingly, for sulfonate analogues of ABT-751, the pyridine nitrogen causes a drastic potency reduction compared to the phenyl analogues.[158-163] Unfortunately, so far clinical trials for ABT-751 (**11**) suggest that it is not sufficiently potent to warrant further development,[18, 19, 164] but its oral bioavailability, an acceptable toxicity profile and its insensitivity to multidrug resistance pumps (MDR) justify recent research aimed at finding more potent analogues of ABT-751. The X-ray crystal structure of ABT-751 (**11**) in complex with tubulin[42] showed a previously unpredictable binding mode which for the first time confirmed that the pyridine ring acts as a replacement of the trimethoxyphenyl ring and partially uncovered the new binding zone in the colchicine site. This structure provided a rationale for the binding of related structures to tubulin and explained the important role played by the pyridine nitrogen on the potency of ABT-751 (**11**) against xenograft models when compared to phenyl analogues.

The recognition of the structural equivalence of the pyridine ring of ABT-751 (**11**) and the trimethoxyphenyl ring of combretastatin A-4 (**5**) when bound to tubulin led to the synthesis of hybrid molecules with a sulfonamide linker connecting a carbazole ring (deemed as an expanded B ring) and a trimethoxyphenyl ring (**62**) (Fig. 19).[165] The resulting hybrid molecule (**62**) presented antiproliferative activity *in vitro* and *in vivo* antitumour effectiveness, an activity surprisingly not due to inhibition of tubulin polymerization. Further elaboration of the hypothesis led to sulfonamide IG-105 (**63**) by the replacement of the 3,4,5-trimethoxyaniline with a 2,6-dimethoxypyridin-3-amine, which strongly inhibited tubulin polymerization and showed a potent activity against human leukemia and solid tumours in vitro and in vivo.[166, 167] The potency of the 2,6-dimethoxy-3-pyridyl moiety seems to be confined to the sulfonamides as it inclusion in combretastatin analogues results in a significant potency reduction.[167] The chameleonic nature of the sulfonamide group showed up in other structurally related sulfonamides acting on targets different from tubulin, such as for instance indisulam (**64**)[168] or the Polo-like Kinase inhibitors HMN-176 (**66**) and its prodrug HMN-214 (**65**).[169]

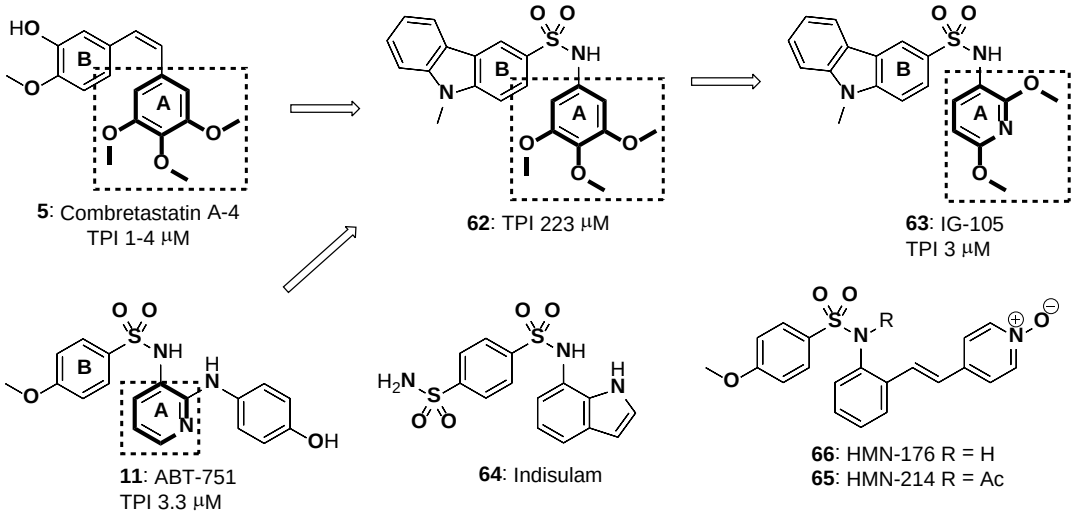


Fig. (19). Rationale for the design of combretastatin A-4 (**5**) and ABT-751 (**11**) hybrids and structures of the related non tubulin inhibitors HMN-176 (**66**), its prodrug HMN-214 (**65**) and indisulam (**64**).

The formation of an additional cycle incorporating the sulfonamide nitrogen and the pyridine ring of ABT-751 (**11**) has also led to conformationally restricted analogues with increased anti-tubulin potency (Fig. 20). A pyridine nitrogen is favored in the new A rings when the additional ring is either aromatic (indole[170] and 6-azaindole[171] analogues (**68**-**75**)) but partially reduced indoline[170, 172] analogues are also very potent. For these conformationally restricted analogues of ABT-751 (**11**), the aniline substituent directed towards the deep groove in β tubulin can be efficiently replaced by a 3- or 4-pyridyl[172] or isonicotinamido[170] substituent or even the corresponding *N*-oxide.[173] In a related strategy, ABT-751 (**11**) has been cyclized to benzopyridothiadiazepine (**67**)[174] and benzopyridooxathiazepine [175] derivatives with a pendant phenethyl group and a substantial potency reduction was observed. Although moving the phenethyl substituent to the pyridine nitrogen with concomitant double bond reorganization and ring substitutions led again to highly potent inhibitors of tubulin polymerization. *In vitro* stability studies on this new class of sulfonamide and sulfonate analogues suggest that their chemical and metabolic stability are sufficient enough to further pursue in vivo development of their use as potential antitumour drugs.[176-178]

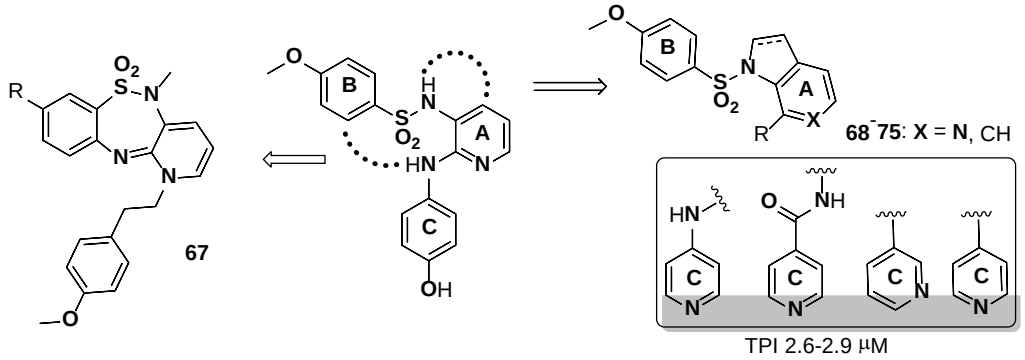


Fig. (20). Design of conformationally restricted analogues of ABT-751 (**11**) and successful pyridine substituents occupying the zone 3.

In the sulfonamide series the pyridine A ring has proven important for the achievement of oral bioavailability, probably due to its high polarity. The diaminopyridines are expected to be basic centers, but the impact of protonation on tubulin binding seems to be limited, as systems with reduced basicity, such as the azaindoles or even lacking it as the indoles or the phenyl analogues, are still potent cytotoxic and tubulin polymerization inhibitory compounds.[179]

4.2.2. Pyrrolidinediones and piperazindiones

Since the discovery of phenylahistin (**76**) and plinabulin (**17**) (Fig. 21) in the late nineties,[180-183] the diketopyperazines (DKP) have been developed as a new family of antitubulin agents, binding at the colchicine site. These compounds are moderately potent in comparison with reference combretastatin A-4 (**5**), but recently the coupling of the benzophenone[184] moiety to the DKP has increased the potency of these compounds and the structure activity relationships demonstrated that a 2-pyridyl ring bonded to the DKP is highly convenient for the activity (**78**). The benzophenone can also be replaced by a pyridine. For all of them, a

hydrogen bond between de pyridine type Nitrogen and the DKP NH (reversed in TN-16 (**14**)), flattening the structure in this part of the molecule, seems to be essential.

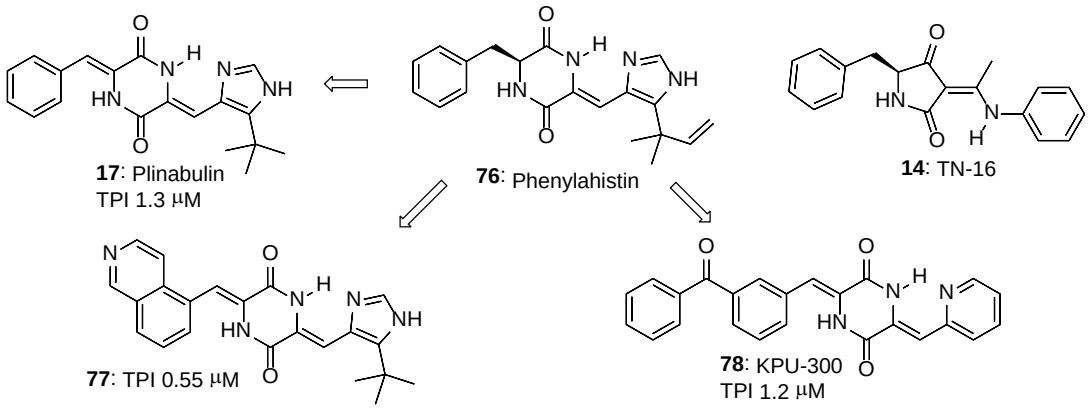


Fig. (21). Structures of (-)-phenylahistin (**76**) and related plinabulin (**17**) and azine derivatives. The structure of the pyrrolidindione TN-16 (**14**) is shown for comparison.

Recently, the structure of plinabulin (**17**) when in complex with tubulin has been solved, and the structural resemblance with TN-16 (**14**)[42] as well as the establishment of the intramolecular hydrogen bond has been confirmed, therefore unveiling the important role of the DKP scaffold.[185]

*4.2.3. Pyrido[3,4-*b*]pyrazines*

Originally conceived as deaza methotrexate analogues, it soon became clear that the pyrido[3,4-*b*]pyrazines lacked antifolic activity and were instead antimitotic agents acting on the colchicine site of tubulin.[186] Structure-activity relationship studies indicated that the basic diaminopyridine is an essential structural requirement.[187, 188] The fused dihydropyrazine increases the pyridine basicity and tolerates diverse substituents in positions 2 and 3.[189]

Clinical trials with mivobulin (CI-980) showed that it lacks sufficient activity in the treatment of disseminated malignant melanoma [190] metastatic colorectal carcinoma[191] progressive malignant gliomas[192] and soft tissue sarcomas.[193]

The X-ray crystal structure of tubulin in complex with mivobulin and its enantiomer[43] provided an explanation for the observed structure – activity relationships. The dihydropyrazine and the 3-substituent occupy the pocket of the trimethoxyphenyl A ring and the protonated diaminopyridine forms a salt bridge with Glu200β, thus explaining the significance of the basic pyridine nitrogen.

4.2.4. Quinolones and acridones

The 2-phenylquinolones were initially synthesized as aza analogues of antitumour flavones (Fig. 22), and found to possess potent cytotoxicity against colon and lung cancer cells by exerting antimitotic effect through the interaction with the colchicine site of tubulin.[194] Their synthesis and biological activity has been recently reviewed,[195] and only the impact of the modifications on the biological activity will be discussed here. The basic pharmacophore of this family of colchicine site binders is an (*aza*)-2-phenyl-1*H*-quinolin-4-one, as evidence potent analogues without the optional nitrogen, such as the phenylquinolinones,[196, 197] as well as with it such as the phenylnaphthiridinones,[198, 199] and the phenylquinazolinones.[200] Interestingly, the reduction of the 2-3 double bond results in more potent compounds, with the *S* enantiomer being the eutomer.[195, 201] With respect to the decoration on the pharmacophore, a 6,7-methylenedioxy or methoxy, halogens and amines (e.g. pyrrolidin-1-yl) substituents at the 6 position[196, 202, 203] and methoxy groups[196, 197] or fluorine atoms[204, 205] at the 3' position of the pendant phenyl ring provide the best results. The basic pharmacophore has also been extended by bioisosteric replacements of the 2-phenyl ring that also lead to potent analogues, with the 2-benzothiazole ring providing optimal results.[206, 207] Recently, the skeleton has been further modified by fused pyrrole rings giving the phenylpyrroloquinolones, which endow high tubulin binding potencies when the pyrrole nitrogen is substituted with small hydrophobic groups. The phenylpyrroloquinolones are

potent TPI but additional mechanisms are also operative, such as inhibition of the aromatase for the pyrrolo[2,3-*h*]quinolinones[208, 209] and kinase inhibition for the pyrrolo[3,2-*f*]quinolinones.[210-212] This manifold mechanistic behaviour probably spreads to other members of the family as well.[213-215]

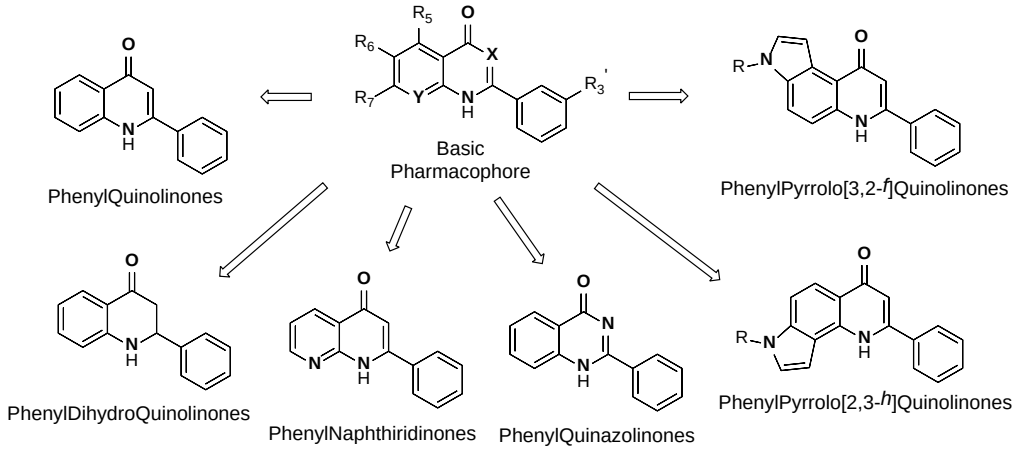


Fig. (22). Generic structures of quinolone inhibitors of tubulin polymerization.

The most potent cytotoxic agents of the different series have been assayed in mice models but neither the quinolones[203, 214] nor their aza-analogues[216] provide enough aqueous solubility for oral dosing studies. Therefore, an unprecedented prodrug formation on the quinolone, i.e. the formation of a phosphate ester on the tautomeric hydroxyquinoline, has allowed p.o. administration with fast regeneration of the active species after absorption.[203] Notably, the effect of the prodrug after oral administration mirrored the antitumour effect of the same intraperitoneal dose of the active compound and the compound showed a promising safety profile. More conventional prodrug approaches implying the formation of sodium phosphates on phenolic hydroxyl groups have also been attempted.[214]

Modification of the basic flavone pharmacophore by translation of the 2-phenyl ring, such as in the 4-phenylquinolin-2(1*H*)-ones, leads to *aza*-podophyllotoxin analogues with much less potent cytotoxic activity,[217] but elongation of the linker to the quinoline has resulted again in nanomolar cytotoxic compounds.[218] Structurally related acridones with pendant phenethyl substituent have also been shown to be potent TPI, an activity which is not accompanied by potent cytotoxicity against cancer cells *in vitro*.[219]

4.3. Polycyclic derivatives and other

The first example of polycyclic derivatives is CYT997 (**79**), whose structure contains pyridyl and pyrimidyl residues.

Screening of Cytopia´s compound library against DU145 and PC3 cancer cell lines followed by exhaustive SAR optimization of the initial hits led to the discovery of CYT997 (**79**) (Fig. 23),[220] which is actually in phase II clinical trials for the treatment of glioblastoma and multiple myeloma because it is able to depolymerize microtubules, it presents cytotoxicity mediated by caspases and PI3K AKt/mTOr and it also shows activity as vascular disrupting agent.[221-224]

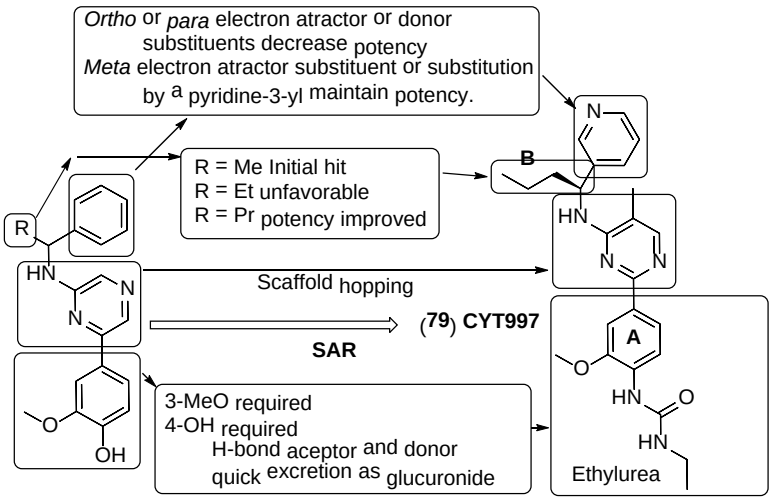


Fig. (23). Structural optimization strategies towards CYT997 (**79**).

Recently, the X-ray crystal structure of CYT997 (**79**) in complex with tubulin has been resolved. The structure shows a completely novel arrangement of the structural elements with respect to previous structures, with the methoxyphenylurea substituting for the trimethoxyphenyl ring and with the aminopyrimidine and the pyridine occupying the intermediate region corresponding to the tetrahydrocycloheptane ring of colchicine and the propyl extending towards the tropolone – binding zone.

The azine systems can also be integrated in polycyclic ligands of the colchicine site in different ways, as a central ring with pending substituents or as substituents of other central structures.

Starting from indole derivatives,[225] designed as ligands of hormone receptors but also showing antimitotic effects by inhibition of cell cycle at G2/M, a family of polycyclic systems based on indole and pyridine was developed (Fig. 24). For these compounds, that are inhibitors of tubulin polymerization by colchicine competitive binding, the unsubstituted indole is required for the activity, but the requirement of the pyridine ring is not demonstrated because most of them contain the pyridine and the few exceptions are also as potent as pyridine containing representatives. Docking studies of these compounds point to binding in a site overlapping the GTP and the colchicine sites and do not justify the requirement of the pyridine system. Among these compounds, the sulfonamide LP-261 (**81**) administered orally has demonstrated its efficacy *in vivo* and used in combination with other anticancer agents and it is currently in clinical development.[226]

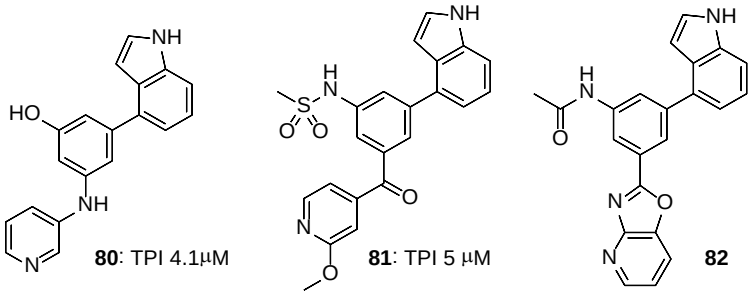


Fig. (24). 3-indol-5-yl-1-pyridylbenzenes.

Several compound families characterized by linear extended polycyclic structures with one or two central heterocycles (for most of them two azoles), and with two ending moieties deemed to occupy zones 1 and 2. Such end rings can be a benzodioxole and a trimethoxyphenyl ring respectively, as those of podophyllotoxin (**4**) but also more divergent modifications (e.g. pyridines at both ends) have been assayed (families I – IV in Fig. 25). As there is no structural information available and they are rather flexible systems, they might accommodate themselves in diverse dispositions in the colchicine site, as suggested by docking results and therefore the indicated correspondences are only tentative.

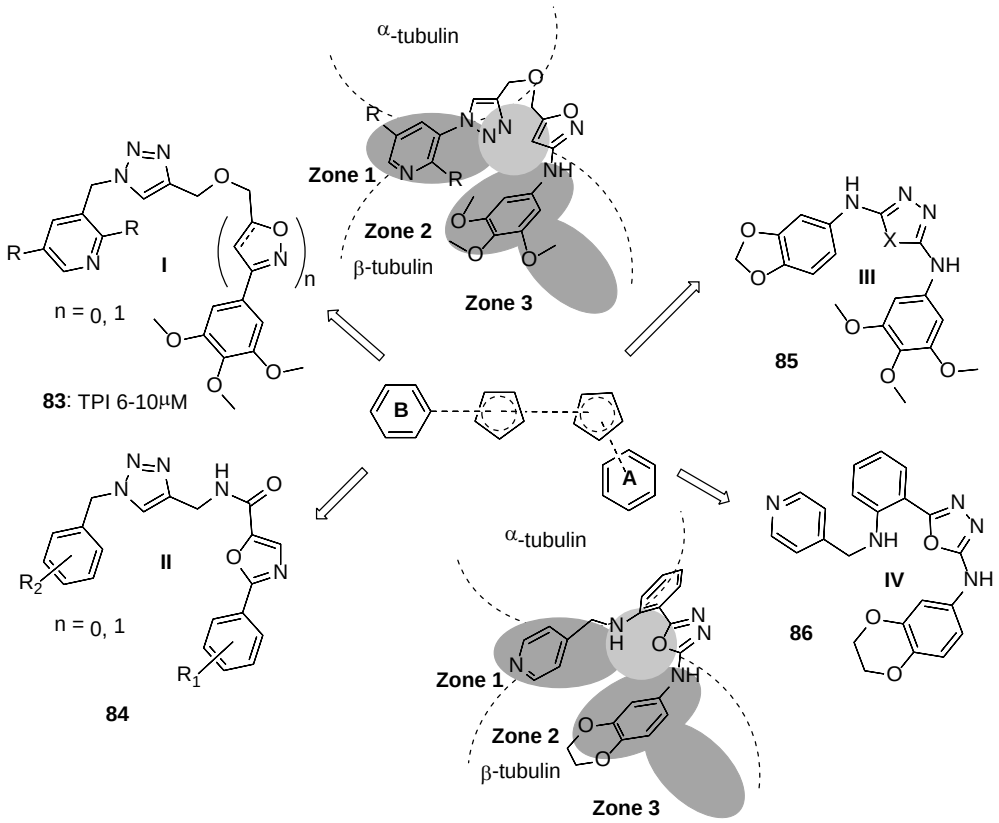


Fig. (25). Representative structures of several families of extended polycyclic inhibitors of tubulin polymerization and proposed binding modes at the colchicine site.

The 1-(3-pyridylmethyl)triazoles (I (**83**) in Fig. 25) were synthesized by click chemistry as a focused chemical library and are moderately active as cytotoxic agents, inhibitors of tubulin polymerization and other typical effects caused by potent ligands of the colchicine site, in accordance with a poor accommodation of these compounds in the site, placing the trimethoxyphenyl ring in Zone 2 and the rest of the chain and ring in the space between tubulin subunits. [227] The structurally related amides of family II (**84**) were designed as a combination in an unique structure of the cytotoxic ABT-751 and nicotinamides using 1,2,3-triazoles.[228] Some of them demonstrated to be moderately cytotoxic (in the micromolar range) but potent TPI, displaying the characteristic effects of CSL.

Family III (**85**) based on the 1,3,5-oxadiazole motif has been tested in the Sea Urchin Embryo Assay and the most potent derivatives have a 4-pyridylmethyl substituent at the amino group, showing higher potency in cell cycle arrest in G2/M and TPI than nocodazole. Docking studies placed the 4-aminophenyl derivative in the colchicine site, occupying Zone 2 with the benzodioxane ring and placing the intermediate azoles in the *inter* dimer zone and the aminopyridine in Zone 1 (Fig. 25).[229]

Family IV (**86-89**) of nicotinic acid derivatives (Fig. 26) with polycyclic structures carrying a triazole or oxadiazole ring have been synthesized and assayed,[230, 231] as well as less active families of related oxadiazole compounds.[232] Many structural variations have been introduced and some conclusions were drawn, as the convenience of the nicotinic Nitrogen *ortho* to the amino substituent, the detrimental effect of alkylation of triazole Nitrogen or the increase in activity introduced by the benzodioxole moiety. The modeling of these compounds suggested accommodation in the colchicine site similar to that of podophyllotoxin (**4**).

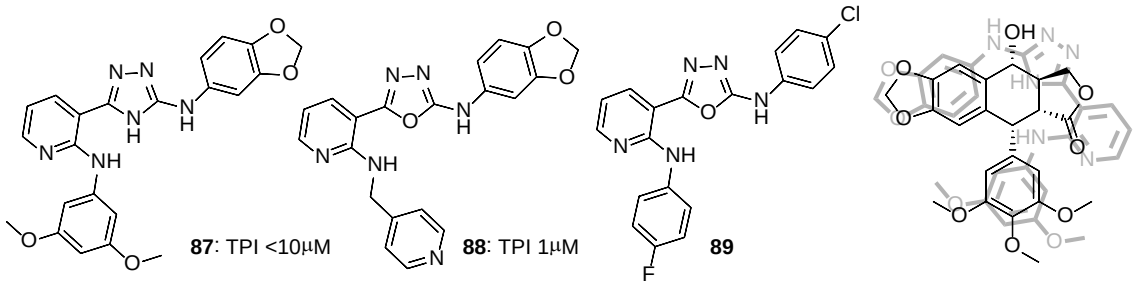


Fig. (26). Representative structures of family IV of extended polycyclic inhibitors of tubulin polymerization and proposed binding modes at the colchicine site.

Pyridine is part of the fused imidazo[1,2-*a*]pyridine scaffold (Fig. 27) of a new family of antimitotic agents lacking the trimethoxyphenyl ring and other common structural characteristics of CSL.[233-237] This scaffold was investigated as a modification of the previously discovered 6*H*-pyrido[2',1':2,3]imidazo[4,5-*c*]isoquinolin-5(6*H*)-ones (**90**). The biological assays demonstrated binding to the colchicine site but the *in vivo* studies were limited by the low water solubility and by the toxicity. For MT119 (**90**), docking studies show the pyrido-imidazole plane lying in the plane A of the pharmacophore (Zone 2), the methoxyphenyl ring in the Zone 1, extending the 3-pyridylmethyl ring close to the acetamide of colchicine towards the interfacial tubulin space. Other structurally related fused tetrahydroisoquinoline system were also found starting from cytostatic isoquinolines (**92**) that bind to the estrogenic receptor, [238] are cytotoxic at submicromolar concentrations and inhibit tubulin polymerization.

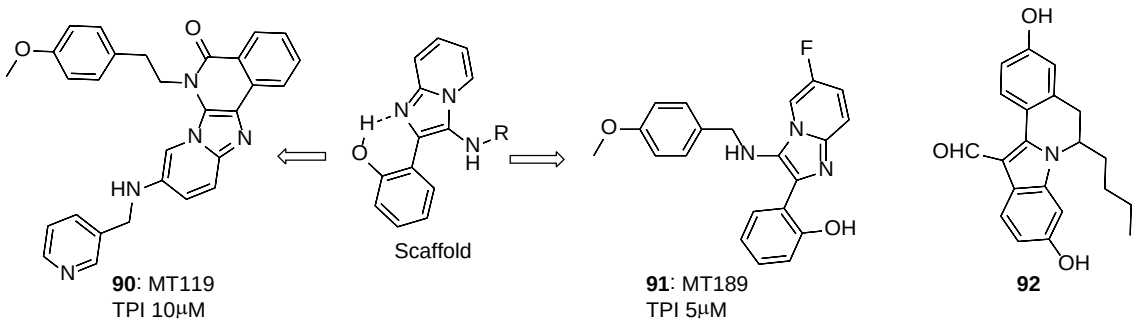


Fig. (27). Fused polycyclic inhibitors of tubulin polymerization.

CONCLUDING REMARKS

The colchicine site of tubulin is a promising target for the development of antimitotic agents with antitumour, anti-parasitic, anti-fungal, herbicidal and other biological activities. Unlike tubulin ligands binding at the taxane or vinca sites of tubulin, no CSL have reached the market yet, but many of them are in clinical trials. The discovery of their vascular disrupting activity against tumour neo-vasculature often at much lower concentrations than their maximal tolerated dose has shifted the focus from the direct cytotoxicity against tumour cells, and many of them are often classified as VDA (vascular disrupting agents).[239, 240] However, it has also been shown that shutting down the tumour neo-vasculature results in the death of the cells of the inner mass of the tumour, but a peripheral rim of survivor cells are responsible for rapid tumour regrowth.[92] Therefore, VDA are often combined with more cytotoxic drugs (as for instance paclitaxel) aimed at killing these cells at the periphery. Most of the failures documented so far have been attributed to a lack of efficacy and therefore more potent and less susceptible to resistance CSL with improved pharmacokinetic properties are actively being pursued. The combination of multiple activities in single chemical entities (the so called multiple drugs) is also emerging as an attractive target in some therapeutic areas, in particular cancer chemotherapy.

In order to improve the existing arsenal of CSL and select the optimal candidates for continuing studies, adequate comparisons between drugs is necessary. However, this comparison is often difficult, as there is a great diversity of experimental conditions

and assays reported depending on the laboratory and even within the same laboratory. Just to mention a few, TPI activities are not always reported and when it is done varying concentrations of tubulin, different buffer conditions, tubulins purified to a different degree, and diverse measuring methods are commonplace. Furthermore, in many families of CSL the TPI potency does not always correlate with cytotoxicity, different relationships in TPI and cytotoxicity potencies are observed for different families of compounds and, in some cases, cytotoxicity varies a lot depending on the cell lines and time lapses used for the studies. These variations suggest, in some cases, multiple mechanisms of action and in others, a resistance which can have multiple origins, as for instance, reduced drug intake, different tubulins or augmented drug metabolism. In turn, the predictive value of *in vitro* cytotoxicity and even of *in vivo* animal models for clinical outcome is less than optimal, thus complicating the decision making.

The highly hydrophobic nature of the colchicine site of tubulin has led in many cases to highly lipophilic and low water soluble compounds which often required the formation of prodrugs in order to improve their pharmacokinetic properties and allow clinical use (e.g. fosbretabulin).[14] These prodrugs are usually inactive compounds and highly hydrophilic and require activation in order to enter the tumour cells and kill them, thus providing the tumours with a means of evading their cytotoxic effects. Other families of more hydrophilic nature have allowed even p.o. administration but at the expense of showing lower anti-tubulin and cytotoxic potencies, which in some cases has resulted in lack of activity in the clinic (e.g. CI-980).[241] The introduction of azines as alternative scaffolds to the benzenic tubulin inhibitors has in many cases improved their pharmacokinetics and in some cases enhanced potency (pharmacodynamics) but very seldom have both gains been simultaneously achieved, thus providing a great room for improvement.

The realization that there are three distinct zones in the colchicine site has shown that most drugs occupy only two of them.[44, 45] Although, we are still far from completely understanding the effects of filling either zone, zone 1 is mainly hydrophobic and the most potent ligands are usually bound within it while zone 3 is more polar and enables more soluble ligands but often with lower atom binding efficiency. Zone 2 admits both kinds of moieties. To date, the majority of described successful azines bind at sites 2 and 3. Therefore, the discovery of new scaffolds or the combination of structural motifs of different families will be a means of combining the beneficial effects of all of them.

An additional area of improvement for CSLs involves the incorporation of different activities in the same molecule (multiple drugs). CSL with topoisomerase (e.g. azatoxin),[242] histone deacetylase (HDAC),[243] Hsp 27,[244] CDK4,[245, 246] or different kinases (e.g. MEK [247] or VEGFR-2 [210, 248]) inhibitory activity are emerging. In this regard, azines are often found in kinase inhibitors, thus providing an opportunity for design of new dual drugs, such as tivantinib.

The achieved successes and the new design opportunities uncovered guarantee further development in this field, as novel chemical scaffolds interacting with the colchicine site of tubulin continue to be discovered in the search for new drugs and biological tools.

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