Journal of Enzyme Inhibition and Medicinal Chemistry, December 2004 Vol. 19 (6), pp. 521–540

Taylor & Francis
healthsciences

Review Article

Naphthalene Combretastatin Analogues: Synthesis, Cytotoxicity and Antitubulin Activity

MANUEL MEDARDE*, ANA B.S. MAYA and CONCEPCIÓN PÉREZ-MELERO

Laboratorio de Química Orgánica y Farmacéutica, Facultad de Farmacia, Universidad de Salamanca, Campus Miguel de Unamuno, E-37007, Salamanca, Spain

(Received 21 January 2004; In final form 16 April 2004)

Synthesis and evaluation of new combretastatin analogues with varied modifications on the bridge and the aromatic rings, have shown that the 2-naphthyl moiety is a good surrogate for the 3-hydroxy-4-methoxyphenyl (B-ring) of combretastatin A-4. Other bicyclic systems, such as 6(7)-quinolyl and 5-indolyl, can replace the B-ring, but they produce less potent analogues in the cytotoxicity and tubulin polymerization inhibition assays. Other modifications are detrimentral for the potency of the studied analogues. The 2-naphthyl combretastatin 53 and the related 6-quinolyl combretastatin 106 analogues are the most potent among the derivatives of this type, whereas 92 and 95 are the most potent among the naphthalene derivatives with a heterocycle in the bridge. Previous and new results in this family of combretastatin analogues are discussed.

Keywords: Combretastatin analogues; S.A.R; Naphthalene derivatives; Quinoline derivatives; Cytotoxicity; Tubulin polymerization inhibition

INTRODUCTION

Cancer is one of the major health concerns worldwide, being the second cause of death after heart diseases in developed countries, with lung and breast cancer as the most devastating malignancies. It has been estimated that there are six million new cases a year in the world.[1] Due to this problem, it is of foremost importance to have a better knowledge of the nature, origin and molecular basis of cancer.

Cancer is a group of malignancies including more than one hundred diseases. They are characterized by an uncontrolled proliferative growth, invasiveness of surrounding tissues and dissemination of the aberrant cells from the point of origin.[2] In cancer, cells have lost communication with the surroundings, ignoring the signals that control the cell cycle.

One of the possibilities for cancer treatment is to interfere with the cell cycle of the cancer cells. The cell cycle[3] is a highly coordinated process that allows the cell to divide into two daughter cells in an ordered manner. It is divided into several phases, mitosis being one of them. Mitosis is a dramatic, coordinated change in the architecture of the cell, in which two identical daughter cells are formed from the parent one. The basic events that take place during mitosis are the formation of the chromosomes by condensation of chromatin, their correct arrangement by means of the mitotic spindle, separation of the chromatids and formation of the two new nuclei. Finally, the cell division is physically accomplished through the cytokinesis process, by the formation of a septum between both daughter cells.

Tubulin as a Target for Anticancer Agents

One of the structures highly implicated in mitosis are the microtubules,[4] playing also important roles in the maintenance of cell shape, cell transport and cell motility. During cell division, the microtubules organize themselves as the mitotic spindle around the centrosomes, capture the chromosomes, place them in an ordered manner in the centre of the mitotic spindle and finally pull the chromatids away

*Corresponding author. Tel.: +34-923-294528. Fax: +34-923-294515. E-mail: medarde@usal.es

ISSN 1475-6366 print/ISSN 1475-6374 online © 2004 Taylor & Francis Ltd
DOI: 10.1080/14756360412331280473

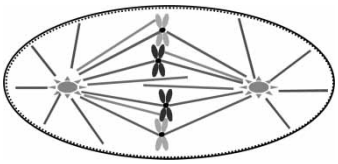


FIGURE 1 Arrangement of chromosomes in the centre of the mitotic spindle during metaphase (microtubules are depicted as straight lines).

from each other and towards opposite poles (Figure 1).

Microtubules are structures of about 25 nm diameter with the appearance of hollow tubes formed by 10–16 protofilaments (the most common being 13). The basic polypeptidic component is a heterodimer of α and β tubulin (a globular protein), that polymerizes to build up the protofilaments.[5] *In vivo*, a complex and diverse set of additional proteins are associated, in a permanent or transient way, with microtubules.

γ-Tubulin participates as a starting point for polymerization of αβ tubulin dimers from the centrosome. α and β tubulins are very similar 55 kDa proteins, sharing about 35–40% similarity to each other. In the α, 72% of all residues are conserved, whilst in the β 78% of all positions are conserved. The individual sequences show that many of the non-conserved residues involve highly conservative aminoacid substitutions. The similarity between the α and β tubulin sequences suggests that the two subunits must have closely related tertiary structures.

The architecture of the microtubules is formed by lateral contacts between protofilaments, which are in turn formed by longitudinal contacts between αβ tubulin dimers (Figure 2). Microtubules are dynamic structures, which polymerize and depolymerize depending on the conditions, thus presenting adequate properties for their cellular functions.

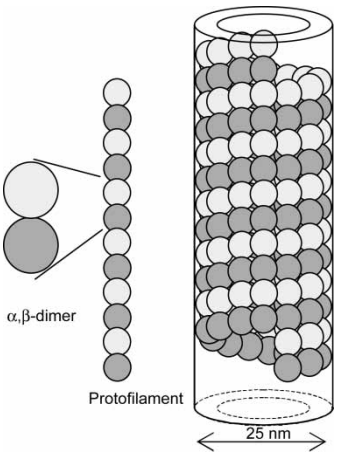


FIGURE 2 Structure of microtubules.

The structure of the tubulin heterodimer was obtained by electron crystallography of zinc-induced tubulin sheets. The structure contains information at 3.7 Å on all but the final 10–18 carboxy tail residues.[6] The major recent progress in the field has been the docking of the high-resolution structure of tubulin into a microtubule lattice.[7]

Tubulin Sites

Tubulin heterodimers have two nucleotide binding sites, named N-site (for *n*on-exchangeable) on the α subunit, always occupied by GTP, and E-site (for *e*xchangeable) on the β subunit, which may have GTP or GDP bound when tubulin is in the heterodimeric form. This structural feature establishes a direct influence of nucleotides on stabilizing straight or curved quaternary structures in these polymers since the E-site is located at the interface between two dimers (Figure 3). A cap of GTP at the E-site of β-tubulin stabilizes the unstable microtubules, which otherwise "peel apart", depolymerizing.

Compounds binding to tubulin are classified depending on their ability to prevent the binding of different representative ligands to the protein. Apart from the aforementioned sites for nucleotide binding, the taxol, the *Vinca* alkaloids and the colchicine sites are distinguished, although there are ligands which bind to other unidentified sites.[8] Those three sites have been localized on β-tubulin, near the E-site in the case of *Vinca* alkaloids site, in the middle region (where lateral contacts between microtubules are produced) in the case of the taxol site and close to the intradimer contact area, near the N-site, for the colchicine site (Figure 4).

Taxol[9] binds to microtubules, but not to isolated αβ heterodimers, at the M loop on the β subunit. The hydrophobic pocket occupied by taxol in β tubulin is filled by 8 extra residues in α tubulin. The peeling apart of protofilaments in taxol-stabilized microtubules is greatly inhibited, which suggests that the mechanism by which taxol stabilizes microtubules is

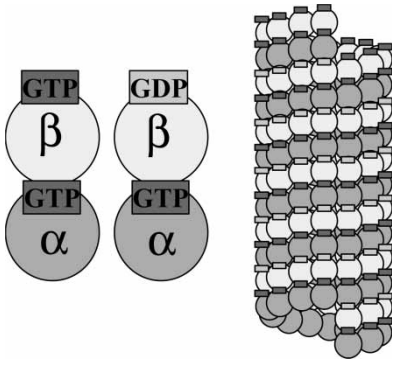


FIGURE 3 Nucleotide binding sites on α and β tubulin.

mainly through strengthening of the conformation of the M loop that favours the lateral contacts, or it could counteract the destabilizing change induced by the loss of the phosphate when GTP is converted into GDP during microtubule formation.[10] Actually, the real effect of taxol could be due to a combination of several effects, producing stronger lateral contacts between protofilaments at the β subunit.

Vinblastine[11] was identified years ago as another drug that inhibits cell division by interfering with the normal activity of the microtubule system. Appreciation has grown that, as with the colchicines, the effect of *Vinca* alkaloids in the cell is more related to stopping the dynamic instability that allows microtubules to explore the cell and catch the chromosomes, than to the disruption of microtubules themselves. At high concentrations, vinblastine disrupts microtubules, inducing the protofilaments to form spirals which can also form paracrystals. A photoactivated crosslink has identified one peptide (residues 177–215 in β tubulin) and the H5 and H6 α-helixes as structural elements that must lie close to the *Vinca* alkaloids site.[12] Because of the formation of spirals, it can be supposed that the presence of the drug does no stop the addition of a new dimer to the microtubule, but produces a kink at the inter-dimer surface, leading to curved protofilaments. Because different compounds binding at the *Vinca* site occupy non-overlapping areas, as it is the case for rhizoxin and maytansine,[13] the site is usually called the *Vinca* domain.

Colchicine[14] was the first drug known to bind to tubulin, which was originally isolated by its ability to bind this simple molecule.[15] Colchicine inhibits microtubule formation by binding to the αβ dimer and also disrupts microtubules at higher concentrations. The tubulin-colchicine complex (TC) substoichiometrically inhibits microtubule assembly and activates the GTPase activity of the dimer. Thus, colchicine binding alters the protein conformation near the longitudinal and lateral protofilament interfaces and potentially destabilizes the normal microtubule lattice.

Cochicine binds in a biphasic manner, with a rapid but weak first step followed by a second slower and stronger one, at a site near the interface between dimers.[16] There is evidence for interaction of colchicines with both α and β monomers, although data suggest that the main interaction is with β. Separate experiments have identified crosslinking between colchicine analogues and residues 1–36 and 216–243 of the β subunit. In addition, crosslinking has also been observed between the colchicine A-ring and cys356 also on β tubulin. The perturbed region was identified by the protection of cys295, 305, 315 and 316 from labeling with *N*-ethylmaleimide (NEM) in the presence of colchicine. These residues, 295–316 on the α-subunit, correspond to the H9 helix and B8 sheet near the top of the α subunit, a region that is very close to the αβ interface with the β-subunit, and directly involved in lateral contacts between protofilaments.

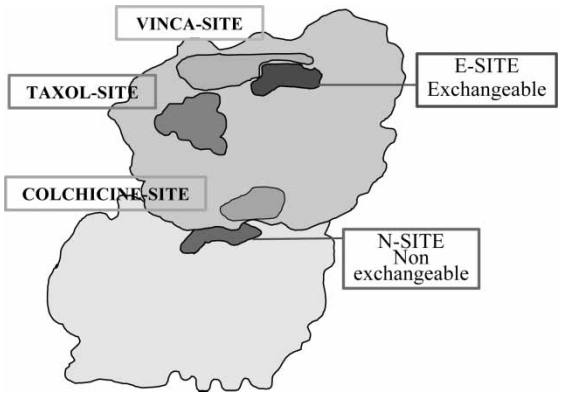


FIGURE 4 Localization of *Vinca* alkaloids, taxol and colchicine binding sites.

Agents Binding at the Colchicine Site

The largest number of antimitotic agents described so far belong to the group of ligands binding to the colchicine site. Among them are colchicine and related natural products such as cornigerine. Other related synthetic compounds have a strong effect, as for instance MTC, lacking the B-ring, or the thio analogue of colchicine (Figure 5).

Other synthetic or natural products, with two aromatic systems directly bonded to each other or separated by a 1–3 atoms bridge as common structural features, have been described which inhibit the binding of colchicine to tubulin. Representatives of these structures are depicted in Figure 6, including amphetinile, nocodazole, the trimethoxy derivative 1069C, chalcones, arylquinolones, flavonoids or polyaromatic sulphonamides. Rotenone and sanguinarine, polycyclic natural

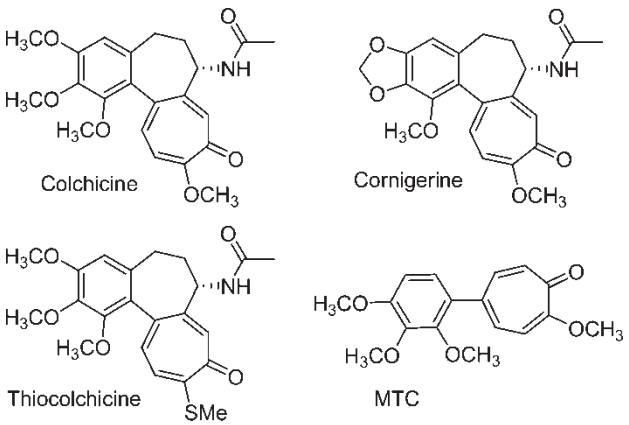


FIGURE 5 Structure of colchicinoids and related synthetic compounds.

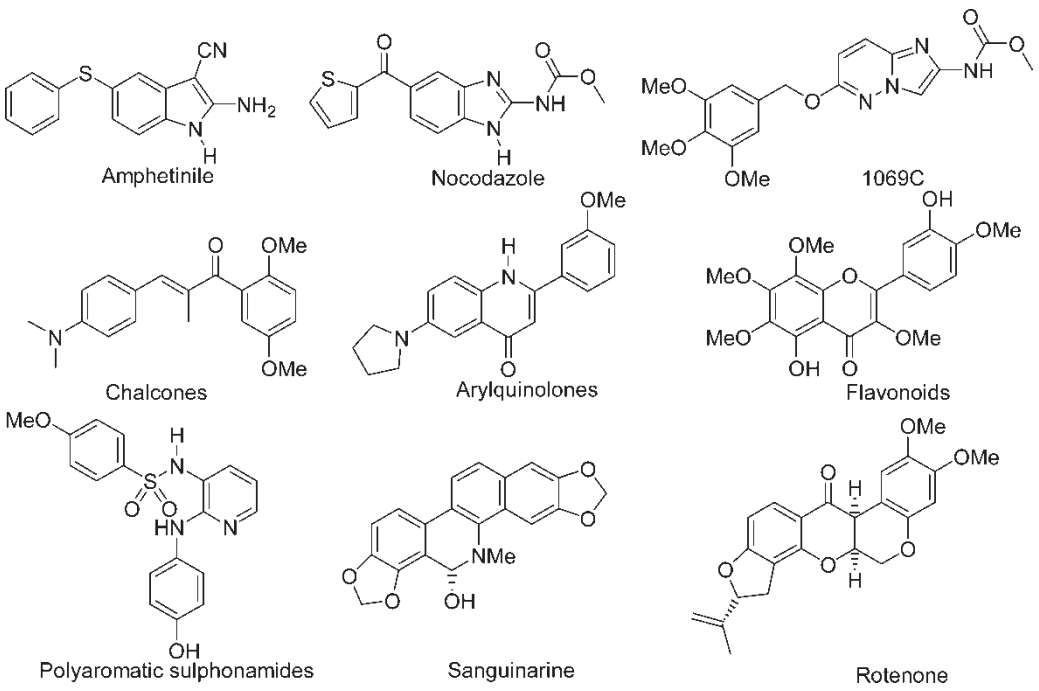


FIGURE 6 Structure of polycyclic compounds that bind to tubulin at the colchicine site.

products, also belong to this broad group of antimitotic agents.

The most representative compounds, structurally close to the colchicines and related to each other, are the natural products podophyllotoxin (and similar lignans), phensatin and our model compound combretastatin A-4. These compounds have two polyoxygenated benzene rings in a non-planar disposition, resembling the arrangement of the aromatic systems of colchicine (Figure 7).

The Combretastatins

The isolation and crystal structure determination of (−)-combretastatin was first reported by Pettit in 1982.[17] The isolation of this substance from the South Africa tree *Combretum caffrum* was based upon its activity in reversing the differentiation of glioma cells into astrocytes. The attention was then focused at uncovering the principal *in vivo* active constituents of *C. caffrum* fractions where combretastatin was not responsible for the biological activity, with two active fractions being obtained from which combretastatin A-1 and combretastatin B-1 were isolated.[18] Afterwards, the most potent of the constituents, combretastatin A-4 (**CSA-4**) was isolated.[19]

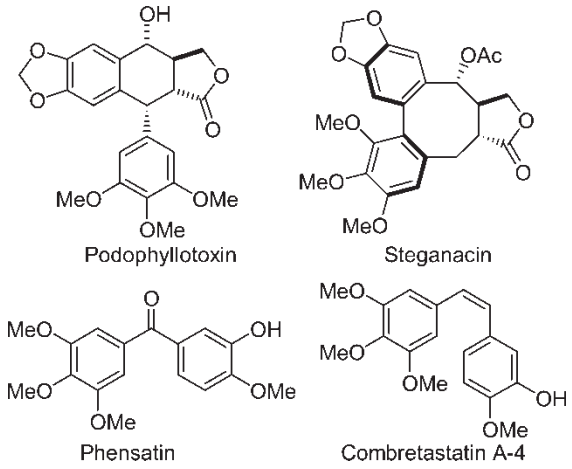


FIGURE 7 Structure of podophyllotoxin, steganacin, phensatin and combretastatin A-4.

Along with the stilbenes or combretastatins A and the dihydrostilbenes or combretastatins B (Figure 8), phenanthrenes (combretastatins C) are also present in *C. caffrum*.[20] A phenanthroquinone, a class of compounds rarely found as biosynthetic products, has been isolated from the same extract and called combretastatin C-1. Combretastatins D-1 and D-2 belong to a different type of compounds isolated from the same source, whose more prominent structural feature is the caffrane ring, a 15-member biaryl ether macrolactone.[21]

The structure of combretastatin and **CSA-4** are reminiscent of that of colchicine and the active colchicine analogue 2-methoxy-5-(2′,3′,4′-trimethoxyphenyl)tropone MTC (Figure 5). Combretastatin structure, as well as its ability in reversing the differentiation of immature glioma cells into mature differentiated astrocytes, prompted its testing for antimitotic and antitubulin activity.[22] Progressive inhibition of microtubule assembly was observed *in vitro* at combretastatin concentrations from

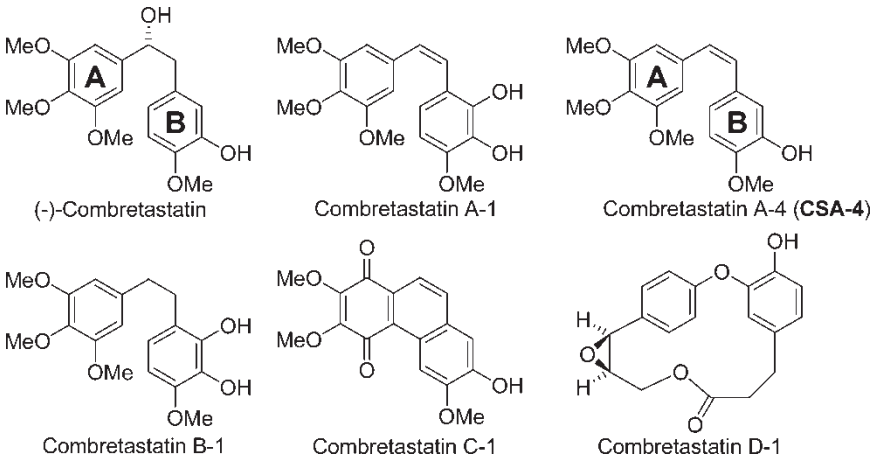


FIGURE 8 Structure of (−)-combretastatin and combretastatins A, B, C and D.

1–10 μM. It also exhibits a significant stimulatory effect on GTP hydrolysis by tubulin. Combretastatin A-4 is active at concentrations in the same order of magnitude as the most potent inhibitors of tubulin polymerization, being also a competitive inhibitor of the binding of colchicine to tubulin with a K_i value of 1.1 μM.

SAR of Combretastatins

SAR studies on combretastatins have been directed at the effect of structural modifications on different parts of the molecule on the cytotoxicity, inhibition of microtubule polymerization or inhibition of the binding of colchicine to tubulin.

The modifications described in the literature affect: a) substituents on ring A; b) substituents on ring B, generally hydroxy, alkoxy or other related substitutions; and c) the structure of the bridge linking both aromatic systems, including length of the bridge, presence or absence of a double bond and its configuration, replacement of carbon atoms by heteroatoms and effect of substituents.

Modifications of Rings A and B

No modification in the substitution pattern of the A ring has yet improved the cytotoxicity, tubulin polymerization inhibition or displacement of colchicine displayed by combretastatin A-4, although some of them retain high potency, as the combretastatin A-2-type 3,4-methylenedioxy derivatives (Figure 9).[23] Accordingly, the exact location of the four methoxy groups is very important for the pronounced cytotoxicity of the tetramethoxy analogue (3,4,5-triOMe-4′-OMe), because changes in their positions (2,3,4-trimethoxy) result in decreased potency. Demethylation at the 4′ position and methylation at the 3′ position contributed to decrease the potency of *cis*-stilbenes. An additional hydroxy group at the 2′ position (combretastatin A-1), although resulting in a marked reduction of

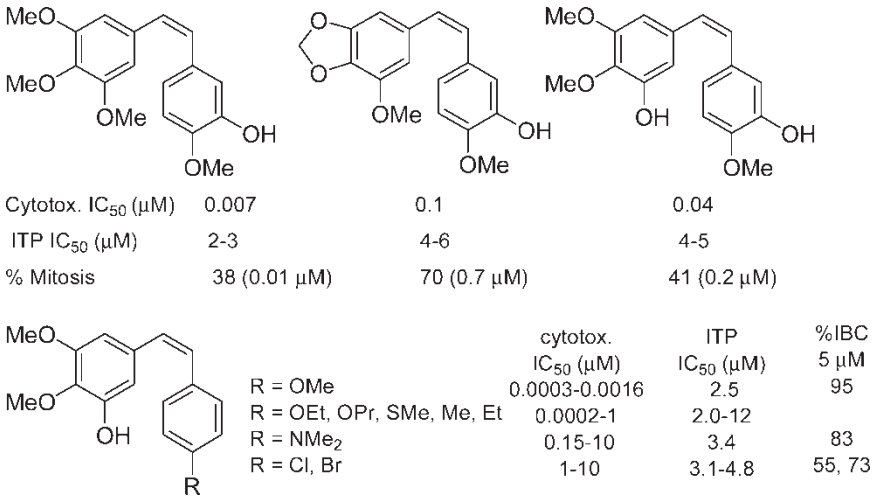


FIGURE 9 Representative modifications at the A and B-rings of combretastatins (ITP: Inhibition of tubulin polymerization; IBC: Inhibition of colchicine binding).

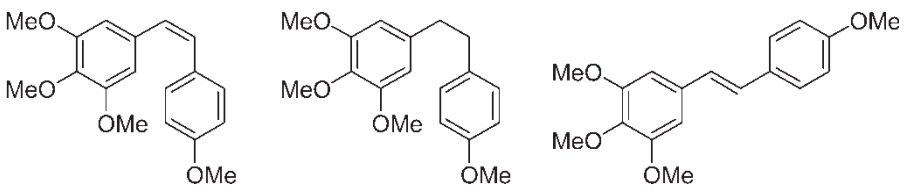


FIGURE 10 Comparison of activities of representative *cis*, dihydro and *trans* combretastatins.

the cytotoxicity, produces an equipotent agent in the assay as inhibitor of tubulin polymerization. In this assay, podophyllotoxin is less potent than both combretastatins A-1 and A-4. The absence of one of the methyl groups on ring A also yields relatively high potent derivatives.[24–26]

Apart from the aforementioned derivatives, any other modification of the B-ring has led to a decrease in the potency of combretastatin A-4 and related compounds in all series. Only 4′-methylthio and 4′-methoxy derivatives retain high cytotoxicity, which decreases upon substitution by Cl, Br or NMe_2 and disappears when substituted by other groups, such as the bulky *t*-butyl.[27]

Modifications of the Bridge

Bridge Configuration

The *cis*-stilbene configuration is optimal for activity and the *trans* configuration was thought to be active as well. Initially it appeared that the *trans* configuration of combretastatin A-4 had about one-fourth the potency of the *cis* isomer as inhibitor of tubulin polymerization, but later it was found that freshly prepared stock solutions were inactive and gained activity with time. This suggested some conversion from the inactive *trans* to the active *cis* form under laboratory storage, and slow conversion could be proven under controlled conditions. In all cases, the saturated compounds were weaker cytotoxic and weaker inhibitors in the tubulin assay *in vitro* than the corresponding *cis*-stilbenes,[24] although the dihydrocombretastatin form retains significant activity as a polymerization inhibitor relative to the *cis*-stilbene cognate, depending on specific substituents on the two phenyl rings. In the dihydrostilbene series, replacement of the B-ring methoxy group by an amino group resulted in loss of activity, but some activity was recovered if the amino group was converted into a dimethylamino group. It is assumed that the flexibility of the dihydro-compounds allows them to adopt a conformation resembling the *cis* isomers, which explains why these compounds are more cytotoxic and potent as tubulin polymerization inhibitors than the *trans* isomers. The relative potencies of representative compounds (Figure 10) of the *cis*- > dihydro- > *trans*- series as inhibitors of tubulin polymerization paralleled the results of the cytotoxicity assays.[27]

SAR Studies on the Bridge Length and Substitution Pattern

Modifications on the bridge are always detrimental for activity. To check if there is an optimal separation between the two aromatic moieties, a series of compounds which differ only in the type of bridge separating the two ring systems were prepared.[28] All of the analogues with one carbon bridge were less potent than the 3,4,5,4′-tetramethoxy derivative in the cytotoxicity assays, although their inhibitory effect on tubulin polymerization was essentially identical to that of the model compound.[29] With the exception of the biphenyl compound (3′,4′,5′,4-tetramethoxy-3-hydroxybiphenyl), all the synthesized combretastatin analogues had activity as inhibitors of both tubulin polymerization and colchicine binding. There is a clear correlation between bridge length and potency in this series of analogues. Maximum potency was achieved with the two-carbon bridge analogue, whilst the one-carbon and three-carbon bridge ones were somewhat less potent and nearly equipotent.

Introduction of substituents on the bridge of dihydrostilbenes greatly reduced the inhibitory potency of this class of agents. In combretastatin analogues with the *cis*-ethylene bridge, only the small cyano group produced derivatives of comparable potency to that of **CSA-4**.[30]

SAR Studies on the Bridge: Carbon Replacement

When the ethylene bridge in the tetramethoxy derivative (3,4,5-triOMe-4′-OMe) was replaced by an amide, no significant activity was observed, maybe due to the electrostatic effect of the electron withdrawing amide carbonyl group.[31] However, when an aminomethylene linkage was introduced, the *N*-benzylaniline with the 3,4,5,4′-tetraOMe substitution pattern was active.[32] The enhanced activity of N-substituted benzylanilines, such as the N-propyl derivative in Figure 11 (as potent as combretastatin A-4 in the inhibition of tubulin polymerization assay), was atributed to

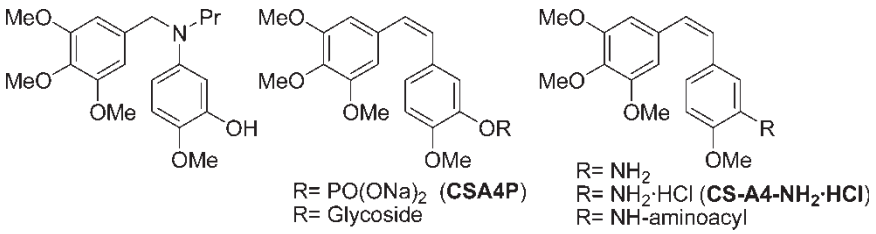


FIGURE 11 Combretastatin analogues with a nitrogen-containing bridge and soluble derivatives.

the induction of a *cis*-orientated active conformer.[31] Phenylbenzyl ethers have also shown interesting antimitotic properties.[33]

Soluble Combretastatin Derivatives and Analogues

Combretastatin A-4 was prevented from entering clinical trials owing to its limited solubility in pharmaceutically acceptable solvents.[34] Therefore, prodrugs having increased aqueous solubility are of great interest. The obvious position available for modification in combretastatin A-4 is the hydroxy group. Several derivatives containing acidic and basic functional groups[35–37] and glycosides[38] were prepared in an attempt to make compounds that were more water soluble and could therefore be administered more readily. The most promising of those derivatives, the phosphate prodrug of combretastatin A-4 (**CSA4P**. Figure 11), has entered phase II clinical trials due to its antiangiogenic properties.[39]

Other analogues obtained in searching for more soluble compounds are the amino derivatives prepared by Oshumi's group, carrying a 3′-NH_2 group instead the 3′-OH of **CSA-4**.[30] Compound **CS-A4-NH_2·HCl**, 3′-aminocombretastatin A-4 hydrochloride, is a potent cytotoxic agent and tubulin polymerization inhibitor in the same potency range as **CSA-4**, as are other related amino derivatives carrying a cyano group on the bridge. **CS-A4-NH_2·HCl** is also a highly potent antitumor agent *in vivo*, shown to be more efficacious than *cis*-platin in the treatment of several model tumor in animal assays. Its marked antitumor activity may be due to increased hydrophillicity of the compound.

The interest in the replacement of the phenolic OH by the amino group and amino acid derivatives has been extended to the preparation of these types of compounds with other combretastatins, such as combretastatin A-2. In this case, amine, amine hydrochloride, glycine and tyrosine derivatives are the most cytotoxic. Amine and amine hydrochloride analogues inhibit tubulin polymerization by binding at the colchicine site, while the amides had little activity against purified tubulin. Nevertheless, most of the amides caused a marked increase in the mitotic index of treated cells, indicating that tubulin was the intracellular target and suggesting that they are converted to the amines by amidases.[23]

New Heterocyclic and Naphthalene Combretastatins

Some time ago we reported a versatile approach to the synthesis of combretastatins and combretastatin analogues (Figure 13), starting from a common 1,3-dithiane intermediate,[40] which is suitable for the preparation of diversely functionalized derivatives on the ethane bridge. This methodology was applied to the synthesis of combretastatin, combretastins B (dihydrostilbenes) and keto analogues. The presence of the keto group is very interesting for the generation of new analogues carrying a variety of substituents on the bridge between both aromatic rings.

Next, we decided to commence the synthesis of new analogues, not previously described. We took into consideration that:

(1) a *cis* disposition between both aromatic rings, followed by a saturated ethane bridge, is preferred for highly potent cytotoxic and antimitotic agents, over the *trans* disposition between the aromatic moieties
(2) substituted benzenes had been studied for almost all the variations with different substitution patterns (the best combination being the 3,4,5-trimethoxyphenyl and the 3-hydroxy-4-methoxyphenyl), but the replacement by other aromatic systems had been less exploited.

With this in mind, we chose the replacement of the A and B rings of combretastatins by heteroaromatic and naphthalene systems carrying substituents or not, and the introduction of heterocycles on the bridge in order to lock the *cis* disposition. The general structure of the initially planned analogues is depicted in Figure 12. The results from these new derivatives are presented in the Results and Discussion sections, whereas the preparation and

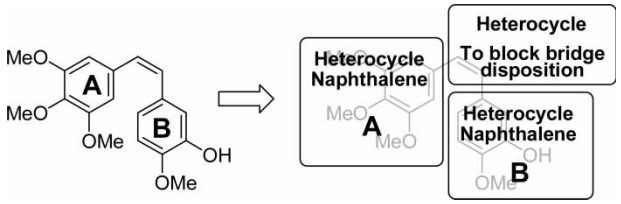


FIGURE 12 Design of new combretastatin analogues.

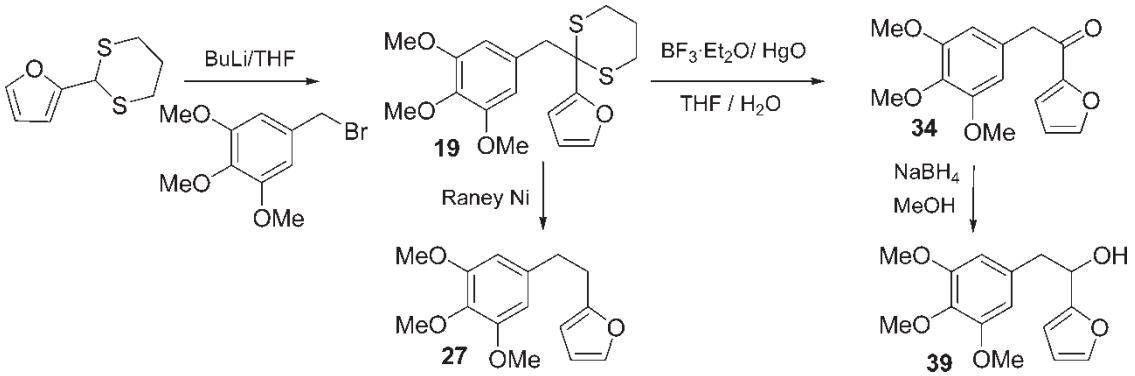


FIGURE 13 Synthetic methodology for combretastatins B and their analogues.

some representative experimental details are included in the Materials and Methods section.

MATERIALS AND METHODS

General Synthetic Procedures

Aryl^A and Aryl^B represent different moieties in compounds **1**–**109**, which include substituted or unsubstituted phenyl, benzyl, heteroaryl, heteroarylmethyl, naphthyl and naphthylmethyl.

Ethane (dihydro) Derivatives (Figure 13)

Synthesis of 2-aryl^A-1,3-dithianes

To a stirred solution of the appropriate aryl^A-carbaldehyde (0.25 M in $CHCl_3$), 1,3-propanedithiol (1.1 eq) and trimethylsilyl chloride (1.1 eq) were sequentially added at 0°C. The reaction was kept at room temperature overnight, then quenched with saturated aqueous NaOH and extracted with $CHCl_3$. The combined organic layers were washed with brine and dried over Na_2SO_4. Evaporation of the solvent left a brown solid that was purified by crystallization (Hexane-AcOEt).

Alkylation of 2-aryl^A-1,3-dithianes

1.6 M solution of BuLi in hexane (1.1 eq) was added dropwise to a solution of 2-aryl^A-1,3-dithiane (0.15 M in dry THF) at −78°C, under argon. After 1 h the mixture was warmed up to −20°C and the corresponding aryl^B bromide (1.1 eq, 0.5 M in dry THF) was slowly added. After 24 h at that temperature, the reaction was quenched with saturated aqueous NH_4Cl and extracted with AcOEt. The combined organic layers were washed with brine, dried over Na_2SO_4 and the solvent evaporated under vacuum. The solid residue was purified by flash chromatography.

Deprotection of Dithianes

To a stirred suspension of HgO (2 eq) in 15% THF-H_2O, $BF_3{\cdot}Et_2O$ (2 eq) was added dropwise, at 0°C. After 5 min, a solution of the appropriate 2-aryl^A-2-aryl^B-1,3-dithiane (0.05 M in 15% H_2O-THF) was slowly added and the mixture was allowed to react at room temperature overnight. Then, CH_2Cl_2 was added and the precipitate was filtered. The precipitate was washed with 2 M HCl and exhaustively extracted with AcOEt. The organic solution was then washed with brine and dried over Na_2SO_4. The solvent was removed under vacuum and the crude product was purified by flash chromatography.

Desulphurization of Dithianes

A solution of 2-aryl^A-2-aryl^B-1,3-dithiane (0.01 M in EtOH) was added to a slurry of Raney Ni (80 eq). The mixture was gently refluxed for 1 h, allowed to cool to room temperature, filtered over silica and the organic solvent evaporated under vacuum.

Reduction of Ketones

To a solution of 2-aryl^A-1-aryl^{B}ethanone in anhydrous MeOH, $NaBH_4$ (1.1 eq) was slowly added with vigorous stirring, under argon, at 0°C. The reaction mixture was stirred at this temperature for 1.5 h and then quenched with water. The resulting mixture was extracted with diethyl ether and the organic layer was washed with brine, dried over Na_2SO_4 and evaporated under vacuum. The crude product was purified by flash chromatography.

Indole-bridged Derivatives (Figure 14)

Indole Formation by Fischer Reaction

Glacial AcOH (4 mL per equivalent of ketone) was added to a solution of the corresponding 2-aryl^A-1-aryl^{B}ethanone (1 eq) and *p*-methoxyphenylhydrazine (2 eq) in EtOH, and the mixture refluxed for 1.5 h. The resulting solution was sequentially washed with saturated aqueous Na_2CO_3 and brine, dried over Na_2SO_4 and the solvent eliminated under reduced pressure. The crude product was purified by flash chromatography.

Ethene-bridged Analogues (Figure 15)

Wittig Reaction

A suspension of the required phosphonium bromide (0.08 M in dry THF) was cooled to −15°C, under argon. *n*-Butyllithium (1.6 M in hexane, 1.1 eq) was added dropwise and the resulting solution was stirred at this temperature for 30 min, changing colour from deep-red to yellow-red. Then a solution of the corresponding aldehyde (0.9 eq) in THF was added and the mixture was stirred at −15°C overnight. Once the reaction was completed, water was added and the solution was extracted with dichloromethane. The organic layers were washed with brine, dried and concentrated under vacuum. The residue was chromatographed on silica gel to afford the *E*- and *Z*-isomers in variable yields.

Heterocyclic-bridged Analogues (Figure 16)

Synthesis of Enaminoketones

DMFDMA (15 eq) was added dropwise to 2-aryl^{A}-1-aryl^{B}ethanone (0.04 M in toluene) under argon and the mixture was refluxed for 24 h. After cooling, toluene was removed under vacuum. The crude product was purified by crystallization from Et_2O.

Pyrazoles

A mixture of the appropriate enaminoketone and hydrazine hydrochloride (1.5 eq) in dry EtOH was refluxed for 24 h. After cooling, EtOH was removed under vacuum. Water was added and the pyrazole derivative extracted with CH_2Cl_2. The combined organic layers were dried over Na_2SO_4, the solvent evaporated under vacuum and the resulting residue purified by preparative chromatography on silica.

Isoxazoles

To a solution of the corresponding enaminoketone in 2:1 MeOH–H_2O, a mixture of hydroxylamine hydrochloride (1.1 eq) and Na_2CO_3 (0.5 eq) was added at room temperature. The solution was acidified with glacial AcOH and refluxed for 20 h. The reaction was quenched with saturated aqueous NH_4OH and extracted with CH_2Cl_2. The organic layer was dried over Na_2SO_4 and the solvent evaporated under vacuum.

Pyrimidines

A mixture of enaminoketone, ammonium formate (10 eq), formamide (2.8 eq) and formic acid (2.5 eq) was heated to 165°C until evaporation of water ceased (50 min). Then, the temperature was raised up to 180°C until complete consumption of the starting material was observed. After cooling the reaction mixture to room temperature, the resulting solution was extracted with CH_2Cl_2. Organic extracts were dried over Na_2SO_4, evaporated under reduced pressure and the resulting residue purified by HPLC.

Pyrazine and Quinoxaline Analog

To a solution of 1-(2-naphthyl)-2-(3,4,5-trimethoxyphenyl)ethane-1,2-dione (obtained by DDQ oxidation of the corresponding monoketone) in glacial AcOH, ethylenediamine (2 eq) or O-phenylenediamine was added. The reaction mixture was refluxed for 20 h and then AcOH was removed. AcOEt was added and the organic solution sequentially washed with a solution of saturated aqueous $NaHCO_3$ and brine, dried over Na_2SO_4 and the solvent evaporated under vacuum.

Cytotoxicity Assays

Cytotoxicity assays were carried out as previously described.[41] The cell lines usually employed were P-388 (lymphoid neoplasma from DBA/2 mouse), A-549 (human lung carcinoma) and HT-29 (human colon carcinoma). The cytotoxicity against normal cells was determined using CV-1 cells (monkey fibroblasts).

A suspension culture of P-388 cells was seeded into 16 mm wells (24-well multidishes) at 1×10^4 cells/well in 1 mL aliquot of medium MEM 10FCS (minimum essential medium supplemented with 10% fetal calf serum) containing the indicated concentration of the corresponding compound. The remaining cell lines (monolayer cultures) were seeded into 16 mm wells at 2×10^4 cells/well in 1 mL aliquots of MEM 10FCS containing the indicated concentration of each compound. In both cases a separate set of cultures without any compound was counted daily to ensure that the cells remained in exponential growth. Cells were incubated at 37°C in a 10% CO_2 humid atmosphere. All determinations were carried out in duplicate. After three days of incubation, cells were counted and the IC_{50} for each compound was determined.

Tubulin Polymerization Inhibition Assay

Calf brain microtubule protein (MTP) was purified by two cycles of temperature-dependent assembly/disassembly according to the method of Shelanski,[42] modified as described in the literature.[43] The MTP solution was stored at −80°C. Protein concentration was determined by the method of Bradford,[44] using BSA as standard.

Assembly

The *in vitro* self-assembly of tubulin was monitored turbidimetrically using a thermostated Thermospectronic Helios α spectrophotometer fitted with a Peltier temperature controller and a circulating water bath. The increase in turbidity was followed at 450 nm instead of 350 nm in order to avoid light absorption by the ligands. Each turbidimetry

measure was carried out for a batch of six cuvettes simultaneously, always including a control (i.e., with no ligand) in the batch. Four different MTP preparations were used in these assays. The assayed ligands were dissolved in DMSO and the resultant solutions stored at −20°C.

Cuvettes contained 1.5 mg/mL MTP in 0.1 M MES buffer, 1 mM EGTA, 1 mM $MgCl_2$, 1 mM β-ME, 1.5 mM GTP, pH 6.7, and the corresponding ligand concentration. The maximum amount of DMSO in the assay cuvettes was 4%, which is reported not to interfere with the assembly process.[45]

The samples were pre-incubated for 30 min at 20°C in order to allow binding of the ligand, and subsequently placed on ice for 10 min. The cuvettes were then transferred to the spectrophotometer at 4°C and the baseline registered. The assembly process was started by shifting the temperature to 37°C.

IC_{50} was calculated as the concentration of drug causing 50% inhibition of polymerization after 20 min incubation, and was determined graphically.

RESULTS AND DISCUSSION

Dihydro and Indole-bridged Combretastatin Analogues

Following our versatile synthesis of combretastatins, we initially prepared and assayed for cytotoxicity several derivatives carrying different groupings on the ethane bridge and different substitution patterns on benzene rings A and B.[46] These derivatives were not highly potent, but they maintained an IC_{50} in the micromolar range, as expected for analogues of combretastatins B (Table I). The most potent of the assayed derivatives was the trimethoxyphenyl naphthyl derivative **9**, with an unsubstituted ethane bridge. This finding was interesting in that it could lead to introduction of the naphthyl moiety as substituent in other derivatives with new structures.

According to the design depicted in Figure 12, we prepared a series of derivatives carrying 5-membered and fused 5-membered heterocycles as A or B ring replacement. Following the aforementioned methodology, the dithiane, ethane, ethanone and ethanol derivatives with heterocycles as ring B and 3,4,5-trimethoxyphenyl as ring A or, alternatively, heterocycles as ring A and isovanillin (3-hydroxy-4-methoxyphenyl) as ring B, were obtained. The employed methodology proved to be very convenient for the preparation of such derivatives.

Both families of compounds were assayed for cytotoxicity and the results are presented in Table II. The compound that maintains high cytotoxicity is the derivative carrying the indolyl moiety as ring B equivalent (**37**).

From these and previous results it was concluded that replacement of the aromatic A or B rings of combretastatins B (dihydrostilbenes) was very convenient when they are changed by extended aromatic systems, such as the previously described naphthyl or indolyl ones. It was then suspected that in analogues carrying these moieties and having a locked *cis* disposition between both aromatic rings, a cytotoxicity and, may be, an inhibition of tubulin plymerization close to that of the most potent combretastatins could be expected.

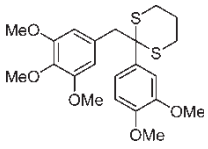
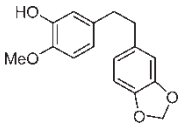
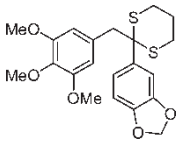
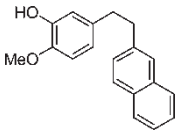
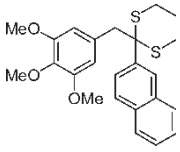
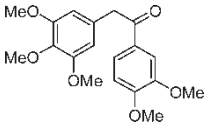
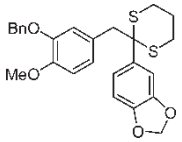
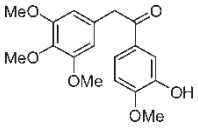
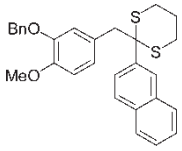
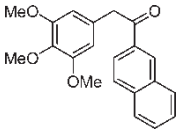
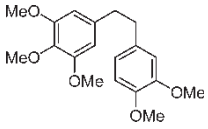
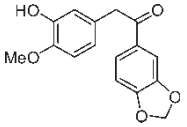
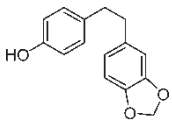
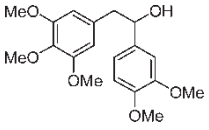
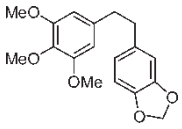
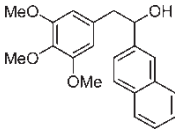
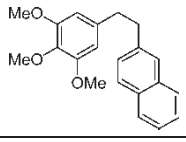
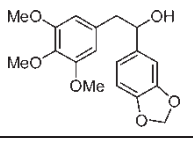
With this in mind, we planned to build up rigid structures on the flexible ethane bridge of the previously synthesized analogues. In these analogues, the presence of a keto group allowed us to introduce an indole system. This moiety was very interesting because it permitted us to easily produce a rigid structure on the bridge, and the indole system, present in many well known antineoplastic agents, could contribute to higher cytotoxic potency. Furthermore, the new derivatives containing three aromatic systems could be accommodated in the colchicine binding site of tubulin, because three different binding domains have been predicted taking into consideration the structure of the inhibitors of tubulin polymerization by binding at this site (podophyllotoxin, quinolones, combretastatins, phensatin, colchicines and their analogues).[47] The treatment of diaryl ethanones with 4-methoxyphenylhydrazine in refluxing EtOH–AcOH for 1.5 h produced the indole derivatives in moderate to good yields (Figure 14).

For most of these derivatives the cytotoxic effect is increased, from 2- to 100-fold, when the rigid indoles were build up on the bridge, the 2-(2-furyl)-3-(3,4,5-trimethoxyphenyl)indole (**41**) being the most potent of these compounds. For the most potent among the ethane-bridge compounds, the 2-naphthyl derivative **9**, there was a small decrease in the activity in the indole analogue **45** (Table III).

Up to that time, only a few examples of heterocycles as a substructure for maintaining the *cis* disposition between both aromatic rings A and B in analogues of combretastatins had been described. The papers by Shirai[48,49] and Ohsumi[50] presented a similar approach, to force a *cis* disposition between both aromatic residues through the construction of a heteroaromatic system. Recently, new examples of such heterocyclic[51–56] (and cyclopentane[57,58]) bridged analogues of combretastatins have appeared. In these cases, the combination of the trimethoxy and the isovanillin (or the 3-amino-4-methoxyphenyl or 4-methoxyphenyl) produced highly potent cytotoxic and inhibitors of tubulin polymerization analogues, of the same order of magnitude as **CSA-4** or 4-amino-**CSA-4**.

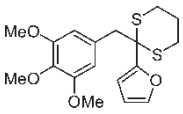
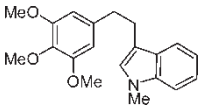
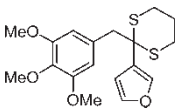
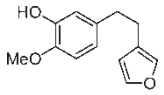
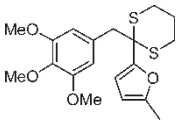
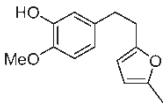
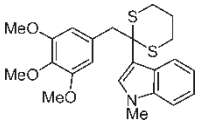
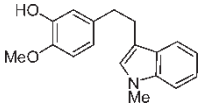
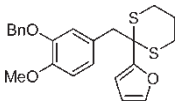
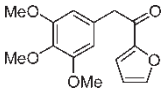
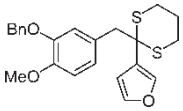
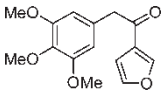
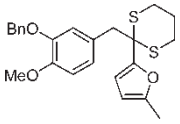
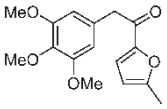
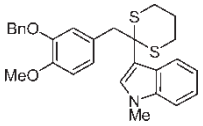
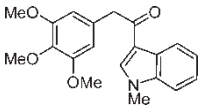
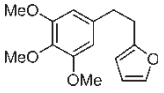
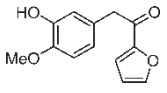
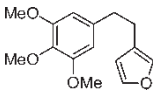
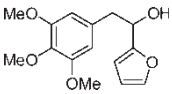
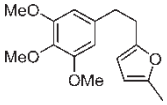
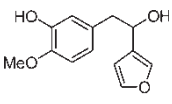
Following our work on this family of cytotoxic agents, we decided to focus our study at the effect

TABLE I Cytotoxic activity of analogues of combretastatins B (dihydrocombretastatins). Ranges correspond to values for assays against several representative cell lines (usually P-388, A-549 and HT-29)

Compound		μM	Compound		μM
1		22.9	**10**		4.4–18.4
2		4.8–5.9	**11**		3.6–9
3		5.9–11.7	**12**		>28.9
4		5.4–10.7	**13**		>30
5		5.3–21.1	**14**		14.8–29.7
6		15	**15**		8.7–35
7		>41.2	**16**		>28.7
8		6.3	**17**		>22.8
9		0.3	**18**		2.9

produced by the replacement of the benzene rings A and B by naphthalene and heterocycles in the first instance. The rationale for the substitution of the oxygenated rings by the non polar naphthalene ring is that: (1) QSAR analysis of combretastatins indicates a good correlation between the cytotoxic activity and the hydrophobicity values of the functional groups on ring B,[59] (2) our previous experience, showing that naphthalene in dihydrocombretastatins and indole derivatives gives compounds which display activities close to those of the most potent compounds of each family (those carrying the 3,4,5-trimethoxyphenyl and 3-hydroxy-4-methoxyphenyl moieties).

TABLE II Cytotoxic activity of analogues of combretastatins B carrying heterocyclic moieties

Compound		μM	Compound		μM
19		6.8–13.7	**30**		15.4
20		6.8–13.7	**31**		22.9– > 45.9
21		>52.6	**32**		21.5–43.1
22		5.8–11.6	**33**		3.6–8.9
23		6.1–12.1	**34**		>72.5
24		2.9–12.1	**35**		7.2
25		2.3–11.7	**36**		8.6–17.2
26		5.3–10.5	**37**		0.3–0.6
27		19.1	**38**		18.5–21.5
28		>76.3	**39**		17.5–36
29		7.2	**40**		>71.9

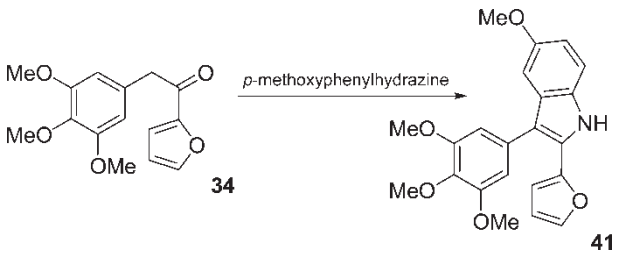


FIGURE 14 Synthetic Fischer methodology for indole-bridged combretastatin analogues.

Naphthalene Combretastatins

To explore the possibilities for introducing the naphthalene moiety into the combretastatin skeleton, as a replacement for the A or B rings, we centred our study on the synthesis and assay of compounds intended (Figure 17):

(1) to determine whether naphthalene is a better replacement for the A ring or the B ring and determine the highest effect on cytotoxicity. Naphthalene combretastatin analogues with an ethylene bridge were prepared with this purpose (Tables IV–VII).
(2) to determine the effect of the naphthalene moiety on the activity of other combretastatin analogues, carrying a heterocycle or other groupings on the bridge between both A and B rings. 5-Membered and 6-membered heterocycles in the bridge of naphthalene combretastatins were obtained in this case (Tables VIII and IX).

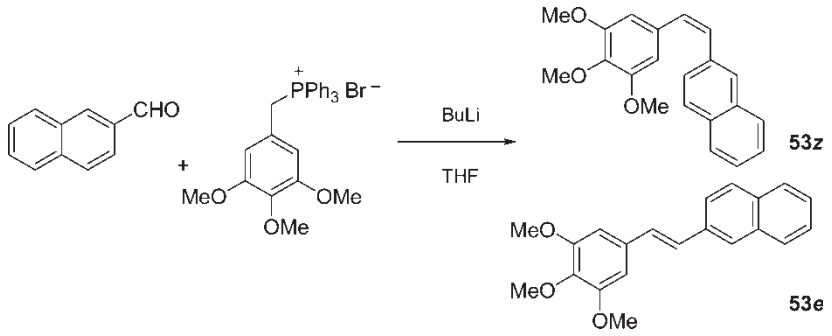


FIGURE 15 Synthetic methodology for ethene-bridged combretastatin analogues.

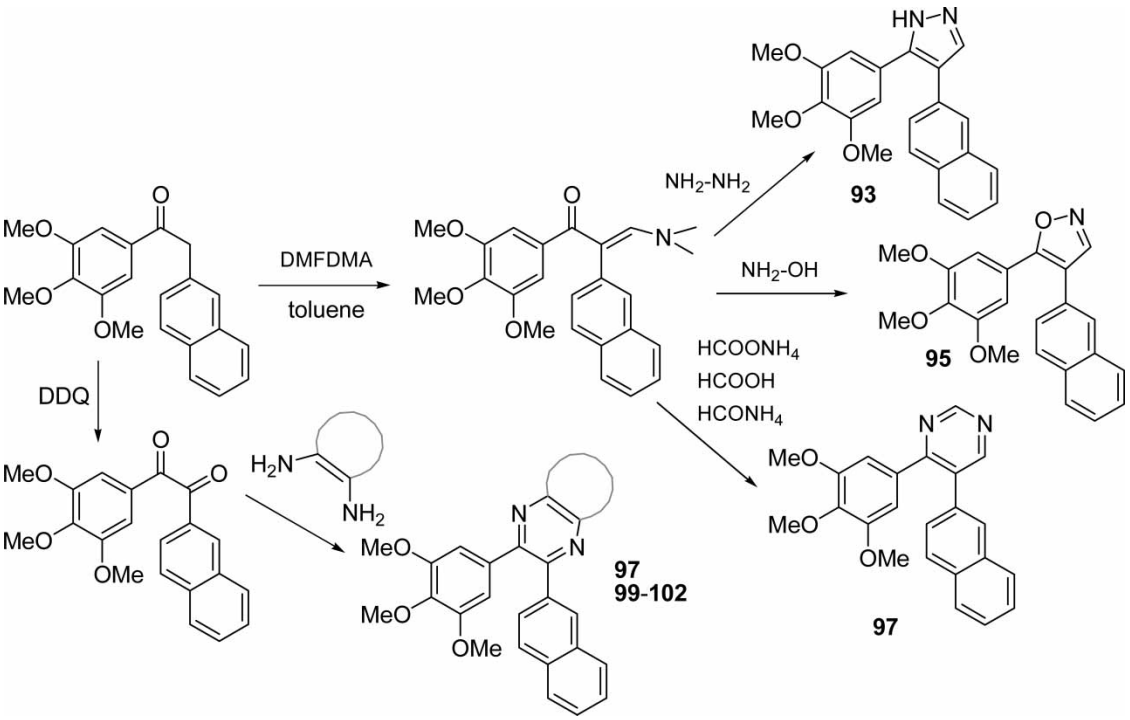
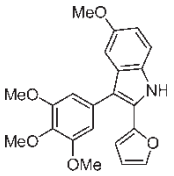
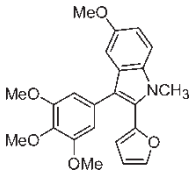
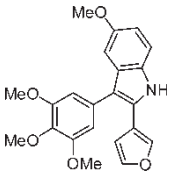
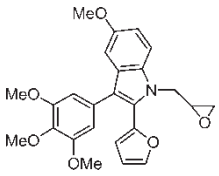
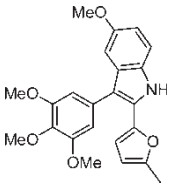
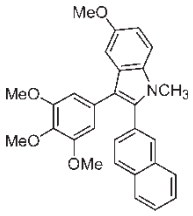
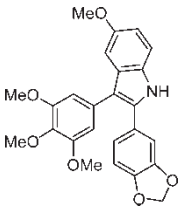
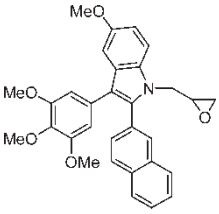
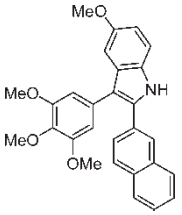
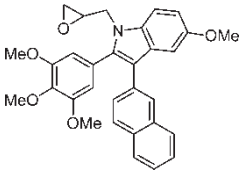
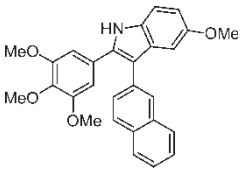
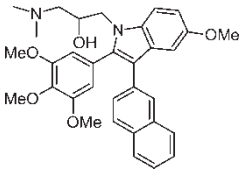


FIGURE 16 Synthetic methodology for heterocyclic-bridged combretastatin analogues.

TABLE III Cytotoxic activity of indole-bridged analogues of combretastatins

Compound		μM	Compound		μM
41		0.25–1	**47**		1–5
42		2.6–6.6	**48**		1–10
43		6.8–8.5	**49**		5–20
44		2.3	**50**		0.5–5
45		1.1–2	**51**		>1
46		>1	**52**		1–10

(3) and to find other aromatic bicyclic systems as replacements for these rings, with the same or better effect than the naphthalene moiety. Quinolines and quinoxalines were used to study this aspect (Table X).

Naphthalene Combretastatin Analogues with Ethylene Bridge

Following the well established Wittig methodology we prepared several compounds bearing the naphthalene moiety. Starting from the corresponding aldehydes, by sequential reduction, bromination, conversion into the phosphonium salts and further transformation to the phosphoranes, followed by reaction with the required aldehyde, mixtures of both *cis* and *trans* isomers of the final products were obtained (Figure 15).

The three initially prepared derivatives[60] were those containing naphthalene and either of the two moieties of combretastatin A-4: the 3,4,5-trimethoxyphenyl and the isovanillin. The high potency of the 3,4,5-trimethoxyphenyl and the isovanillin containing analogues **53z** and **54z** is lost in derivative **55z** (with two naphthalene systems) and the *trans*-isomers **53e** and **54e** (Table IV). These results are indicative of the ability of the naphthyl system to be integrated into the structure of the combretastatins.

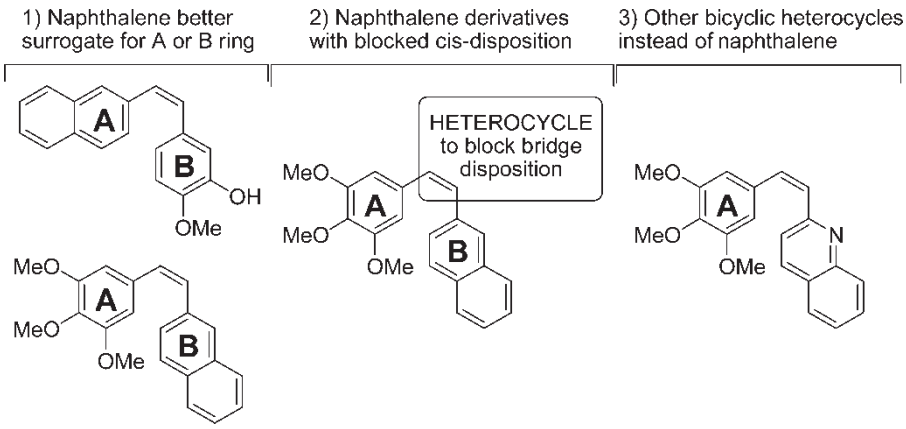
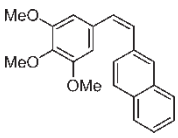
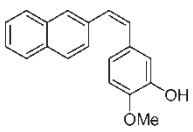
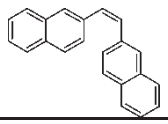


FIGURE 17 Proposed research for naphthalene analogues of combretastatins.

This is in agreement with the usual necessity of the 3,4,5-trimethoxyphenyl group to be present in the structure of the combretastatin to produce highly potent cytotoxic agents. Compound **53z** displays a cytotoxic effect of the same order of magnitude as **CSA-4** although it lacks the 3-phenolic group.

These results were a good starting point to establish the similarity of naphthalene to the A or the B ring of combretastatins, as proposed in Figure 17-1. The next step was to synthesize and assay compounds maintaining the naphthalene as a ring A (3,4,5-trimethoxyphenyl) and other well known surrogates of the ring B (for example the 3-amino-4-methoxyphenyl or the 4-methoxyphenyl). The activity results shown in Table V clearly demonstrate that the naphthyl moiety is not a good choice to produce highly potent derivatives when it is replacing the 3,4,5-trimethoxyphenyl residue. All the compounds were inactive or displayed cytotoxicities above the micromolar range i.e. two or three orders of magnitude lower in potency than the model **CSA-4**.

TABLE IV Naphthalene analogues of combretastatin A-4

Compound		*Z* (μM)	*E* (μM)
53		0.02– > 5	0.39
54		0.04–3.6	3.6
55		3.6–5	–

Once it was observed that the naphthalene moiety is a worse replacement of the 3,4,5-trimethoxyphenyl, in order to prove its ability for replacing isovanillin, two types of derivatives were prepared: compounds **76**–**85**, several of them carrying polycyclic aromatic systems closely resembling naphthalene (for example *N*-methyl-5-indolyl), and compounds **86**–**91**, including in their structures the 2-naphthalene moiety as ring **B** and other systems closely resembling the 3,4,5-trimethoxyphenyl as ring **A**.

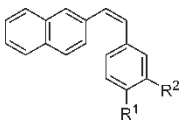
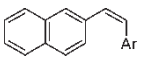
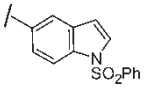
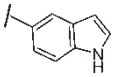
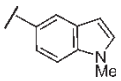
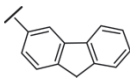
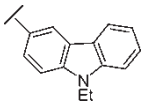
The results of the cytotoxicity assays are presented in Table VI (for compounds **76**–**85**) and in Table VII (for compounds **86**–**91**). From the activity displayed by both types of compounds it can be concluded that: 1) when the 3,4,5-trimethoxyphenyl is ring **A**, the derivative **83** with the 5-indolyl bicyclic system (although not other indole derivatives bonded through other positions) displays the closest potency to that of compound **53**; and 2) when the 2-naphthalene is maintained as ring B, the potency increases with the similarity of the ring A to the 3,4,5-trimethoxyphenyl, the 3,4-dimethoxy-5-nitrophenyl naphthalene derivative **86** being the most potent of these analogues.

From the results of Tables IV–VII, it is deduced that the combination of 3,4,5-trimethoxyphenyl (ring A) and a 2-naphthalene (ring B) in a *cis*-locked disposition is a good approach to other potent combretastatin analogues.

Naphthalene Analogues of Combretastatins with Heterocycles in the Bridge

Following the research on the title analogues, we continued with the synthesis of derivatives proposed in Figure 17-2 carrying heterocyclic systems of different size, that could complete the information obtained from the indole derivatives included in Table III. According to the synthetic methodology presented in Figure 16, 5-membered **92**–**96** (Table

TABLE V Analogues of combretastatin A-4 with naphthalene as ring A

		*Z* (μM)	*E* (μM)			*Z* (μM)	*E* (μM)
56	R^1=OMe R^2=EOM	3	3– > 5	**69**		>5	>5
57	R^1=AcO R^2=H	–	>5				
58	R^1=OH R^2=H	–	4.1	**70**		>5	>5
59	R^1=OMe R^2=H	1.9	>5				
60	R^1=OMe R^2=F	0.45	>5	**71**		>5	>5
61	R^1=OCF_3 R^2=H	3.18	>5				
62	R^1=Me R^2=H	>5	>5	**72**		>5	–
63	R^1=CF_3 R^2=H	>5	>5				
64	R^1=$N(CH_3)_2$ R^2=H	3.7	3.7	**73**		>5	>5
65	R^1=OMe R^2=NO_2	>20	>5				
66	R^1=OMe R^2=NH_2	0.36– > 20	1.8– > 5	**74**		>5	>5
67	R^1=OMe R^2=NHAc	1.6– > 20	>5				
68	R^1=OMe R^2=$N(Et)_2$	–	>5	**75**		>5	>5
CSA4		0.003–0.032					

VIII) and 6-membered **97**–**99**/extended 6-membered **100**–**102** heterocyclic analogues (Table IX) were obtained.

In agreement with the combretastatin analogues carrying the 3,4,5-trimethoxyphenyl and the isovanillin moieties on a pentagonal heterocyclic bridge, three derivatives (**92**, **93** and **95**) in Table VIII are highly potent cytotoxic agents. For derivative **96**, with an additional methyl group on the heterocycle bridge, the activity disappears, as it is the case for larger derivatives with the hexagonal/extended hexagonal heterocycles in the bridge (Table IX). Nevertheless, these results support the previous conclusion on the ability of 2-naphthalene in replacing the isovanillin moiety of combretastatins.

Quinoline Analogues of Combretastatins

To complete this study, the synthesis and evaluation of derivatives with other bicyclic systems as ring B was undertaken. The quinoline bicyclic system was the selected choice, because: 1) it has the same size as the naphthalene system, 2) instead of the highly hydrophobic character of naphthalene ring it also has a nitrogen able to establish hydrogen bonds, and 3) it could increase the water solubility, the major drawback of the combretastatins for entering clinical phases. The quinoline system also offers the possibility of preparing different regioisomeric structures, depending on the relative binding position of the quinoline to the ethylene bridge.

These compounds were obtained by Wittig methodology, requiring the preparation of the quinoline carbaldehydes by oxidation of the corresponding methylquinoline derivatives. The 2-, 3-, 4-, 6- and 7-quinoline derivatives (**103**–**107**) were synthesized by this procedure. The related quinoxaline analogues **108**–**109** were prepared starting from 3,4-diaminobenzoic acid, which was transformed into the quinoxaline

TABLE VI 3,4,5-Trimethoxyphenyl derivatives with heterocyclic and related systems as ring B replacement

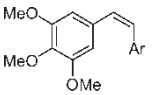
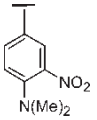
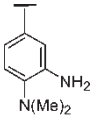
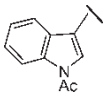
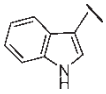
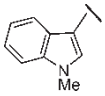
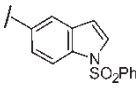
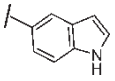
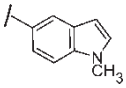
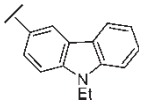
		*Z* (μM)	*E* (μM)
76		1.8–3.7	3.7
77		>5	>5
78		0.03–0.15	0.30–1.5
79		2.9	>5
80		3.23	3.2
81		3.1	3.1
82		>5	>5
83		0.16–0.32	0.16–0.32
84		>5	0.15
85		0.06–0.13	0.03

TABLE VII Naphthalene analogues with other phenyl moieties as ring A surrogates

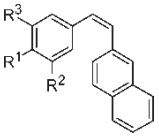
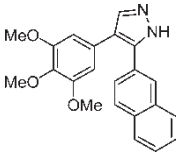
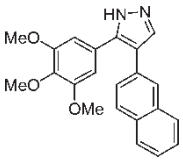
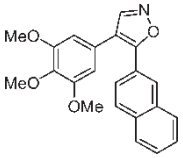
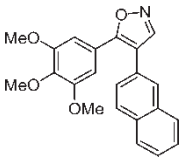
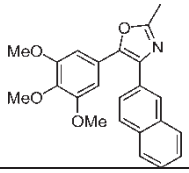
		*Z* (μM)	*E* (μM)
86	R^1=R^2=OMe R^3=NO_2	0.15	1.5– > 5
87	R^1=R^2=OMe R^3=NH_2	0.3–1.6	0.16–3.3
88	R^1=R^2=OMe R^3=NHAc	>5	>5
89	R^1=OMe R^2=R^3=NO_2	>5	>5
90	R^1=OMe R^2=R^3=NH_2	>5	>5
91	R^1=R^2=NO_2 R^3=$N(OMe)_2$	>5	>5

TABLE VIII Naphthalene combretastatins with 5-membered heterocycles on the bridge

Compound		μM
92		0.014–0.28
93		0.14–1.4
94		>5
95		0.028–0.14
96		>5

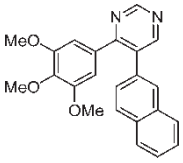
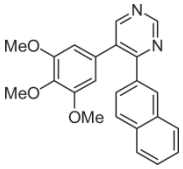
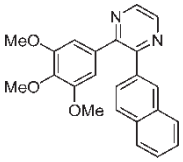
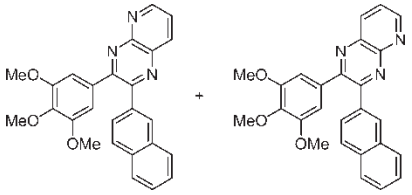
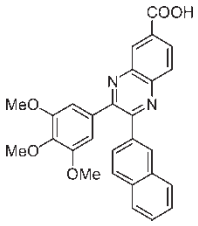
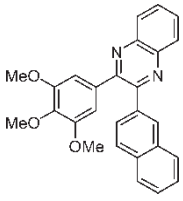
system, followed by reduction and oxidation to the aldehyde and Wittig reaction. Their cytotoxicities are presented in Table X, where values in the submicromolar range can be observed for compounds **103**, **104** and **107**. The most potent and close to the most cytotoxic naphthalene analogues is the 6-quinoxaline derivative **106**.

These results are of great interest in view of the similarity of the bicyclic systems (bonded through position 2 in naphthalene or 6/7 in quinolines) to the isovanillin system of combretastatins. Such similarity is less evident for analogues not carrying the 3,4,5-trimethoxyphenyl ring, but is clearly observed when this moiety is present in *cis*-disposition with the bicyclic system.

Inhibition of Tubulin Polymerization by Naphthalene Combretastatins and Related Compounds

The potency of the assayed compounds as inhibitors of tubulin polymerization (Table XI) was compared with that of **CSA4** and the naphthylcombretastatin **53z**, which elicits antitubulin potency close to that of **CSA4**.[61] Compound **54z**, which retains the isovanillin

TABLE IX Naphthalene combretastatins with 6-membered heterocycles on the bridge

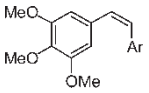
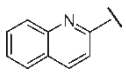
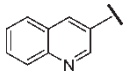
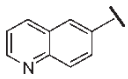
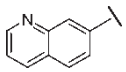
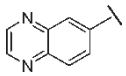
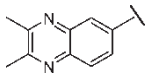
Compound		μM
97		>5
98		>5
99		1.3–2.7
100		>5
101		>5
102		>5

ring and carries the 2-naphthyl system instead of the trimethoxyphenyl moiety, is slightly less potent, in agreement with cytotoxicity results.

Among the different families of compounds that were subsequently synthesized and tested in cytotoxicity assays, in order to elucidate whether the trimethoxyphenyl or the isovanillin rings are better replaced by the 2-naphthyl moiety, some of the most representative analogues were also tested as inhibitors of tubulin polymerization.

For compounds in Table V, in which the naphthyl moiety replaces ring A, compounds **59z** and **60z** did not elicit a significative antitubulin potency, their IC_{50} values being considerably higher than 30 μM.

TABLE X Cytotoxicities of quinoline and quinoxaline-combretastatins

		*Z* (μM)	*E* (μM)
103		0.31	0.39
104		0.39	3.1
105		2.5–5	–
106		0.031	–
107		0.16–1.6	1.6
108		>5	>5
109		>5	>5

For compounds in Tables VI and VII, with a variety of cyclic systems instead of ring B, compounds displaying higher potency were found, **86z** (in which a nitro group replaces one of the methoxy substituents of ring A) being the best one. When the replacing moiety is a 5-indolyl (**83z**), potency

TABLE XI Inhibition of tubulin polymerization by selected compounds, expressed as IC_{50} (μM)

Compound	IC_{50} (μM)
53z	10
54z	16
59z	⩾ 50
60z	⩾ 30
78z	⩾ 30
83e	⩾ 30
83z	25
84e	30
86z	13
95	12
96	⩾ 50
103z	⩾ 40
104z	⩾ 40
105z	⩾ 40
106z	⩾ 40
107z	18
108z	⩾ 40
CSA-4	3

decreases by 2-fold, and activity is abolished in the case of the *E* isomer **83e**.

For derivatives bearing a heterocycle on the bridge, those with a 5-membered heterocycle (pyrazole or isozaxole ring) showed high inhibitory potency, comparable in some cases to that of **53z**. Among them, the most potent was isoxazole **95**, with $IC_{50} = 12\,\mu M$. The introduction of a substituent on these rings, even a small one such a methyl group, produced much less potent derivatives: 2-methyloxazole **96** was neither an inhibitor of tubulin polymerization nor cytotoxic. When the ring size is increased up to 6-membered heterocyclic derivatives, potency decreased even further: the pyrimidine analogues **97** and **98** exerted no polymerization inhibition even at 50 μM. Open synthetic intermediates in Figure 16 were also assayed but were inactive.

The quinoline and quinoxaline families also displayed a broad range of polymerization inhibitory potencies. The high cytotoxic potency elicited by 6- and 7-quinolyl analogues was paralleled by a high antipolymerization potency only in the case of the 7-quinolyl **107z**, suggesting that the mechanism of cytotoxic activity for compound **106z** could be a different one. The rest of the quinolyl derivatives, bearing the nitrogen atom on the ring directly attached to the ethylene bridge (through positions 2-, 3- or 4-), were inactive. Two other derivatives with 6-quinoxalyl- and 2,3-dimethyl-6-quinoxalyl- moieties were also inactive, showing that an additional nitrogen is detrimental for antitubulin activity as was also observed in the cytotoxicity results.

CONCLUSION

From more than one hundred analogues of combretastatins presented in this paper several conclusions can be drawn. The most interesting is the ability of two bicyclic aromatic systems, the 2-naphthalene and the 6(7)-quinoline, to replace the isovanillin moiety of the most potent combretastatins. This fact has been observed for different structural types of analogues, carrying *cis*-double bonds or small heterocycles as a bridge between the 3,4,5-trimethoxyphenyl and the aforementioned systems. Although these analogues are slightly less potent as cytotoxic agents than the natural model compound **CSA-4**, they are also inhibitors of tubulin polymerization *in vitro* with IC_{50} values in the micromolar range. The most potent compound **53z** also disrupts the microtubule system of the cell.

Two of the most cytotoxic quinoline analogues **106z** and **107z** have a noticeable difference. Although they display cytotoxic potency in the same range, the results of tubulin polymerization are very different, with the former being inactive and the latter displaying remarkable activity. This suggests the possibility of an alternative mechanism for the cytotoxicity of these combretastatin analogues.

Moreover, there is a difference between the naphthalene and another bicyclic system, the indole, which led to less potent analogues as cytotoxic agents. Among the indole derivatives there are noticeable differences in potency depending on the position at which it is attached to the bridge.

Finally, these results are of interest for the synthesis of further analogues of antimitotic agents that bind to tubulin at the colchicine site. The quinoline and naphthalene moieties are adequate substructures to be integrated into these analogues, in order to prepare highly potent inhibitors of tubulin polymerization with different physicochemical properties from the polysubstituted phenyl moieties usually present.

Addendum

During the revision process of this manuscript a model of the colchicine binding site of tubulin has been published,[62] from X-ray data of crystallized tubulin with bound colchicine.

Acknowledgements

The authors thank financial support from Spanish DGI-MCyT (PPQ2001-1977), Junta de Castilla y León (SA073/02) and european FEDER funds. A. B. S. Maya thanks Universidad de Salamanca for a predoctoral grant. Cytotoxicity assays were performed by Instituto Biomar S. L. (León, Spain) and PharmaMar (Madrid, Spain).

References

[1] Ojiva, I., Vite, G.D. and Altmann, K.-H. (2001) *Anticancer Agents* (American Chemical Society, Washington).
[2] Cooper, G.M. (1993) *The Cancer Book* (Jones & Barlett Publishers, New York).
[3] McIntosh, J.R. and McDonald, K.L. (1989) *Sci. Am.* **261**, 48–56.
[4] Hyams, J. and Lloyd, C.W. (1994) *Microtubules* (Wiley-Liss, New York).
[5] Shelanski, M.L. and Taylor, E.W. (1967) *J. Cell. Biol.* **34**, 549–554.
[6] Nogales, E., Wolf, S.G. and Downing, K.H. (1998) *Nature* **391**, 199–203.
[7] Nogales, E., Whittaker, M., Milligan, R.A. and Downing, K.H. (1999) *Cell* **96**, 79–88.
[8] Jordan, A., Hadfield, J.A., Lawrence, N.J. and McGown, A.T. (1998) *Med. Chem. Res. Rev.* **18**, 259.
[9] Schiff, P.B. and Horwitz, S.B. (1980) *Proc. Natl Acad. Sci. USA* **77**, 1561–1565.
[10] Rao, S., He, L., Chakravarty, I., Ojima, G.A. and Horwitz, S.B. (1999) *J. Biol. Chem.* **274**, 37990–37994.
[11] Jordan, M.A., Thrower, D. and Wilson, L. (1991) *Cancer Res.* **51**, 2212–2222.
[12] Rai, S.S. and Wolff, J. (1996) *J. Biol. Chem.* **271**, 14707–14711.
[13] Bai, R.B., Pettit, G.R. and Hamel, E. (1990) *J. Biol. Chem.* **265**, 17141–17149.
[14] Hastie, S.B. (1991) *Pharmacol. Ther.* **512**, 377–401.
[15] Weisenberg, R.C., Borisy, G.G. and Taylor, N.E. (1968) *Biochemistry* **7**, 4446–4478.

[16] Uppuluri, S., Knipling, L., Sackett, D.L. and Wolff, J. (1993) *Proc. Natl Acad. Sci. USA* **90**, 11598–11602.
[17] Pettit, G.R., Cragg, G.M., Herald, D.L., Schmidt, J.M. and Lohavanijaya, P. (1982) *Can. J. Chem.* **60**, 1374–1376.
[18] Pettit, G.R., Singh, S.B., Niven, M.L., Hamel, E. and Schmidt, J.M. (1987) *J. Nat. Prod.* **50**, 119–131.
[19] Lin, C.M., Ho, H.H., Pettit, G.R. and Hamel, E. (1989) *Biochemistry* **28**, 6984–6991.
[20] Singh, S.B. and Pettit, G.R. (1989) *J. Org. Chem.* **54**, 4105–4114.
[21] Rychnovsky, S.D. and Hwang, K. (1994) *J. Org. Chem.* **59**, 5414–5418.
[22] Hamel, E. and Lin, C.M. (1983) *Biochem. Pharmacol.* **32**, 3864–3867.
[23] Pettit, G.R., Anderson, C.R., Herald, D.L., Jung, M.K., Lee, D.J., Hamel, E. and Pettit, R.K. (2003) *J. Med. Chem.* **46**, 525–531.
[24] Lin, C.M., Singh, S.B., Chu, P.S., Dempcy, R.O., Schmidt, J.M., Pettit, G.R. and Hamel, E. (1988) *Mol. Pharmacol.* **34**, 200–208.
[25] Nandy, P., Banerjee, S., Gao, H., Hui, M.B. and Lien, E.J. (1991) *Pharm. Res.* **8**, 776–781.
[26] Brown, M.L., Rieger, J.M. and Macdonald, T.L. (2000) *Bioorg. Med. Chem.* **8**, 1433–1441.
[27] Cushman, M., Nagarathnam, D., Gopal, D., Chakraborti, A.K., Lin, C.M. and Hamel, E. (1991) *J. Med. Chem.* **34**, 2579–2588.
[28] Getahun, Z., Jurd, L., Chu, P.S., Lin, C.M. and Hamel, E. (1992) *J. Med. Chem.* **35**, 1058–1067.
[29] Cushman, M., Nagarathnam, D., Gopal, D., He, H.-M., Lin, C.M. and Hamel, E. (1992) *J. Med. Chem.* **35**, 2293–2306.
[30] Ohsumi, K., Nakagawa, R., Fukuda, Y., Hatanaka, T., Morinaga, Y., Nihei, Y., Ohishi, K., Suga, Y., Akiyama, Y. and Tsuji, T. (1998) *J. Med. Chem.* **41**, 3022–3032.
[31] Shirai, R., Tokuda, K., Koiso, Y. and Iwasaki, S. (1994) *Bioorg. Med. Chem. Lett.* **4**, 699–704.
[32] Cushman, M., He, H.M., Lin, Ch.M. and Hamel, E. (1993) *J. Med. Chem.* **36**, 2817–2821.
[33] Lawrence, N.J., Rennison, D., Woo, M., McGown, A.T. and Hadfield, J.A. (2001) *Bioorg. Med. Chem. Lett.* **11**, 51–54.
[34] Bedford, S.B., Quarterman, C.P., Rathbone, D.L. and Slack, J.A. (1996) *Bioorg. Med. Chem. Lett.* **6**, 157–160.
[35] Dark, G.G., Hill, S.A., Prise, V.E., Tozer, G.M., Pettit, G.R. and Chaplin, D.J. (1997) *Cancer Res.* **57**, 1829–1834.
[36] Pettit, G.R., Temple, C., Jr., Narayanan, V.L., Varma, R., Simpson, M.J., Boyd, M.R., Rener, G.A. and Bansal, N. (1995) *Anticancer Drug. Des.* **10**, 299–309.
[37] Ohsumi, K., Hatanaka, T., Nakagawa, R., Fukuda, Y., Morinaga, Y., Suga, Y., Nihei, Y., Ohishi, K., Akiyama, Y. and Tsuji, T. (1999) *Anticancer Drug. Des.* **14**, 539–548.
[38] Brown, R.T., Fox, B.W., Hadfield, J.A., McGown, A.T., Mayalarp, S.P., Pettit, G.R. and Woods, J.A. (1995) *J. Chem. Soc. Perkin Trans.* **1**, 577–581.
[39] Stevenson, J.P., Rosen, M., Sun, W., Gallagher, M., Haller, D.G., Vaughn, D., Giantonio, B., Zimmer, R., Petros, W.P., Stratford, M., Chaplin, D., Young, S.L., Schnall, M. and O'Dwyer, P.J. (2003) *J. Clin. Oncol.* **21**, 4428–4438.
[40] Medarde, M., Peláez Lamamié de Clairac, R., López, J.L. and San Feliciano, A. (1994) *J. Nat. Prod.* **57**, 1136–1144.
[41] San Feliciano, A., Medarde, M., Peláez Lamamié de Clairac, R., López, J.L., Puebla, P., Grávalos, M.D., Ruiz, P. and García de Quesada, M.T. (1993) *Arch. Pharm. (Weinheim)* **326**, 421–426.
[42] Shelanski, M.L., Gaskin, F. and Cantor, C.R. (1973) *Proc. Natl Acad. Sci. USA* **70**, 765–768.
[43] Dumortier, C., Gorbunoff, M., Andreu, J.M. and Engelborghs, Y. (1996) *Biochemistry* **35**, 4387–4395.
[44] Bradford, M.M. (1976) *Anal. Biochem.* **72**, 248–254.
[45] Han, Y., Chaudhary, A.G., Chordia, M.D., Sackett, D.L., Perez-Ramirez, B., Kingston, D.G.I. and Horwitz, S.B. (1996) *Biochemistry* **35**, 14173–14183.
[46] Medarde, M., Peláez Lamamié de Clairac, R., Ramos, A.C., Caballero, E., López, J.L., Gª Grávalos, D. and San Feliciano, A. (1995) *Bioorg. Med. Chem. Lett.* **5**, 229–232.
[47] ter Haar, E., Rosenkranz, H.S., Hamel, E. and Day, B.W. (1996) *Bioorg. Med. Chem.* **4**, 1659–1671.
[48] Shirai, R., Okabe, T. and Iwasaki, S. (1997) *Heterocycles* **46**, 145–148.
[49] Shirai, R., Takayama, H., Nishikawa, A., Koiso, Y. and Hashimoto, Y. (1998) *Bioorg. Med. Chem. Lett.* **8**, 1997–2000.
[50] Ohsumi, K., Hatanaka, T., Fujita, K., Nakagawa, R., Fukuda, Y., Nihei, Y., Suga, Y., Morinaga, Y., Akiyama, Y. and Tsuji, T. (1998) *Bioorg. Med. Chem. Lett.* **8**, 3153–3158.
[51] Wang, L., Woods, K.W., Li, Q., Barr, K.J., McCroskey, R.W., Hannick, S.M., Gherke, L., Credo, R.B., Hui, Y.H., Marsh, K., Warner, R., Lee, J.Y., Zielinski Mozng, N., Frost, D., Rosenberg, S.H. and Sham, H.L. (2002) *J. Med. Chem.* **45**, 1697–1711.
[52] Nam, N.H., Kim, Y., You, Y.J., Hong, D.H., Kim, H.M. and Ahn, B.Z. (2001) *Bioorg. Med. Chem. Lett.* **11**, 3073–3076.
[53] Flynn, B.L., Flynn, G.P., Hamel, E. and Jung, M.K. (2001) *Bioorg. Med. Chem. Lett.* **11**, 2341–2343.
[54] Chen, Z., Mocharla, V.P., Farmer, J.M., Pettit, G.R., Hamel, E. and Pinney, K.G. (2000) *J. Org. Chem.* **65**, 8811–8815.
[55] Kim, Y., Nam, N.H., You, Y.J. and Ahn, B.Z. (2002) *Bioorg. Med. Chem. Lett.* **12**, 719–722.
[56] Flynn, B.L., Hamel, E. and Jung, M.K. (2002) *J. Med. Chem.* **45**, 2670–2673.
[57] Nam, N.H., Kim, Y., You, Y.J., Hong, D.H., Kim, H.M., Ahn, B.Z., *Bioorg. Med. Chem. Lett.* **12**, 1955–1958.
[58] Westwell, A.D. (2003) *Drug Discovery Today* **8**, 421–422.
[59] Nandy, P., Banerjee, S., Gao, H., Hui, M.B. and Lien, E.J. (1991) *Pharm. Res.* **8**, 776–781.
[60] Maya, A.B.S., del Rey, B., de Clairac, R.P.L., Caballero, E., Barasoain, I., Andreu, J.M. and Medarde, M. (2000) *Bioorg. Med. Chem. Lett.* **10**, 2549–2551.
[61] Maya, A.B.S., Pérez-Melero, C., Mateo, C., Alonso, D., Fernández, J.L., Peláez, R., Caballero, E. and Medarde, M., *J. Med. Chem.*, under revision.
[62] Ravelli, R.B.G., Gigant, B., Curmi, P.A., Jourdain, I., Lachkar, S., Sobel, A. and Knossow, M. (2004) *Nature* **428**, 198–202.