Improving the potency and reducing the susceptibility to MDR resistance of combretastatins by means of 3-substituted indole moieties.

Substitution at the indole 3 position yields highly potent indolecombretastatins with reduced susceptibility to MDR resistance.

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Abstract: Resistance to combretastatin A-4 is mediated by metabolic modification of the phenolic hydroxyl and ether groups of the 3-hydroxy-4-methoxyphenyl (B ring). Replacement of the B ring of combretastatin A-4 by a N-methyl-5-indolyl reduces tubulin polymerization inhibition (TPI) and cytotoxicity against human cancer cell lines but cyano, methoxycarbonyl, formyl, and hydroxyiminomethyl substitutions at the indole 3-position restores potent TPI and cytotoxicity against sensitive human cancer cell lines. These highly potent substituted derivatives displayed low nanomolar cytotoxicity against several human cancer cell lines due to tubulin inhibition, as shown by cell cycle analysis, confocal microscopy, and tubulin polymerization inhibitory activity studies and promoted cell killing mediated by caspase-3 activation. Binding at the colchicine site was suggested by molecular modeling studies. Substituted combretastatins displayed higher potencies than the isomeric isocombretastatins and the highest potencies were achieved for the hydroxyiminomethyl (21) and cyano (23) groups, with TPI values in the submicromolar range and cytotoxicities in the subnanomolar range. Dose-response and time-course studies showed that drug concentrations as low as 1 nM (23) or 10 nM (21) led to a complete G2/M cell cycle arrest after 15 h treatment followed by a high apoptosis-like cell

\[\text{These authors contributed equally to this work.}\]
response after 48-72 h treatment. The P-glycoprotein and calcium antagonist verapamil increased 21 and 23 cytotoxicity to IC\textsubscript{50} values of 10^{-10} M, and highly potentiated the cytotoxic activity in 100-fold of the CHO derivative (17), in A549 human non-small cell lung cancer cells. The differences in cytotoxic potency observed between the highly potent cyano (23) and hydroxyiminomethyl (21) groups and other substituents with similar TPI values (17) were very much reduced upon co-treatment with verapamil. A 3,4,5-trimethoxyphenyl ring always afforded more potent derivatives than a 2,3,4-trimethoxyphenyl ring.

**INTRODUCTION**

Microtubules and their main constituents the αβ-tubulin heterodimers are the targets of many anti-tumor and anti-parasitic drugs that disrupt their dynamical equilibrium. Microtubule-targeting agents (MTAs) can affect cell shape maintenance, trafficking, cilia and flagella functioning, signaling, and mitosis as some of the many functions of microtubules in the cells. Several drug binding sites different from the nucleotide binding sites have been described, with at least seven structurally characterized, including the taxanes, the vinca alkaloids, the colchicine, the laulimalide/peloruside, the maytansine, the pironetin, and the hemiasterlin sites located in both the α and β tubulins. Some MTA stabilize the microtubules, as for example paclitaxel, while other destabilize them, as for instance colchicine, but at low concentrations all of them affect the polymerization/depolymerization dynamics.\textsuperscript{1}

The pivotal role of microtubules in mitosis, together with their role as scaffolds for signaling molecules modulating apoptosis, makes them a major target in cancer chemotherapy, and thus MTAs lead to cell cycle arrest at G\textsubscript{2}/M phase and late apoptosis.\textsuperscript{2} Inhibition of the mitotic spindle is the most common signature of MTAs in cell treatment, and therefore they are often referred to as anti-mitotic agents, although other mechanisms are probably important in their in vivo anti-tumor effects.\textsuperscript{3, 4} In fact, colchicine site binding drugs as for instance the combretastatins (Figure 1) are often considered vascular disrupting agents (VDA), because in vivo they cause a rapid collapse of the tumor associated
vasculature and blood flow shutdown which is mainly responsible for tumor death.\textsuperscript{5, 6} The emergence of VDAs holds great potential in the treatment of malignant solid tumors, and the 3-O-phosphate prodrug of combretastatin A-4 (CA4P), the more effective CA1P (Oxi4503) and the amino analogue AVE8062 are undergoing preclinical and clinical development.\textsuperscript{7} However, despite a rapid initial central tumor necrosis after VDAs treatment, a peripheral rim of resistant viable cancer cells remains and eventually grow to the necrotic area causing recurrence and metastasis.\textsuperscript{8, 9} Several strategies have been applied to increase the clinical efficiency of combretastatins, including combinations with antiangiogenic drugs or with other antitumor agents or the development of more potent derivatives.

\textbf{Figure 1. Representative combretastatin type ligands}

![Combretastatin A-4 (CA4)](image1)

![Naphthylcombretatins](image2)

![Indoleisocombretastatin](image3)

Innate or acquired resistance mechanisms employed by cancer cells against treatment with combretastatin A-4 have been described, including adaptation to the hypoxic conditions and reestablishment of the vasculature in response to the reduction in blood supply as well as direct resistance to the drug. The B ring of combretastatin A4 through its hydroxyl group provides the attachment point for prodrug formation thus allowing pharmacokinetic optimization but it is also its Achilles’ heel struck by phase I and II metabolic reactions associated with its inactivation, as shown for resistant hepatocellular carcinoma or colon adenocarcinoma-derived HT-29 cells.\textsuperscript{10-12}

We have previously shown that an indole ring is a suitable replacement for the B ring of combretastatin A4 in the context of a 1,1-diarylethene (isocombretastatin) skeleton (Fig. 1).\textsuperscript{13} Substitutions of the indole 3 position did not result in improvements with respect to the parent unsubstituted indole. Surprisingly, the isomeric indole combretastatin (1,2-diarylethene) showed much lower potency.\textsuperscript{14} Here we show that introduction of substituents at the 3 position of indole combretastatin improves on the tubulin inhibitory
activity. Furthermore, the cytotoxicity of compounds with similar tubulin inhibitory potency against combretastatin A4 resistant colon adenocarcinoma (HT-29) and lung adenocarcinoma (A-549) cell lines is dependent on minor structural changes and can be further improved upon combination with verapamil. Our results suggest that MDR efflux is an important factor in the observed cytotoxicity differences and that substitution at the indole 3 position with appropriate substituents such as the cyano or hydroxyimino groups greatly reduces the compounds’ efflux and result in apoptotic-like cell death after complete G2/M cell cycle arrest after very low dose (1nM) drug treatments.

RESULTS AND DISCUSSION

Chemical synthesis

The synthesis of combretastatins was carried out by the usual Wittig procedure, followed by derivatization at 3-position of the indoles. The indole aldehydes used as starting materials 1-5, were obtained from commercial 1H-indole-5-carboxaldehyde (Scheme 1), whereas 1H-benzo[d]imidazole-5-carboxylic acid (6) was used to prepare the corresponding 1-methyl-1H-benzo[d]imidazole-5(6)-carbaldehydes (9 and 10, Scheme 2). Indole-combretastatins unsubstituted at 3-position 13, 15 and 16 were respectively synthesized by Wittig olefination of 1 and 2 with 3,4,5-trimethoxybenzyl bromide (11) and 2 with 2,3,4-trimethoxybenzyl bromide (12) (Scheme 1). 15 and 16 were readily formylated at 3-position,15 to yield 17 and 18 (Scheme 3), the two latter used to prepare derivatives at this position 19-34.

To complete the series of compounds to assay, the aldehyde 14, bromoderivatives 35 and 36 (Scheme 1), and the benzimidazole-combretastatins (Scheme 2) seemed adequate to fulfil the structural modifications of indole related derivatives. The unresolved mixture of 1-methyl-1H-benzimidazole-5(6)-carbaldehydes (9+10), obtained by methylation of 1H-benzimidazole-5(6)-carbaldehyde (8), was used to produce the combretastatins 37+38/Z+E, which could only be partially separated into 37+38/Z and 37+38/E.
Once separated from the \(E\)-aldehydes, several transformations aimed at increasing solubility by means of additional nitrogen or oxygen atoms were made, \(Z\)-aldehydes 17 and 18 were reduced to the alcohols 19 and 20, transformed into the oximes\(^{16,17}\) 21 (\(Z+E\)) and 22 (\(Z+E\)) and oxidized to carboxylic acids\(^{18}\) 25 and 26. In addition, the oximes by treatment with acetic anhydride in pyridine, were converted into the nitriles 23 and 24 and the carboxylic acid 25 was transformed into its methyl ester 27.

**Scheme 1: Synthesis of 3-indolecarbaldehyde combretastatin derivatives 13-16 and bromoderivatives 35 and 36.**

(a) (i) \((3,4,5-(\text{MeO})_{3}\text{PhCH}_{2})\text{Ph}_{3}\text{PBr} (11)\), THF, BuLi (1.6M), THF, -40\(^\circ\)C, 1h; (ii) 1-ethyl-1\(H\)-indole-5-carbaldehyde (1), 1-methyl-1\(H\)-indole-5-carbaldehyde (2), 1-ethyl-1\(H\)-indole-3,5-dicarbaldehyde (3), 1-methyl-3-bromo-1\(H\)-indole-5-carbaldehyde (4) or 1-methyl-2,3-dibromo -1\(H\)-indole -5-carbaldehyde (5), THF, -40 °C to rt, 24 h. (b) (i) \((2,3,4-(\text{MeO})_{3}\text{PhCH}_{2})\text{Ph}_{3}\text{PBr} (12)\), THF, BuLi (1.6M), THF, -40\(^\circ\)C, 1h; (ii) 1-methyl-1\(H\)-indole-5-carbaldehyde (2), THF, -40 °C to rt, 24 h.

**Scheme 2: Preparation of benzimidazole combretastatin derivatives 37+38.**
Reagents and conditions: (a) (i) Ph₃P(3,4,5-MeO₃PhCH₂)I, THF, nBuLi (1.6M), THF, -40 ºC, 1h; (ii) 1-methyl-1H-benzimidazole-5(6)-carbaldehydes, THF, -40 ºC to rt, 24 h.

Scheme 3: Preparation of 3-substituted indole-combretastatin derivatives 17-34.

Reagents and conditions: (a) (i) POCl₃, DMF, 0 ºC, 1 h; (ii) 15 or 16, 60 ºC, 2 h. (b) NaBH₄, MeOH, rt, 30 min. (c) NH₂OH·HCl, MeOH, pyr (2 drops), rt, 24 h. (d) Ac₂O, pyr, rt, 4 h. (e) 17 or 18 in tBuOH, 2-methyl-2-butene, THF; add NaClO₂, NaH₂PO₄, H₂O, rt, 12 h. (f) TMSCHN₂, MeOH, 15 min. (g) (i) NH₂CH₂COOEt·HCl; EDCI; 4-DMAP; rt, 48 h, inert atmosphere. (h) CH₂NHCH₂COOMe·HCl; EDCI; 4-DMAP; rt, 48 h, inert atmosphere. (i) NH₂CH₂CH₂COOEt; EDCI; 4-DMAP; rt, 48 h, inert atmosphere. (j) (COOMe)₂CHNH₂·HCl; EDCI; 4-DMAP; rt, 48 h, inert atmosphere. (k) KOH/MeOH, rt, 30 min.
Other transformations include the formation of several amides from the carboxylic acid 25, using different aminoesters (Scheme 3). Thus, the amides 28, 29, 30 and 31 were obtained by treatment with glycine ethyl ester, sarcosine methyl ester, β-alanine ethyl ester and dimethyl aminomalonate. By saponification with KOH/MeOH the carboxylic acids 32, 33 and 34 were obtained.

With these compounds, representative structures of the 3-substituted 1-methyl-1H-indole combretastatins were at hand in order to explore the effect of this substitution on their activities.

_Aqueous solubility_

Aqueous solubility in phosphate buffer at pH 7.0 was determined using a shake-flask method, microfiltration and UV absorbance measurement of soluble compound. _N_-methylindole combrestatins with hydrogen at C-3 (13, 15 and 16) display aqueous solubility similar to Combretastatin A-4 (Table 1). Cyano or bromine substituents do not improve aqueous solubility but a carboxylic acid or amides of aminoacids increase the solubility between 40 and 150 times.

<table>
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<th>Compound</th>
<th>Solubility (µg/mL)</th>
<th>Compound</th>
<th>Solubility (µg/mL)</th>
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<td>23</td>
<td>1.0</td>
<td>36</td>
<td>&lt;1.0</td>
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_Biology._

The effect of the synthesized compounds on cell viability against four types of human cancer cell lines: A-549 human lung carcinoma, HeLa human cervix epithelioid carcinoma, HL-60 human acute myeloid leukemia, and HT-29 human colon adenocarcinoma was assayed by the XTT method. The results are summarized in Table 2. The compounds are cytotoxic at concentrations between micromolar
and subnanomolar, thus indicating that the combination of a trimethoxyphenyl and a 5-indolyl forming a Z stilbene skeleton is a very favorable scaffold for cytotoxicity. The most potent compounds of the series inhibited proliferation of the indicated human cancer cell lines at very low drug concentrations, with IC\textsubscript{50} values in the 10\textsuperscript{-9} M and 10\textsuperscript{-10} M range. These IC\textsubscript{50} values were up to 10–100 times lower than those shown by doxorubicin (IC\textsubscript{50} at a range of 10–8 M). For most compounds the IC\textsubscript{50} are similar for HL60 and HeLa whereas they are higher for A-549 and HT-29, a difference in sensitivity which has been previously observed for other colchicine site ligands.\textsuperscript{10, 13}

Table 2. Tubulin Polymerization Inhibitory Activity, Cytotoxic Activity against Human Cancer Cell Lines, and Results of the Docking Studies.

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<th>R\textsubscript{N}</th>
<th>X</th>
<th>Y</th>
<th>Z</th>
<th>R\textsubscript{2}</th>
<th>%TPI (at 20 \mu M)\textsuperscript{a}</th>
<th>TPI IC\textsubscript{50} \textsuperscript{b} (10\textsuperscript{-6} M)</th>
<th>HL-60 (10\textsuperscript{-6} M)\textsuperscript{c}</th>
<th>A-549 (10\textsuperscript{-6} M)\textsuperscript{c}</th>
<th>HeLa (10\textsuperscript{-6} M)\textsuperscript{c}</th>
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<th>Binding E (Cluster number // % of conf)\textsuperscript{d}</th>
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<td></td>
<td>OMe</td>
<td>H</td>
<td>NMe</td>
<td>H</td>
<td>49</td>
<td>&gt;20</td>
<td>0.03</td>
<td>0.22</td>
<td>0.028</td>
<td>0.029</td>
</tr>
</tbody>
</table>

* Percentage tubulin polymerization inhibitory (TPI) activity at the indicated concentration.  
* Tubulin polymerization inhibitory (TPI) IC₅₀.  
* Values are derived from concentration-response curves using the XTT assay as described in the Experimental Section. Data are shown as the mean values of three experiments performed in triplicate.  
* AutoDock Binding Energies in kcal/mol for the best pose of the cluster with number representing the indicated number of poses out of a total of 100.  
* At 5µM.  
* At 10µM.

For all the pairs differing only in the disposition of the methoxy groups of ring A (e.g., compare the matched couples 15/16Z, 17/18, 19/20, 21/22, 23/24 and 25/26), the compounds with a 2,3,4-trimethoxyphenyl (like that of colchicine) were less potent than those with a 3,4,5-trimethoxyphenyl
ring (like those of podophyllotoxin and combretastatin A4), similar to what has been reported previously.\textsuperscript{22} Comparison of 13 and 14 with 15 and 17 respectively, shows that a methyl is slightly preferred over an ethyl on the indole nitrogen.

Substitutions at the indole 3 position (e.g. 17, 19, 21, 23 and 25) result in an overall potency increase with respect to the unsubstituted 15 for the 3,4,5-trimethoxyphenyl substituted compounds whereas an opposite trend is seen for compounds with a 2,3,4-trimethoxyphenyl (e.g., compare 18, 20, 22, 24 and 26 with 16Z). This different behavior probably arises from steric hindrance, as larger groups result in more reduced potencies, in agreement with previous results on the isocombretastatins where indole substitution causes a reduction in potency.\textsuperscript{13} We have previously shown that substituents protruding out of the plane of ring B in isocombretastatins result in less potent compounds.\textsuperscript{23}

The optimal substitutions at the indole 3 position are the hydroxyiminomethyl (21) and the cyano (23) groups, which are nanomolar or subnanomolar in the four cell lines assayed. The formyl substituent (17) provides also high potency against HeLa and HL60, but there is a significant potency drop down to the micromolar range for A549 and HT-29. For the second potency level (two digit nanomolar), carboxy (25), methoxycarbonyl (27) and hydroxymethyl (19) indoles and the \(N\)-methylbenzimidazoles (37, 38) show similar potencies in the four cell lines. Larger substitutions at the three position (29, 34) result in a significant potency reduction, although most of them are still in the submicromolar range.

The effect of the compounds on in vitro bovine brain tubulin assembly was studied at concentrations between 5 and 20 \(\mu\)M (Table 2). For those compounds inhibiting tubulin polymerization more than 50% at 20 \(\mu\)M we determined the IC\textsubscript{50} values of tubulin polymerization inhibition (TPI). These values allowed us to compare the potencies of different compounds and to analyze the relationship between structure and TPI. Most of the compounds showed potent TPI inhibitory activity with values below 20 \(\mu\)M, many better than combretastatin A4 (IC\textsubscript{50} = 3 \(\mu\)M). As seen for the cytotoxicity assays, the 3,4,5-
trimethoxyphenyl and 3 substituted N-methylindole combination was optimal, with several compounds (e.g. 17, 21, 23 and 27) showing submicromolar values. The compounds with a 2,3,4-trimethoxyphenyl ring showed reduced potencies, typically a ten-fold reduction as for example from 19 to 20. Substitution of the indole N-methyl by an N-ethyl modestly reduces the TPI potency. The TPI results show that aldehyde (17), hydroxyiminomethyl (21), nitrile (23), and methoxycarbonyl (27) substituents at the 3-position of the indole are optimal for tubulin inhibition. These groups share a sp2 carbonyl or equivalent conjugated with the indole ring and both hydrogen-bond donors and acceptors are allowed. Comparison of the TPI and cytotoxicity IC50 values shows that there is a good overall agreement between the TPI and the cytotoxic potencies against HeLa and HL60 cells, while the effects against A-549 and HT-29 show in some cases significant deviations. These results suggest either a difference in the target (different tubulin isotype expression) or the involvement of an additional effect (drug efflux, drug metabolism, or other) to account for the observed potency reduction in these cell lines.

**Figure 2.** Effects of compounds 17, 21 and 23 on the microtubule network of Hela cells. Cells were incubated in the absence (Control) or in the presence of 10 or 100 nM of compounds 17, 21 and 23 for 15 h and then fixed and processed to analyze microtubules (red fluorescence) and nuclei (blue fluorescence) as described in the Experimental Section. Bar: 25 µm. The photomicrographs shown are representative of at least three independent experiments performed.
The three compounds that showed a lower IC$_{50}$, namely 17, 21 and 23, led to a drastic and severe disruption of the microtubule network of HeLa cells at nanomolar concentrations (Figure 2), further confirming that the observed effects were due to interaction with tubulin. Dose-response and time-course analyses of the most active compounds synthesized in this work (16E, 17, 21, 23, 25, 27, 30) showed a major cell cycle arrest at G$_2$/M phase (Figure 3), followed by the induction of apoptotic cell death, as assessed by an increase in the percentage of cells at the sub-G$_0$/G$_1$ region, representing cells undergoing chromosomal DNA degradation (Supplementary Table S1).

**Figure 3.** Effect of different compounds on the distinct cell cycle phases in HeLa cells. Compounds 16E, 17, 21, 23, 25, 27 and 30 were incubated for 15 h and then their DNA content was analyzed by flow cytometry as described in the Experimental Section. The different cell cycle phases were
quantified and represented in pie charts to easily visualize the changes in G2/M arrest. Untreated control cells were run in parallel. Data shown are representative of at least three independent experiments performed.
Compound 23 was the most active one in arresting most of the cells (74.2%) at the G2/M phase at only 1 nM concentration and after 15 h incubation, followed by compound 21 (79.9%) and 17 (59.3%), which required 10 nM concentration (Supplementary Table S1, and Figure 3).

Figure 4. Time-course of the effects of compounds 21 and 23 on cell cycle in HeLa cells. Cells were incubated with 1 or 10 nM of 21 and 23 for the indicated times, and their DNA content was analyzed by fluorescence flow cytometry. The positions of the G0/G1 and G2/M peaks are indicated by arrows, and the proportion of cells in each phase of the cell cycle was quantified by flow cytometry. The cell population in the sub-G0/G1 region represents cells with hypodiploidal DNA, an indicator of apoptosis, and the percentages of cells at this region are indicated in each histogram. Untreated control cells were run in parallel. The experiments shown were representative of three performed.
Figure 4 shows a time-course of the effects of the most active analogs 23 (active at 1 nM) and 21 (active at 10 nM) on the cell cycle profile of HeLa cells. G2/M cell cycle arrest was evident even at only 9 h treatment, and the onset of apoptosis took place after 24 h incubation, and the cell death response was almost complete after 72 h treatment (Figure 4). Taken together, these data indicate that the actions of 23 and 21 on microtubules lead to a total disruption of microtubules that triggers a potent apoptotic response, rendering a complete cell demise in the drug-treated population. Caspase-3 activation was observed following treatment of HeLa cells with 23, as assessed by the processing of procaspase-3 into the p17/p19 active form, and the cleavage of the typical caspase-3 substrate PARP into the 89-kDa cleaved form (Supplementary Figure S1). Interestingly, the combined incubation of 21 or 23 and 10 µM verapamil, a concentration that had been previously reported not to decrease cell viability,24, 25 highly potentiated the cytotoxic activity of both 21 and 23 to IC50 of 10-10 M in A549 cells (Figure 5). Furthermore, the compound 17 became very active against A549 cells (IC50 = 5.98 x 10^-8 M) when combined with 10 µM verapamil. These data suggest that the P-glycoprotein and calcium antagonist verapamil highly potentiate the cytotoxicity rendered by these newly synthetized combretastatin A-4 derivatives.

Figure 5. Effect of verapamil on the sensitivities of A549 cancer cells to 21 and 23. Cells were co-incubated with verapamil and different concentrations of 21 and 23. The dose–response curves were drawn using the GraphPad Prism 5 software. Results are mean values ± S.D. of a representative experiment carried out in triplicate, out of three performed.
The different effect of the 3-substituents on the indole ring of isomeric combretastatins and isocombretastatins on their potency could be explained by their topological differences. Therefore, the preferred conformations of the compounds were calculated, DFT-optimized and those conformations better superimposing onto the X-ray crystal structure of combretastatin A-4 when in complex with tubulin (pdb ID 1LYJ)\textsuperscript{26} were selected as the putative conformations binding to the colchicine site of tubulin. Figure XRAY shows the superimposition of the unsubstituted (a, 15) and 3-cyano indole combretastatins (b, 23 with a 3,4,5-trimethoxyphenyl ring and c, 23 with a 2,3,4-trimethoxyphenyl ring) and isocombretastatins (d and e) onto combretastatin A4 (carbon atoms colored in cyan). All of them place the phenyl rings close to those of combretastatin A4 to comply with the essential role assigned by pharmacophoric models for ligands of the colchicine site to their spatial arrangement. The single atom sp\textsuperscript{2} hybridized connection between the phenyl rings of isocombretastatins results in a wider opening
angle between the ring centroids and the bridge than in a stilbene (Fig d and e). When in complex with tubulin this disposition of the rings either projects the 3 substituents at positions further away probably colliding with the site walls or forces the displacement of the trimethoxyphenyl ring in the opposite direction towards the site walls thus resulting in the observed potency reduction. Similarly, a 2,3,4-trimethoxyphenyl ring is more sterically demanding (Fig c) in the plane of the B – ring and trying to accommodate it in the colchicine site pushes the indole ring in the opposite sense. Substituents at the indole 3-position further worsen the steric problem and explains the potency decrease proportional to substituent size.

**Figure 6.** Best superpositions of the X-ray structure of combretastatin A4 (in cyan) in the complex with tubulin and the conformations of representative compounds showing the effects of the structural modifications and the corresponding docking results (the protein is shown in grey): a) with 15; b) with 23; c) with 24; d) with the 11,diarylethene isomer (isocombretastatin) of 15 and e) with the 11,diarylethene isomer (isocombretastatin) of 24 and f) a combination of b,c and e.
Docking experiments using AutoDock 4.2 on the X-ray structure of tubulin in complex with combretastatin A4 (pdb ID: 5LYJ) yielded results in excellent agreement with the experimental TPI and cytotoxicity results (Table 2) and with the results of the conformational study. When other X-ray structures of tubulin (15 different structures obtained from X-ray crystal structures of tubulin in complex with less structurally related ligands of the colchicine site or structures derived from molecular dynamics simulations, as previously described\textsuperscript{13}) yielded worse results. The docking binding energy differences between compounds with a 3,4,5-trimethoxyphenyl (15, 17, 19, 21, 23, and 25) and their matched pairs with a 2,3,4-trimethoxyphenyl ring (16, 18, 20, 22, 24, and 26, respectively) is ca 0.6 kcal/mol, in good agreement with the differences observed for TPI IC\textsubscript{50} values. Also, for the former group the lowest energy clusters correspond to the same ring dispositions as seen for combretastatin A4, whereas for the second group the clusters with this ring disposition are usually second or worse by energy, being usually surpassed by dispositions with the two phenyl rings switched. For the most potent analogues (15, 17, 19, 21, 23, 25 and 27) the rank order of binding energies (21, 23, 19, 17, 27, 15, 25, 19, CA-4) roughly matches the TPI values (23, 21, 17, 27, 19, 25, 15, CA-4), in spite of the fact that the energy differences between them are probably too small to be safely trusted. For the extended amides (29-34) the results are similar to those described for the 2,3,4-trimethoxyphenyl rings.

**CONCLUSIONS**

The replacement of the B ring of combretastatin A4 by 3 substituted indoles results in highly potent tubulin inhibition and cytotoxicity against several human cancer cell lines. This effect of the indole 3
substituents is highly context dependent, as it has been shown to be detrimental in the isomeric isocombretastatins or in compounds with a 2,3,4-trimethoxyphenyl ring. The optimal substituents are the cyano and hydroximinomethyl groups, whose compounds display high potency in all the studied cell lines, whereas compounds with other substituents show different potencies in different cell lines. Co-treatment with verapamil restores high potency in these later compounds, thus suggesting a resistance based on MDR efflux proteins. The lower effect observed of the co-treatment for the former compounds suggest a lower sensitivity to MDR efflux. The more potent compounds displayed low nanomolar cytotoxicity against several human cancer cell lines due to tubulin inhibition, complete G2/M cell cycle arrest after 15 h followed by a high apoptosis-like cell response after 48-72 h mediated by caspase-3 activation. Molecular modeling studies support binding at the colchicine site and provide an explanation for the aforementioned SAR. These compounds are highly potent against cell lines intrinsically resistant to combretastatin A4 and therefore could reduce treatment resistance.

EXPERIMENTAL SECTION

Chemical synthesis

1-Methyl-1H-indole-5-carbaldehyde (1) and 1-Ethyl-1H-indole-5-carbaldehyde (1)

1.04 g (26 mmol) of NaOH and 20 mg of n-Bu₄N’HSO₄ were added to a stirred solution of 1H-indole-5-carbaldehyde (2.0 g, 13,8 mmol) in 40 mL of dry CH₂Cl₂. After 1 hour at atmosphere temperature 3 mL (40.2 mmol) of methyl iodide or ethyl bromide were added and the reaction was heated at 50 ºC. After 3 days the reaction mixture was concentrated, re-dissolved in CH₂Cl₂, washed with brine, dried over anhydrous Na₂SO₄, filtered and concentrated in vacuum to obtain 1,10 g (61%) of 1 and 1.48 g (62.0%) of 2.

1-Ethyl-1H-indole-5-carbaldehyde (1): M.p.: 64-66 ºC (CH₂Cl₂/Hex). IR (KBr): 1703, 1605, 1307, 769 cm⁻¹. ¹H NMR (200 MHz, CDCl₃): δ 1.49 (3H, t, J = 7.2), 4.22 (2H, m), 6.66 (1H, d, J = 3.3); 7.22
(1H, d, J = 3.3), 7.42 (1H, d, J = 8.4), 7.78 (1H, dd, J = 8.4 and 1.4), 8.15 (1H, d, J = 1.4), 10.0 (1H, s).

$^{13}$C NMR (50 MHz, CDCl$_3$): δ 15.4 (CH$_3$), 41.2 (CH$_2$), 103.4 (CH), 109.9 (CH), 121.6(CH), 126.5 (CH), 128.4 (C), 128.5 (CH), 129.3 (C), 139.0 (C), 192.5 (CH).

**1-Methyl-1H-indole-5-carbaldehyde (2):** M.p.: 85-86ºC (Ether). $^1$H NMR (200 MHz, CDCl$_3$): δ 3.76 (3H, s), 3.76 (3H, s) 6.55 (1H, d, J = 3.3), 7.10 (1H, d, J = 3.3), 7.41(1H, d, J = 8.8), 7.80 (1H, dd, J= 8.8 and 1.9), 8.05 (1H, d, J = 1.9), 9.92 (1H, s). $^{13}$C NMR (50 MHz, CDCl$_3$): δ 32.6 (CH$_3$), 103.1 (CH), 109.8 (CH),121.4 (CH), 126.1 (CH), 128.2(C), 129.1 (C), 130.9 (CH), 139.8 (C), 192.3 (CH).

**1-Ethyl-1H-indole-3,5-dicarbaldehyde (3):** IR (film): 1687, 1662, 1191 cm$^{-1}$. $^1$H NMR (200 MHz, CDCl$_3$): δ 1.54 (3H, t, J = 7.2), 4.25 (2H, c, J = 7.2),7.44 (1H, d, J = 8.6), 7.84 (1H, s), 7.85 (1H, dd, J = 8.6 and 1.6), 8.73 (1H, d, J = 1.6), 9.99 (1H, s), 10.04 (1H, s). $^{13}$C NMR (50 MHz, CDCl$_3$): δ 15.0 (CH$_3$), 42.1 (CH$_2$), 110.8 (CH), 118.8 (C), 123.4 (CH),125.0 (C), 126.8 (CH), 131.7 (C), 139.7 (CH), 140.1 (C), 184.5 (CH), 192.1 (CH).

**3-Bromo-1-methyl-1H-indole-5-carbaldehyde (4) and 2,3-Bibromo-1-methyl-1H-indole-5-carbaldehyde (5)**

To a solution of 1-methyl-1H-indole-5-carbaldehyde (2.0 g, 12.58 mmol) in 4 mL of chloroform, 2.9 g (16.35 mmol) of N-bromosuccinimide were slowly added. After 24 h stirring at 0 ºC, the reaction mixture was evaporated to obtain 5.38 g of crude. After column chromatography on silica gel (Hex/EtOAc 7/3) 611mg (1.93mmol, 15.34%) of compound 5 and 540 mg (2.27 mmol, 18.04%) of compound 4 were obtained.

**3-Bromo-1-methyl-1H-indole-5-carbaldehyde (4):** IR $^1$H NMR (200 MHz, CDCl$_3$): δ 3.84 (3H, s), 7.26 (1H, s), 7.40 (1H, d, J = 8.8), 7.84 (1H, dd, J = 8.8 and 1.9), 8.09 (1H, d, J = 1.9), 10.07 (1H, s). $^{13}$C NMR (50 MHz, CDCl$_3$): δ 33.3 (CH$_3$), 91.7 (C), 110.2 (CH), 122.4 (CH), 124.9 (CH), 127.3 (C), 129.5 (CH), 129.8 (C), 139.4 (C), 192.0 (CHO).
2,3-Dibromo-1-methyl-1H-indole-5-carbaldehyde (5): $^1$H NMR (200 MHz, CDCl$_3$): $\delta$ 3.84 (3H, s), 7.40 (1H, d, $J = 8.4$), 7.85 (1H, dd, $J = 1.0$ and 7.4), 8.05 (1H, d, $J = 0.8$), 10.06 (1H, s). $^{13}$C NMR (50 MHz, CDCl$_3$): $\delta$ 32.7 (CH$_3$), 94.9 (C), 100.2 (C), 110.3 (CH), 117.0 (C), 118.4 (C), 122.8 (CH), 126.9 (CH), 130.3 (C), 191.8 (CHO).

Preparation of compounds 7-10

To a suspension of 2.0 g (12.3 mmol) of 1H-benzo[d]imidazole-5-carboxylic acid in 50 mL of dry THF, in inert atmosphere was added 983 mg (24.7 mmol) of LiAlH$_4$ and stirred 48 h at 65°C. The reaction mixture was poured into EtOAc/ EtOH and was filtered, by evaporation to dryness 1.4 g (75%) of compound 7 was obtained.

4.94 g (56.7 mmol) of MnO$_2$ were added to a solution of 1.40 g (9.46 mmol) of 7 in 50 mL of MeOH. After 48 h at rt, the mixture was filtered over silica and by evaporation 1.24 g (90%) of compound 8 was isolated.

To a solution of 1.24 g (8.49 mmol) of 8 in 50 mL of CH$_2$Cl$_2$:H$_2$O (3:2) was added 700 mg (17.5 mmol) of NaOH and and 40 mg of $n$-Bu$_4$NHSO$_4$. After 1 h stirring 1.33 mL (21.2 mmol) of MeI were added. After 24 h After 3 days the reaction mixture was concentrated, re-dissolved in CH$_2$Cl$_2$. After chromatography on silica gel (CH$_2$Cl$_2$/EtOAc 85/15), 600 mg (44%) of a mixture 9 and 10 were obtained

1H-benzo[d]imidazol-5-yl)methanol (7): $^1$H NMR (200 MHz, CDCl$_3$): $\delta$ 4.60 (2H, s), 7.08 (1H, dd; $J = 8.4$ and 1.6); 7.45 (1H, d, $J = 8.4$), 7.49 (1H, bs), 7.94 (1H, s). $^{13}$C NMR (50 MHz, CDCl$_3$): $\delta$ 65.9, (CH$_2$), 115.2 (CH), 116.3 (CH), 121.3 (CH), 136.1 (C), 140.6 (C), 141.0 (C), 146.0 (CH).

1H-Benzof[d]imidazole-5-carbaldehyde (8): $^1$H NMR (200 MHz, CDCl$_3$): $\delta$ 7.55 (1H, d, $J = 8.4$), 7.65 (1H, dd, $J = 8.4$ and 1.4), 8.01 (1H, bs), 8.24 (1H, s), 9.85 (1H, s). $^{13}$C NMR (50 MHz, CDCl$_3$): $\delta$ 116.3 (CH), 120.5 (CH), 124.5 (CH), 133.1 (C), 139.7 (C), 142.8 (C), 145.9 (CH), 194.0 (CH).
1-Methyl-1H-benzo[d]imidazole-6-carbaldehyde (9) and 1-Methyl-1H-benzo[d]imidazole-5-carbaldehyde (10): \(^1\)H NMR (200 MHz, CDCl\(_3\)): \(\delta\) 3.60 (3H, s); 3.63 (3H, s), 7.17 (1H, d, \(J = 8.4\)), 7.56 (1H, dd, \(J = 8.4\) and 1.6), 7.56 (1H, dd, \(J = 8.4\) and 1.6), 7.59 (1H, d, \(J = 8.4\)), 7.61 (1H, s), 7.77 (1H, s), 7.82 (1H, d, \(J = 1.6\)), 7.98 (1H, d, \(J = 1.6\)), 9.78 (1H, s), 9.79 (1H, s). \(^{13}\)C NMR (50 MHz, CDCl\(_3\)): \(\delta\) 31.2 (2) (CH\(_3\)), 110.1 (CH), 111.8 (CH), 120.2 (CH), 123.2 (CH), 123.5 (CH), 123.8 (CH), 131.3 (C), 131.5 (C), 134.5 (C), 138.7 (C); 143.4 (C), 145.9 (CH), 147.0 (CH), 148.1 (C), 191.7 (CH), 191.9 (CH).

(Z)-1-Ethyl-5-(3,4,5-trimethoxystyryl)-1H-indole (13)

To a suspension of triphenyl(3,4,5-trimethoxybenzyl)phosphonium bromide (1.67 g, 3.19 mmol) in dry THF (40 mL), at -40 ºC in argon atmosphere, nBuLi (1.6 M in hexane, 2.0 mL, 3.2 mmol) was added and stirred for 1 h. A solution of 1-ethyl-1H-indole-5-carbaldehyde (500 mg, 2.89 mmol) in dry THF (10 mL) was slowly added at -40 ºC and progressively warmed to room temperature. After 24 h, the reaction mixture was poured into ammonium chloride solution and extracted with CH\(_2\)Cl\(_2\). The organic layers were washed to neutrality with brine, dried over anhydrous Na\(_2\)SO\(_4\), filtered and evaporated to dryness. After chromatography on silica gel (Hex/AcOEt 85/15), 130 mg (0.40 mmol, 14%) of 13Z and 161 mg (0.51 mmol, 18%) of a mixture 13Z/13E were obtained.

(Z)-1-Ethyl-5-(3,4,5-trimethoxystyryl)-1H-indole-3-carbaldehyde (14)

IR (film): 1685, 1579, 1127, 1007, 796, 722 cm\(^{-1}\). \(^1\)H NMR (200 MHz, CDCl\(_3\)): \(\delta\) 1.51 (3H, t, \(J = 7.3\)), 3.62 (6H, s), 3.85 (3H, s), 4.18 (2H, c, \(J = 7.3\)), 6.41 (1H, d, \(J = 3.1\)); 6.43 (1H, d, \(J = 12.3\)), 6.57 (2H, s), 6.72 (1H, d, \(J = 12.3\)), 7.08 (1H, d, \(J = 3.1\)), 7.20 (1H, bs), 7.26 (1H, dd), 7.60 (1H, bs). \(^{13}\)C NMR (50 MHz, CDCl\(_3\)): \(\delta\) 15.5 (CH\(_3\)), 41.1 (CH\(_2\)), 55.9 (2) (CH\(_3\)), 60.9 (CH\(_3\)), 103.3 (CH), 106.1 (CH), 108.9 (CH), 121.7 (CH), 122.8 (CH), 127.4 (CH), 128.3 (C), 128.7 (C), 129.8 (CH), 131.4 (CH), 133.3 (C), 135.0 (C), 135.6 (C), 152.8 (2) (C). HRMS (C\(_{21}\)H\(_{23}\)NO\(_3\)): calcd. 337.1678 (M\(^{+}\)), found falta. HPLC
To a suspension of triphenyl(3,4,5-trimethoxybenzyl)phosphonium bromide (466 mg, 0.89 mmol) in dry THF (20 mL), at -40°C in argon atmosphere, nBuLi (1.6 M in hexane, 0.56 mL, 0.89 mmol) was added and stirred for 1 h. The mixture was slowly added to a solution of 1-ethyl-1H-indole-3,5-dicarbaldehyde (189 mg, 0.89 mmol) in dry THF (10 mL) at -40 °C and progressively warmed to room temperature. After 24 h, the reaction mixture was poured into ammonium chloride solution and extracted with CH₂Cl₂. The organic layers were washed to neutrality with saturated NaCl, dried over anhydrous Na₂SO₄, fileted and evaporated to dryness. By repeated chromatography with Hex/AcOEt 7/3 and CH₂Cl₂/AcOEt 96/4, aldehyde 14 (27 mg, 5.1%) was obtained. IR (film): 1655, 1459, 1397, 1124, 784 cm⁻¹. ¹H NMR (400 MHz, CDCl₃): δ 1.51 (3H, t, J = 7.2), 3.61 (6H, s), 3.82 (3H, s), 4.19 (2H, c, J = 7.2), 6.51 (1H, d, J = 12.2), 6.50 (2H, s), 6.73 (1H, d, J = 12.2), 7.19 (1H, d, J = 8.8), 7.30 (1H, dd, J = 8.8 and 2.0), 7.72 (1H, s), 8.26 (1H, bs), 9.96 (1H, s). ¹³C NMR (100 MHz, CDCl₃): δ 15.1 (CH₃), 42.0 (CH₂), 55.9 (2) (CH₃), 61.0 (CH₂), 106.2 (2) (CH), 109.4 (CH), 118.2 (C), 122.8 (CH), 125.2 (CH), 125.6 (C), 129.4 (CH), 130.4 (CH), 132.3 (C), 132.9 (C), 136.0 (C), 137.2 (C), 137.6 (CH), 152.8 (2) (C), 184.4 (CH). MS: 365 (M⁺). HPLC: C₁₈ tr: 19.9 min.

(Z)-1-Methyl-5-(3,4,5-trimethoxystyryl)-1H-indole (15). Referencia 16c

(Z)-1-Methyl-5-(2,3,4-trimethoxystyril)-1H-indole (16Z)

To a stirred suspension of 2,3,4-Trimethoxybenzyltriphenylphosphonium bromide (1.8 g, 3.46 mmol) in dry THF (10 mL), BuLi (1.6 M in hexane, 2.4 mL, 3.80 mmol) was added at -40 °C under argon. After 1 h, 1-methyl-1H-indole-5-carbaldehyde (500 mg, 3.14 mmol) was added and the mixture was allowed to reach room temperature. After 12 h, the reaction mixture was poured onto ice with ammonium chloride and extracted with CH₂Cl₂. The combined organic layers were dried over Na₂SO₄ and the solvent was evaporated in vacuo. The residue was purified by flash chromatography on silica with hexane/EtOAc (7:3) obtaining 16Z (590 mg, 58%) and 16E (112 mg, 11%).
(Z)-1-Methyl-5-(2,3,4-trimethoxystyryl)-1H-indole (16Z): IR: 1596, 1493, 730 cm\(^{-1}\). \(^1\)H NMR (400 MHz, CDCl\(_3\)): \(\delta\) 3.75 (3H, s), 3.83 (3H, s), 3.94 (6H, s), 6.41 (1H, bs), 6.45 (1H, d, \(J = 7.9\)), 6.58 (1H, d, \(J = 12.2\)), 6.78 (1H, d, \(J = 12.2\)), 6.98 (1H, d, \(J = 7.9\)), 7.02 (1H, bs), 7.16 (2H, m), 7.56 (1H, bs). \(^1\)C NMR (100 MHz, CDCl\(_3\)): \(\delta\) 32.8 (CH\(_3\)), 56.0 (CH\(_3\)), 61.1 (CH\(_3\)), 61.2 (CH\(_3\)), 101.3 (CH), 107.1 (CH), 108.8 (CH), 121.5 (CH), 122.8 (CH), 123.0 (CH), 124.6 (CH), 124.9 (C), 128.4 (C), 128.7 (C), 129.1 (CH), 131.1 (CH), 135.9 (C), 142.2 (C), 152.1 (C), 152.7 (C). HRMS (C\(_{20}\)H\(_{21}\)NO\(_3\) + Na): calcd 346.1413 (M\(^+\) + Na), found 346.1401. HPLC: C\(_{18}\) tr.: 22.4 min.

(E)-1-Methyl-5-(2,3,4-trimethoxystyryl)-1H-indole (16E): Mp 110-111ºC (Hexane). IR: 1492, 850 cm\(^{-1}\). \(^1\)H NMR (400 MHz, CDCl\(_3\)): \(\delta\) 3.78 (3H, s), 3.90 (3H, s), 3.94 (3H, s), 3.95 (3H, s), 6.51 (1H, d, \(J = 3.4\)), 6.73 (1H, d, \(J = 8.6\)), 7.04 (1H, d, \(J = 3.4\)), 7.19 (1H, d, \(J = 16.5\)), 7.31 (1H, d, \(J = 8.6\)), 7.35 (1H, d, \(J = 8.6\)), 7.37 (1H, d, \(J = 16.5\)), 7.52 (1H, dd, \(J = 8.6\) and \(J = 1.8\)), 7.76 (1H, d, \(J = 1.8\)). \(^1\)C NMR (100 MHz, CDCl\(_3\)): \(\delta\) 32.9 (CH\(_3\)), 56.1 (CH\(_3\)), 61.0 (CH\(_3\)), 61.4 (CH\(_3\)), 101.3 (CH), 107.9 (CH), 109.5 (CH), 119.5 (CH), 120.0 (CH), 120.2 (CH), 120.3 (CH), 120.4 (CH), 125.3 (C), 128.8 (C), 129.4 (CH), 129.6 (C), 136.5 (C), 142.2 (C), 151.5 (C), 152.8 (C). HRMS (C\(_{20}\)H\(_{21}\)NO\(_3\) + Na): calcd 346.1413 (M\(^+\) + Na), found 346.1401. HPLC: C\(_{18}\) tr.: 23.1 min.

(Z)-1-Methyl-5-(3,4,5-trimethoxystyryl)-1H-indole-3-carbaldehyde (17)

A solution of POCl\(_3\) (0.12 mL, 1.3 mmol) in DMF (1.0 mL) was stirred at 0 ºC for 1 h and then (Z)-5-(3,4,5-trimethoxystyryl)-1-methyl-1H-indole (395 mg, 1.23 mmol) was added and heated at 60 ºC for 2 h. After that, the reaction mixture was poured onto ice with sodium acetate, water (100 mL) was added and the mixture was kept at 4 ºC. After 24 h the obtained precipitate was re-dissolved in CH\(_2\)Cl\(_2\), dried over anhydrous Na\(_2\)SO\(_4\), filtered, and concentrated in vacuum to obtain (Z)-N-methyl-5-[2-(3,4,5-trimethoxyphenyl)vinyl]indole-3-carbaldehyde (17) (400 mg, 93%). M.p.: 144-146ºC. (CH\(_2\)Cl\(_2\)/Hex). IR (KBr): 1650, 1456, 1128 cm\(^{-1}\). \(^1\)H NMR (400 MHz, CDCl\(_3\)): \(\delta\) 3.62 (6H, s), 3.84 (3H, s), 3.86 (3H, s),
6.52 (2H, s), 6.52 (1H, d, J = 12.4), 6.73 (1H, d, J = 12.4), 7.18 (1H, d, J = 9.2), 7.32 (1H, dd, J = 9.2 and 2.2), 7.66 (1H, bs), 8.27 (1H, s), 9.98 (1H, s). \(^{13}\)C NMR (100 MHz, CDCl\(_3\)): \(\delta\) 33.5 (CH\(_3\)), 55.8 (2 (CH\(_3\))), 60.8 (CH\(_3\)), 106.2 (2 (CH)), 109.6 (CH), 117.9 (C), 122.4 (CH), 125.1 (CH), 125.2 (C), 129.2 (CH), 130.3 (CH), 132.1 (C), 132.8 (C), 137.0 (C), 137.2 (C), 140.1 (CH), 152.8 (2 (C)), 184.3 (CH). HRMS (C\(_{21}\)H\(_{21}\)NO\(_4\) + Na): calcd 374.1368 (M\(^+\) + Na), found 374.1343 HPLC: C\(_{18}\) t\(_R\): 14.66 min. C\(_8\) t\(_R\): 13.42 min. Phenylc t\(_R\): 14.24 min.

(Z)-1-Methyl-5-(2,3,4-trimethoxystyryl)-1H-indole-3-carbaldehyde (18)

POCl\(_3\) (360 mg, 2.3 mmol) in dry DMF (1 mL) at 0ºC was stirred for 30 min, then compound 16Z (720 mg, 2.2 mmol) was added and the reaction was heated at 60ºC for 2 h. The reaction mixture was poured onto ice with sodium acetate, after 24 h at 2ºC, the solid obtained was filtered, solved in CH\(_2\)Cl\(_2\), dried over Na\(_2\)SO\(_4\) and the solvent evaporated in vacuo. The residue was purified by flash chromatography on silica gel with hexane/EtOAc (8:2) aldehyde 18 (661 mg, 85%). \(^1\)H NMR (400 MHz, CDCl\(_3\)): \(\delta\) 3.64 (3H, s), 3.70 (3H, s), 3.90 (6H, s), 6.41 (1H, d, J = 8.6), 6.62 (1H, d, J = 12.1), 6.24 (1H, d, J = 12.1), 6.84 (1H, d, J = 8.6), 7.11 (1H, d, J = 8.6), 7.20 (1H, d, J = 8.6), 7.51 (1H, s), 8.18 (1H, bs), 9.92 (1H, s). \(^{13}\)C NMR (100 MHz, CDCl\(_3\)): \(\delta\) 33.6 (CH\(_3\)), 55.9 (CH\(_3\)), 60.8 (CH\(_3\)), 61.0 (CH\(_3\)), 107.0 (CH), 109.3 (CH), 118.1 (C), 122.5 (CH), 124.1 (CH), 124.3 (CH), 124.3 (C), 124.4 (CH), 124.7 (C), 130.1 (CH), 132.5 (C), 136.8 (C), 139.2 (CH), 142.2 (C), 152.0 (C), 152.9 (C), 184.2 (CH). HRMS (C\(_{21}\)H\(_{21}\)NO\(_4\) + Na): calcd. 374.1368 (M\(^+\) + Na), found 374.1349. HPLC: C\(_{18}\) t\(_R\): 19.7 min.

(Z)-(1-Methyl-5-(3,4,5-trimethoxystyryl)-1H-indol-3-yl)methanol (19)

NaBH\(_4\) (20 mg, 0.07 mmol) was added to a stirred solution (Z)-N-methyl-5-[2-(3,4,5-trimethoxyphenyl)vinyl]indole-3-carbaldehyde (24.6 mg, 0.07 mmol) in MeOH (10 mL). After half an hour, the methanol was evaporated and the residue was re-dissolved in CH\(_2\)Cl\(_2\) and washed with brine. The organic layers were dried over anhydrous Na\(_2\)SO\(_4\), filtered, and evaporated under vacuum to give (Z)-[N-methyl-5-[2-(3,4,5-trimethoxyphenyl)vinyl]indol-3-yl]methanol (19) (22 mg, 89%). IR (film):
3413, 1579, 1124 cm\(^{-1}\). \(^1\)H NMR (400 MHz, CDCl\(_3\)): δ 3.64 (6H, s), 3.74 (3H, s), 3.84 (3H, s), 4.79 (2H, bs), 6.44 (1H, d, \(J = 12.2\)), 6.57 (2H, s), 6.71 (1H, d, \(J = 12.2\)), 7.02 (1H, s), 7.14 (1H, d, \(J = 8.6\)), 7.25 (1H, dd, \(J = 8.6\) and 1.8), 7.67 (1H, d, \(J = 1.8\)). \(^{13}\)C NMR (100 MHz, CDCl\(_3\)): δ 32.8 (CH\(_3\)), 56.0 (2) (CH\(_2\)), 57.0 (CH\(_2\)), 61.0 (CH\(_3\)), 106.1 (2) (CH), 109.0 (CH), 115.0 (C), 120.0 (CH), 123.4 (CH), 127.0 (C), 128.1 (2) (CH), 128.5 (C), 131.1 (CH), 133.3 (C), 136.6 (C), 137.0 (C), 152.9 (2) (C). HRMS. Calcd. for (C\(_{21}\)H\(_{23}\)NO\(_4\) + Na): calcd 376.1325 (M\(^{+}\) + Na), found 376.1525. HPLC: C\(_{18}\) tr: 14.82 min. C\(_8\) tr: 13.50 min. Phenyllic tr: 13.96 min.

\((Z)-(1\text{-Methyl-5-(2,3,4-trimethoxystyryl)-1H-indol-3-yl})\text{methanol (20)}\)

To a solution of aldehyde 18 (78 mg, 0.22 mmol) in MeOH (10 mL) at 0ºC and under argon, an excess of NaBH\(_4\) (62 mg) was added. After 15 min the MeOH was evaporated and the residue dissolved in CH\(_2\)Cl\(_2\), the organic layer was washed with water, dried over Na\(_2\)SO\(_4\) and the solvent evaporated in vacuo and by crystallization in MeOH, to produce alcohol 20 (40 mg, 50%) as a white solid. Mp: 135-136ºC (CHCl\(_3\)/ether). IR (KBr) 3424, 1600, 1490 cm\(^{-1}\). \(^1\)H NMR (400 MHz, CDCl\(_3\)): δ 3.69 (3H, s), 3.81 (3H, s), 3.93 (6H, s), 4.76 (2H, s), 6.42 (1H, d, \(J = 8.6\)), 6.56 (1H, d, \(J = 12.1\)), 6.72 (1H, d, \(J = 12.1\)), 6.93 (1H, d, \(J = 8.6\)), 6.98 (1H, s), 7.10(1H, d, \(J = 8.5\)), 7.16 (1H, d, \(J = 8.5\)), 7.60 (1H, s), 7.82 (1H, bs). \(^{13}\)C NMR (100 MHz, CDCl\(_3\)): δ 32.6 (CH\(_3\)), 55.9 (CH\(_3\)), 56.8 (CH\(_2\)), 60.9 (CH\(_3\)), 61.0 (CH\(_3\)), 107.0 (CH), 108.9 (CH), 115.0 (C), 119.7 (CH), 123.0 (CH), 123.2 (CH), 124.4 (CH), 124.7 (C), 126.8 (C), 127.9 (CH), 128.7 (C), 130.8 (CH), 136.3 (C), 142.2 (C), 152.0 (C), 152.7 (C). HRMS. Calcd. for (C\(_{21}\)H\(_{23}\)NO\(_4\) + Na): calcd 376.13519 (M\(^{+}\) + Na), found 376.1512. HPLC: C\(_{18}\) tr: 19.3 min.

\((Z+E)-1\text{-Methyl-5-(3,4,5-trimethoxystyryl)-1H-indole-3-carbaldehyde oxime (21Z+E)}\)

To a solution of aldehyde 17 (500 mg, 1.42 mmol) in MeOH (30 mL) hydroxylamine hydrochloride (987 mg, 14.2 mmol) and two drops of pyridine were added. After 24 at reflux, the solvent was evaporated, the obtained residue was solved in CH\(_2\)Cl\(_2\) and extracted with H\(_2\)O. The combined organic
layers were dried over Na$_2$SO$_4$ and evaporated to give a residue that was purified by silica gel flash chromatography using hexane/EtOAc (8:2) to yield the oximes 21 (280 mg, 73%) as a mixture (3:2) of Z and E isomers. $^1$H NMR (400 MHz, CDCl$_3$): δ 3.62 (6H, s), 3.67 (6H, s), 3.75 (3H, s), 3.83 (3H, s), 3.84 (3H, s), 3.86 (3H, s), 6.50 (1H, d, J = 12.6), 6.51 (1H, d, J = 12.6), 6.53 (2H, s), 6.58 (2H, s), 6.68 (1H, d, J = 12.6), 6.73 (1H, d, J = 12.6), 7.13-7.40 (6H, m), 7.72 (1H, s), 7.73 (1H, s), 8.23 (1H, s), 8.24 (1H, s). $^{13}$C NMR (100 MHz, CDCl$_3$): δ 33.0 (CH$_3$), 33.4 (CH$_3$), 55.8 (2) (CH$_3$), 55.9 (2) (CH$_3$), 60.9 (2) (CH$_3$), 106.0 (2) (CH), 106.1 (2) (CH), 108.8 (CH), 109.3 (CH), 109.1 (2) (C), 118.7 (CH), 122.7 (CH), 124.2 (CH), 124.8 (CH), 128.3 (CH), 128.9 (CH), 129.8 (C), 130.3 (C), 130.5 (CH), 130.5 (C), 131.0 (CH), 131.5 (CH), 132.9 (C), 133.5 (C), 135.2 (C), 135.9 (CH), 135.9 (C), 136.8 (C), 137.0 (C), 137.2 (C), 145.3 (2) (CH), 152.8 (2) (C), 153.0 (2) (C). HPLC: C$_{18}$ tr: 19.5 and 20.3 min.

(Z+E)-1-Methyl-5-(2,3,4-trimethoxystyryl)-1H-indole-3-carbaldehyde oxime (22Z+E): $^1$H NMR (400 MHz, CDCl$_3$): δ 3.72 (3H, s), 3.77 (3H, s), 3.82 (6H, s), 3.92 (12H, s), 6.44 (1H, d, J = 8.8), 6.46 (1H, d, J = 8.8), 6.60 (1H, d, J = 12.1), 6.62 (1H, d, J = 12.1), 6.75 (1H, d, J = 12.1).6.88 (1H, d, J = 8.8), 6.91 (1H, d, J = 8.8), 7.15-7.35 (4H, m), 7.17 (1H, s), 7.26 (1H, s), 7.66 (1H,s), 7.68 (1H, s), 8.18 (1H, s), 8.26 (1H, s). $^{13}$C NMR (100 MHz, CDCl$_3$): δ 33.1 (CH$_3$), 33.2 (CH$_3$), 55.9 (CH$_3$), 56.0 (CH$_3$), 60.9 (CH$_3$), 61.0 (CH$_3$), 105.5 (C), 107.1 (CH), 107.3 (CH), 109.0 (CH), 109.2 (C), 109.6 (CH), 118.6 (CH), 120.5 (C), 121.4 (C), 122.7 (CH), 123.6 (CH), 123.7 (CH), 123.8 (CH), 124.2 (CH), 124.3 (C), 124.4 (CH); 124.9 (CH), 125.2 (C), 125.5 (C), 127.2 (C), 130.2 (C), 130.3 (CH), 130.4 (CH), 130.9 (CH), 134.9 (C), 135.3 (CH), 136.6 (C), 139.5 (CH), 142.2 (C), 145.5 (CH), 151.9 (C), 152.0 (C), 152.7 (C), 152.8 (C).

HPLC: C$_{18}$ tr: 19.5 and 20.5 min.

(Z)-1-Methyl-5-(3,4,5-trimethoxystyryl)-1H-indole-3-carbonitrile (23) and (Z)-1-Methyl-5-(2,3,4-trimethoxystyryl)-1H-indole-3-carbonitrile (24)
To a mixture of (Z+E) oximes \( 21 \) (80 mg, 0.21 mmol) or \( 22 \) (250 mg, 0.65 mmol) dissolved in pyridine (0.4 mL) or (1.25 mL) acetic anhydride (0.4 mL) or (1.25 mL) was added. After 9 h at room temperature, the reaction was treated with HCl 2N, extracted with CH\(_2\)Cl\(_2\), washed with NaOH 2%, dried over anhydrous Na\(_2\)SO\(_4\) and the solvent evaporated. The residue was purified by flash chromatography with hexane/EtOAc (1:1) yielding compound \( 23 \) (30 mg, 41%) or \( 24 \) (35 mg, 16%).

**\((Z)\)-1-methyl-5-(3,4,5-trimethoxystyryl)-1\(H\)-indole-3-carbonitrile (23)**: IR (film): 2217, 1579, 1504 cm\(^{-1}\). \(^1\)H NMR (400 MHz, CDCl\(_3\)): \( \delta \) 3.63 (3H, s), 3.83 (3H, s), 3.85 (6H, s), 6.50 (2H, s), 6.53 (1H, d, \( J = 12.2 \)), 6.68 (1H, d, \( J = 12.2 \)), 7.21 (1H, d, \( J = 8.6 \)), 7.30 (1H, d, \( J = 8.6 \)), 7.52 (1H, s), 7.69 (1H, s). \(^{13}\)C NMR (100 MHz, CDCl\(_3\)): \( \delta \) 33.6 (CH\(_3\)), 55.9 (2) (CH\(_3\)), 60.9 (CH\(_3\)), 85.6 (C), 106.1 (2) (CH), 109.8 (CH), 115.6 (C), 120.3 (CH), 125.1 (CH), 127.8 (C), 129.7 (2) (CH), 131.5 (C), 132.6 (C), 135.0 (C), 135.7 (CH), 137.3 (C), 152.9 (2) (CH). HRMS (C\(_{21}\)H\(_{20}\)N\(_2\)O\(_3\) + Na): calcd 371.1366 (M\(^+\) + Na), found 371.1353. HPLC: C\(_{18}\) tr: 20.9 min.

**\((Z)\)-1-Methyl-5-(2,3,4-trimethoxystyryl)-1\(H\)-indole-3-carbonitrile (24)**: IR: 2216, 1596, 1490 cm\(^{-1}\). \(^1\)H NMR (400 MHz, CDCl\(_3\)): \( \delta \) 3.73 (3H, s), 3.90 (9H, s), 6.43 (1H, d, \( J = 8.6 \)), 6.65 (1H, d, \( J = 12.1 \)), 6.71 (1H, d, \( J = 12.1 \)), 6.83 (1H, d, \( J = 8.6 \)), 7.16 (1H, d, \( J = 8.6 \)), 7.24 (1H, d, \( J = 8.6 \)), 7.50 (1H, s), 7.63 (1H, s). \(^{13}\)C NMR (100 MHz, CDCl\(_3\)): \( \delta \) 33.5 (CH\(_3\)), 55.9 (CH\(_3\)), 60.8 (CH\(_3\)), 60.9 (CH\(_3\)), 85.6 (C), 107.1 (CH), 109.8 (CH), 115.6 (C), 120.1 (CH), 124.0 (C), 124.2 (CH), 124.8 (CH), 125.0 (CH), 127.8 (C), 129.1 (CH), 131.8 (C), 135.0 (C), 135.7 (CH), 142.3 (C), 152.0 (C), 153.0 (C). HPLC: C\(_{18}\) tr: 20.5 min.

**\((Z)\)-1-Methyl-5-(3,4,5-trimethoxystyryl)-1\(H\)-indole-3-carboxylic acid (25) and \((Z)\)-1-Methyl-5-(2,3,4-trimethoxystyryl)-1\(H\)-indole-3-carboxylic acid (26)**

A solution of 80% NaClO\(_2\) (1.03 g, 11.4 mmol) and NaH\(_2\)PO\(_4\)·2H\(_2\)O (1.02 g, 8.53 mmol) in water (15 mL) was added at 0°C to a suspension of aldehyde \( 17 \) (200 mg, 0.57 mmol) or \( 18 \) (200 mg, 0.57 mmol) in tert-butyl alcohol (15 mL) and 2-methylbut-2-ene (15 mL). THF (5 mL) was added to dissolve starting aldehyde \( 17 \) or \( 18 \) and the mixture was stirred at room temperature for 12 h. After dilution with
water (30 mL), the product was extracted with CH₂Cl₂, washed with brine, dried over anhydrous Na₂SO₄ and evaporated. By crystallization in MeOH (Z)-1-methyl-5-(3,4,5-trimethoxystyryl)-1H-indole-3-carboxylic acid (25) (150 mg, 72%) or 26 (180 mg, 86%) were obtained.

(Z)-1-Methyl-5-(3,4,5-trimethoxystyryl)-1H-indole-3-carboxylic acid (25): M.p.: 164-165°C (MeOH). IR (KBr): 3450, 1620, 1502 cm⁻¹. ¹H NMR (400 MHz, CDCl₃): δ 3.64 (6H, s), 3.82 (3H, s), 6.50 (1H, d, J = 12.1), 6.51 (2H, s), 8.76 (1H, d, J = 12.1), 7.18 (1H, d, J = 8.5), 7.28 (1H, d, J = 8.5), 7.83 (1H, s), 8.20 (1H, s). ¹³C NMR (100 MHz, CDCl₃): δ 33.5 (CH₃), 55.8 (2) (CH₃), 60.8 (CH₃), 106.1 (2) (CH), 106.3 (C), 109.3 (CH), 122.4 (CH), 124.3 (CH), 126.8 (C), 128.9 (CH), 130.6 (CH), 131.4 (C), 132.9 (C), 136.4 (C), 136.5 (CH), 137.1 (C), 152.8 (2) (C), 169.8 (C). HRMS (C₂₁H₂₁NO₅ + Na): calcd. 390.1311 (M⁺ + Na), found 390.1318. HPLC: C₁₈ tR: 23.2 min.

(Z)-1-Methyl-5-(2,3,4-trimethoxystyryl)-1H-indole-3-carboxylic acid (26): M.p.: 189-190°C (MeOH). IR (KBr): 1660, 1520 cm⁻¹. ¹H NMR (400 MHz, CDCl₃): δ 3.92 (s, CH₃), 3.93 (s, CH₃), 3.94 (s, CH₃), 3.96 (s, CH₃), 6.72 (1H, d, J = 8.7), 7.20 (1H, d, J = 12.4), 7.33 (1H, d, J = 12.4), 7.31 (1H, d, J = 8.7), 7.35(1H, d, J = 8.7), 7.37 (1H, s), 7.57 (1H, d, J = 8.7), 7.84 (1H, s), 8.29 (1H, bs). ¹³C NMR (100 MHz, CDCl₃): δ 33.6 (CH₃), 56.0 (CH₃), 60.8 (CH₃), 61.3 (CH₃), 106.3 (C), 107.8 (CH), 110.0 (CH), 120.4 (CH), 120.6 (CH), 121.1 (CH), 121.6 (CH), 124.9 (C), 127.1 (C), 128.9.1 (CH), 132.5 (C), 136.6 (CH), 136.8 (C), 142.4 (C), 151.5 (C), 152.9 (C), 169.9 (C). HPLC: C₁₈ tR: 20.6 min.

(Z)-Methyl 1-methyl-5-(3,4,5-trimethoxystyryl)-1H-indole-3-carboxylate (27)

To a solution of the acid 25 (50 mg, 0.136 mmol) in MeOH (10 mL) (trimethylsilyl)diazomethane (2 mL) was added. After 15 min at room temperature, the solvent was evaporated obtaining compound 27 in quantitative yield. IR (film: 1699, 1570, 1503 cm⁻¹. ¹H NMR (400 MHz, CDCl₃): δ 3.62 (6H, s), 3.78 (3H, s), 3.84 (3H, s), 3.85 (3H, s), 6.49 (1H, d, J = 12.2), 6.54 (s, 2H), 6.65 (1H, d, J = 12.2), 7.20 (1H, d, J = 8.9), 7.25 (1H, d, J = 8.9), 7.73 (1H, s), 8.14 (1H, s). ¹³C NMR (100 MHz, CDCl₃): δ 33.5 (CH₃),
51.0 (CH₃), 55.9 (2) (CH₃), 60.9 (CH₃), 106.1 (2) (CH), 107.1 (C), 109.3 (CH), 122.2 (CH), 124.4 (CH), 126.4 (C), 128.8 (CH), 130.7 (CH), 131.0 (C), 133.1 (C), 135.6 (CH), 136.3 (C), 137.0 (C), 152.8 (2) (C), 165.4 (C). HRMS (C₂₂H₂₃NO₅ + Na): calcd. 404.1468 (M⁺ + Na), found 404.1463. HPLC: C₁₈ tr: 21.6 min.

**Preparation of compounds 28-31**

A mixture of carboxilic acid (25) (1 mmol); 1 mmol of the corresponding aminoester: glycine ethyl ester hydrochloride, sarcosine methyl ester, β-alanine ethyl ester or dimethylamino malonate hydrochloride; 2 mmol of EDCI and 0.5 mmol of 4-DAMP were disolved in dry CH₂Cl₂ in inert atmosphere. After 72 h at room temperature, the reaction was treated with HCl 2N, extracted with CH₂Cl₂, washed with NaOH 2%, dried over anhydrous Na₂SO₄ and the solvent evaporated. The residue was purifed by flash chromatography with hexane/EtOAc or crystallization yielding the compounds 28 (72%), 29 (55 %), 30 (54%) and 31 (30%) + 25 (20%).

(Z)-**Ethyl 2-(1-methyl-5-(3,4,5-trimethoxystyryl)-1H-indole-3-carboxamido)acetate (28):** IR (film): 3347, 1744, 1638, 1578 cm⁻¹. ¹H NMR (200 MHz, CDCl₃): δ 1.22 (3H, t, J = 7.2), 3.52 (3H, s), 3.76 (3H, s), 3.90 (2H, m ), 4.14 (2H, c, J = 7.2), 6.41 (1H, d, J = 12.2), 6.46 (2H, s), 6.62 (1H, d, J = 12.2), 6.97 (1H, d, J = 8.6), 7.16 (1H, d, J = 8.6), 7.54 (1H, s), 8.01 (1H, s). ¹³C NMR (50 MHz, CDCl₃): δ 14.2 (CH₃), 33.2 (CH₃), 41.3 (CH₃), 55.8 (CH₃), 60.9 (CH₃), 61.4 (CH₂), 106.0 (CH), 109.3 (C), 109.8 (CH), 121.5 (CH), 123.7 (CH), 125.8 (C), 128.7 (CH), 130.5 (C), 130.8 (CH), 132.5 (CH), 133.1 (C), 136.2 (C), 152.8 (C), 165.2 (C), 170.9 (C). HRMS (C₂₅H₂₈N₂O₆ + Na): calcd. 475.1839 (M⁺ + Na), found 475.1861.

(Z)-**Methyl 2-(N,1-dimethyl-5-(3,4,5-trimethoxystyryl)-1H-indole-3-carboxamido)acetate (29):** IR (film): 3052, 2834, 1746, 1617, 1581 cm⁻¹. ¹H NMR (200 MHz, CDCl₃): δ 3.09 (3H, s), 3.58 (6H, s), 3.71 (3H, s), 3.78 (3H, s), 4.20 (2H, s), 6.44 (1H, d, J = 12.2), 6.47 (2H, s), 6.67 (1H, d, J = 12.2), 7.12 (1H, d, J = 8.8), 7.20 (1H, d, J = 8.8), 7.34 (1H, s), 7.72 (1H, s). ¹³C NMR (50 MHz, CDCl₃): δ 33.1 (CH₃), 50.0 (CH₂), 52.0 (CH₃), 58.8 (CH₃), 60.7 (CH₃), 106.0 (CH), 109.2 (CH), 109.5 (C), 121.7 (CH), 137.5 (CH), 165.1 (CH).
123.7 (CH), 126.6 (C), 128.5 (CH), 130.1 (C), 130.8 (CH), 131.7 (CH), 133.1 (C), 135.6 (C), 136.8 (C), 157.7 (C), 167.5 (C), 170.0 (C). HRMS (C_{25}H_{28}N_{2}O_{6} + Na): calcd. 475.1839 (M^+ + Na), found 475.1846.

(Z)-Ethyl 3-(1-methyl-5-(3,4,5-trimethoxystyryl)-1H-indole-3-carboxamido)propanoate (30): M.p.: 133-134°C (MeOH). IR (KBr): 3359, 1727, 1611, 1579, 1512 cm\(^{-1}\). \(^1\)H NMR (200 MHz, CDCl\(_3\)): δ 1.28 (3H, t, \(J = 7.2\)), 2.64 (2H, t, \(J = 6.1\)), 3.61 (6H, s), 3.73 (2H, t, \(J = 6.1\)), 3.76 (3H, s), 3.82 (3H, s), 4.16 (2H, c, \(J = 7.2\)), 6.49 (1H, d, \(J = 12.2\)), 6.51 (2H, s), 6.73 (1H, d, \(J = 12.2\)), 7.15 (1H, d, \(J = 8.6\)), 1.25 (1H, d, \(J = 8.6\)), 7.59 (1H, s), 7.92 (1H, s). \(^{13}\)C NMR (50 MHz, CDCl\(_3\)): δ 14.2 (CH\(_3\)), 33.2 (CH\(_3\)), 34.3 (CH\(_2\)), 34.8 (CH\(_2\)), 55.8 (CH\(_3\)), 60.6 (CH\(_2\)), 60.8 (CH\(_3\)), 106, 0 (CH), 109.4 (CH), 121.4 (CH), 123.7 (CH), 125.7 (C), 128.7 (CH), 10.8 (CH), 132.2 (CH), 133.1 (C), 136.3 (C), 152.8 (C), 164.9 (C), 172.7 (C). HRMS (C_{26}H_{30}N_{2}O_{6} + Na): calcd. 489.1996 (M^+ + Na), found 489.2021.

(Z)-Dimethyl 2-(1-methyl-5-(3,4,5-trimethoxystyryl)-1H-indole-3-carboxamido)malonate (31). IR (KBr): 1742, 1629, 1576 cm\(^{-1}\). \(^1\)H NMR (200 MHz, CDCl\(_3\)): δ 3.61 (6H, s), 3.76 (3H, s), 3.81 (3H, s), 3.86 (6H, s), 6.42 (1H, d, \(J = 6.6\)), 6.48 (1H, d, \(J = 12.0\)), 6.50 (2H, s), 6.68 (1H, d, \(J = 12.0\)), 6.88 (1H, d, \(J = 6.6\)), 7.13 (1H, d, \(J = 8.6\)), 7.24 (1H, d, \(J = 8.6\)), 7.66 (1H, s), 8.19 (1H, s).

Preparation of compounds 32-34

The compounds 28-30 were saponificated by treatment with KOH/MeOH 10% during 30’ at rt. Yielding the compounds 32 (77%), 33 (84%) and 34 (90%) were obtained.

(Z)-2-(1-methyl-5-(3,4,5-trimethoxystyryl)-1H-indole-3-carboxamido)acetic acid (32): M.p.: 144-145°C (MeOH). IR (KBr): 3412, 1735, 1640, 1578 cm\(^{-1}\). \(^1\)H NMR (200 MHz, CD\(_3\)OD): δ 3.45 (3H, s), 3.62 (3H, s), 3.72 (3H, s), 3.97 (2H, s), 6.40 (1H, d, \(J = 12.2\)), 6.46 (2H, d), 7.09 (1H, d, \(J = 8.6\)), 7.24 (1H, d, \(J = 8.6\)), 7.71 (1H, s), 7.99 (1H, s). \(^{13}\)C NMR (50 MHz, CD\(_3\)OD): δ 33.4 (CH\(_3\)), 41.7 (CH\(_2\)), 56.2 (CH\(_3\)), 61.0 (CH\(_3\)), 107.4 (CH), 110.6 (CH), 110.0 (C), 122.6 (CH), 124.9 (CH), 127.6 (C), 129.9 (CH), 131.9 (CH), 133.8 (CH), 134.6 (C), 137.8 (C), 154.0 (C), 167.8 (C), 173.6 (C). HRMS
(C₂₃H₂₄N₂O₆ + Na): calcd. 447.1526 (M⁺ + Na), found 447.15.39.

(Z)-2-(N,1-dimethyl-5-(3,4,5-trimethoxystyril)-1H-indole-3-carboxamido)acetic acid (33): IR (KBr): 3399, 3431, 1584, 1500 cm⁻¹. ¹H NMR (200 MHz, CD₃OD): δ 3.02 (3H, s), 3.40 (3H, s), 3.61 (3H, s), 3.68 (3H, s), 3.95 (3H, s), 6.37 (1H, d, J = 12.2), 6.43 (2H, s), 6.60 (1H, d, J = 12.2), 7.08 (1H, d, J = 8.6), 7.20 (1H, d, J = 8.6), 7.40 (1H, bs), 7.70 (1H, s). ¹³C NMR (50 MHz, CD₃OD): δ 33.2 (CH₃), 37.5 (CH₃), 51.0 (CH₂), 56.2 (CH₃), 61.0 (CH₃), 107.4 (CH), 110.3 (CH), 110.5 (C), 122.5 (CH), 124.8 (CH), 128.2 (C), 129.8 (CH), 131.5 (C), 131.9 (CH), 134.7 (C), 137.1 (C), 138.0 (C), 154.0 (C), 169.8 (C), 177.7 (C). HRMS (C₂₄H₂₆N₂O₆ + Na): calcd. 461.1683 (M⁺ + Na), found 461.1658.

(Z)-3-(1-methyl-5-(3,4,5-trimethoxystyril)-1H-indole-3-carboxamido)propanoic acid (34): M.p.: 169-170°C (CH₂Cl₂/MeOH). IR (KBr): 3378, 1726, 1606, 1577, 1506 cm⁻¹. ¹H NMR (200 MHz, CDCl₃): δ 2.60 (2H, s), 3.60 (6H, s), 3.70 (2H, m), 3.74 (3H, s), 3.80 (3H, s), 6.48 (1H, d, J = 12.2), 6.52 (2H, s), 6.70 (1H, d, J = 12.2), 7.16 (1H, d, J = 8.2), 7.24 (1H, d, J = 8.2), 7.65 (1H, s), 7.85 (1H, s). ¹³C NMR (50 MHz, CDCl₃): δ 33.8 (CH₃), 34.3 (CH₂), 34.9 (CH₂), 55.9 (CH₃), 60.9 (CH₃), 106.0 (CH), 109.6 (CH), 110.2 (C), 120.2 (CH), 124.0 (CH), 125.1 (C), 128.9 (CH), 130.6 (CH), 130.7 (C), 133.1 (CH), 133.3 (C), 136.4 (C), 152.9 (C), 165.4 (C), 176.0 (C). HRMS (C₂₄H₂₆N₂O₆ + Na): calcd. 461.1683 (M⁺ + Na), found 461.1696.

3-Bromo-1-methyl-5-(3,4,5-trimethoxystyril)-1H-indole (35)

To a suspension of triphenyl(3,4,5-trimethoxybenzyl)phosphonium bromide (3.02 g, 5.80 mmol) in dry THF (40 mL), at -40°C in argon atmosphere, nBuLi (1.6 M in hexane, 3.4 mL, 5.5 mmol) was added and stirred for 1 h. A solution of compound 5 (1.06 g, 4.45 mmol) in dry THF (15 mL) was slowly added at -40 ºC and progressively warmed to room temperature. After 24 h, the reaction mixture was poured into ammonium chloride solution and extracted with CH₂Cl₂. The organic layers were washed to neutrality with brine, dried over anhydrous Na₂SO₄, filtered and evaporated to dryness. After chromatography on silica gel (Hex/AcOEt 7/3), 260 mg (0.65 mmol, 15%) of 35Z and 989 mg (2.46 mmol, 55%) of 35E were obtained.
(Z)-3-Bromo-1-methyl-5-(3,4,5-trimethoxystyryl)-1H-indole (35Z): IR (film): 1H NMR (200 MHz, CDCl3): δ 3.65 (6H, s), 3.75 (3H, s), 3.84 (3H, s), 6.47 (1H, d, J = 12.2), 6.56 (2H, s), 6.70 (1H, d, J = 12.2), 7.04 (1H, s), 7.14 (1H, d, J = 8.6), 7.23 (1H, dd, J = 8.6 and 1.2), 7.55 (1H, d, J = 1.2). 13C NMR (50 MHz, CDCl3): δ 33.3 (CH3), 56.1 (2) (CH3), 61.0 (CH3), 89.8 (C), 106.1 (2) (CH), 109.1 (CH), 119.9 (CH), 124.2 (CH), 128.1 (CH), 128.6 (CH), 129.1 (C), 129.8 (2) (C), 130.6 (CH), 132.9 (C), 135.4 (C), 137.3 (C), 153.5 (2) (C). HRMS HPLC: C18 tR: 26.3 min.

(E)-3-Bromo-1-methyl-5-(3,4,5-trimethoxystyryl)-1H-indole (35E)

IR (film): 1578, 1503, 1170 cm⁻¹. 1H NMR (200 MHz, CDCl3): δ 3.70 (3H, s), 3.88 (3H, s), 3.93 (6H, s), 6.77 (2H, s), 7.04 (1H, d, J = 16.4), 7.14 (1H, s), 7.18 (1H, d, J = 16.4), 7.26 (1H, d, J = 8.8), 7.48 (1H, dd, J = 8.8 and 1.6), 7.66 (1H, d, J = 1.6). 13C NMR (50 MHz, CDCl3): δ 33.2 (CH3), 56.2 (2) (CH3), 61.0 (CH3), 89.7 (C), 103.3 (2) (CH), 109.9 (CH), 117.7 (CH), 121.3 (CH), 126.7 (CH), 127.7 (CH), 128.4 (CH), 129.1 (2) (C), 129.7 (C), 133.7 (C), 136.1 (C), 135.4 (2) (C).

2,3-Dibromo-1-methyl-5-(3,4,5-trimethoxystyryl)-1H-indole (36)

To a suspension of triphenyl(3,4,5-trimethoxybenzyl)phosphonium bromide (1.07 g, 2.05 mmol) in dry THF (40 mL), at -40°C in argon atmosphere, nBuLi (1.6 M in hexane, 1.2 mL, 1.92 mmol) was added and stirred for 1 h. A solution of compound 5 (500 mg, 1.58 mmol) in dry THF (10 mL) was slowly added at -40 °C and progressively warmed to room temperature. After 24 h, the reaction mixture was poured into ammonium chloride solution and extracted with CH2Cl2. The organic layers were washed to neutrality with brine, dried over anhydrous Na2SO4, filtered and evaporated to dryness. After chromatography on silica gel (Hex/AcOEt 8/2 and 6/4), 324 mg (0.67 mmol, 42.4%) of 36Z and 174 mg (0.36 mmol, 23%) of a mixture 36Z/36E were obtained.

(Z)-2,3-Dibromo-1-methyl-5-(3,4,5-trimethoxystyryl)-1H-indole (36Z): IR (film): 1580, 1503, 1170 cm⁻¹. 1H NMR (200 MHz, CDCl3): δ 3.64 (6H, s), 3.77 (3H, s); 3.84 (3H, s), 6.44 (1H, d, J = 12.0), 6.52 (2H, s), 6.68 (1H, d, J = 12.0), 7.16 (1H, d, J = 8.8); 7.21 (1H, dd, J = 8.8 and 1.4), 7.49 (1H, d, J = 1.4).
$^{13}$C NMR (50 MHz, CDCl3): δ 32.3 (CH$_3$), 55.9 (2) (CH$_3$), 60.9 (CH$_3$), 92.7 (C), 106.1 (2) (CH), 109.2 (CH), 115.0 (C), 119.1 (CH), 124.4 (CH), 126.7 (CH), 128.9 (CH), 129.8 (C), 130.2 (C), 132.9 (C), 135.4 (C), 137.3 (C), 152.9 (2) (C). HRMS HPLC: C$_{18}$ tR: 25.4 min.

(E)-2,3-Dibromo-1-methyl-5-(3,4,5-trimethoxystyryl)-1H-indole (36E): $^1$H NMR (200 MHz, CDCl3): δ 3.68 (3H, s), 3.89 (3H, s), 3.91 (6H, s), 6.74 (2H, s), 7.00 (1H, d, $J$ = 16.4), 7.10 (1H, d, $J$ = 16.4), 7.15 (1H, d, $J$ = 8.8), 7.39 (1H, bs, $J$ = 8.8), 7.56 (1H, bs). $^{13}$C NMR (50 MHz, CDCl3): δ 32.3 (CH$_3$), 56.1 (2) (CH$_3$), 60.9 (CH$_3$), 92.8 (C), 103.3 (2) (CH), 109.9 (CH), 115.2 (C), 116.9 (CH), 121.4 (CH), 126.9 (CH), 127.1 (C), 128.5 (CH), 130.3 (C), 133.4 (C), 135.9 (C), 137.7 (C), 153.4 (2) (C) HRMS HPLC: C$_{18}$ tR: 21.5 min.

(Z and E)-1-Methyl-5-(3,4,5-trimethoxystyryl)-1H-benzo[d]imidazole (37Z and 37E) and (Z and E)-1-methyl-6-(3,4,5-trimethoxystyryl)-1H-benzo[d]imidazole (38Z and 38E).

(3,4,5-trimethoxybenzyl)triphenylphosphonium bromide (1.38 g, 2.63 mmol) was suspended in THF (18 mL) and cooled to -40 ºC under Ar, then nBuLi (1.6 M in hexane, 1.8 mL, 2.88 mmol) was added dropwise and the resulting solution was stirred for 1 h. A solution of a 1:1 mixture of 1H-benzimidazol-5-carbaldehyde and 1H-benzimidazol-6-carbaldehyde (383 mg, 2.39 mmol) in THF (15 mL) was added and warm to room temperature. Once completed, the reaction mixture was cooled to 0 ºC, quenched with some drops of water and extracted with dichloromethane. The organic layers were washed with brine, dried over anhydrous Na$_2$SO$_4$, and evaporated to give the crude of the reaction (1.48 g), which after chromatography on silica gel afforded one fraction of 1:1 mixture containing the Z-isomers of 1-methyl-5-(3,4,5-trimethoxystyryl)-1H-benzo[d]imidazole and 1-methyl-6-(3,4,5-trimethoxystyryl)-1H-benzo[d] imidazole (206 mg, 24%) and another 1:1 mixture containing the E-isomers of 1-methyl-5-(3,4,5-trimethoxystyryl)-1H-benzo[d]imidazole (326 mg, 38%).
(Z)-1-methyl-5-(3,4,5-trimethoxy styryl)-1H-benzo[d]imidazole (37Z) and (Z)-1-Methyl-6-(3,4,5-trimethoxy styryl)-1H-benzo[d]imidazole (38Z). IR (film): cm⁻¹. Major isomer: ¹H NMR (400 MHz, CDCl₃): δ 3.60 (6H, s), 3.74 (3H, s), 3.82 (3H, s), 6.49 (2H, s), 6.52 (1H, d, J = 12.1), 6.72 (1H, d, J = 12.1), 7.22 (1H, d, J = 8.6), 7.29 (1H, s), 7.66 (1H, d, J = 8.6), 7.83 (1H, s). ¹³C NMR (100 MHz, CDCl₃): δ 30.9 (CH₃), 55.8 (2) (CH₃), 60.9 (CH₃), 106.0 (2) (CH), 109.6 (CH), 119.8 (CH), 123.7 (CH), 129.6 (CH), 130.2 (CH), 131.6 (C), 132.3 (C), 132.6 (C), 132.8 (C), 137.2 (C), 144.1 (CH), 152.8 (2) (C). Minor isomer: ¹H NMR (400 MHz, CDCl₃): δ 3.61 (6H, s), 3.81 (3H, s), 3.83 (3H, s), 6.50 (1H, d, J = 12.1), 6.50 (2H, s), 6.71 (1H, d, J = 12.1), 7.27 (1H, s), 7.29 (1H, d, J = 8.6), 7.65 (1H, m), 7.75 (1H, s). ¹³C NMR (100 MHz, CDCl₃): δ 31.1 (CH₃), 56.1 (2) (CH₃), 60.9 (CH₃), 106.0 (2) (CH), 108.8 (CH), 120.7 (CH), 124.4 (CH), 129.1 (CH), 130.3 (CH), 144.0 (CH), 153.4 (2) (C), five C were not observed. HRMS (C₁₉H₂₀N₂O₃⁺Na): calcd. 347.1366 (M⁺Na⁺), found 347.1365. HPLC: C₁₈ tR: 13.83 and 13.93 min. (7:3). C₈ tR: 13.08 and 13.14 min. (7:3). Phenylíc tR: 13.52 and 13.68 min. (7:3).

(E)-1-methyl-5-(3,4,5-trimethoxy styryl)-1H-benzo[d]imidazole (37E) or (E)-1-methyl-6-(3,4,5-trimethoxy styryl)-1H-benzo[d]imidazole (38E). IR (film): cm⁻¹. ¹H NMR (400 MHz, CDCl₃): δ 3.84 (3H, s), 3.87 (3H, s), 3.92 (6H, s), 6.76 (2H, s), 7.05 (1H, d, J = 16.4), 7.16 (1H, d, J = 16.4), 7.47 (1H, bs), 7.49 (1H, dd, J = 8.4 and 1.8), 7.75 (1H, d, J = 8.4), 7.85 (1H, s). ¹³C NMR (100 MHz, CDCl₃): δ 31.2 (CH₃), 56.2 (2) (CH₃), 61.0 (CH₃), 106.1 (2) (CH), 107.4 (CH), 120.2 (CH), 121.2 (CH), 128.0 (CH), 128.6 (CH), 132.8 (C), 133.3 (C), 135.1 (C), 137.9 (C), 144.1 (CH), 143.4 (C), 153.5 (2) (C). HRMS (C₁₉H₂₀N₂O₃⁺Na): calcd. 347.1366 (M⁺Na⁺), found 347.1362. HPLC: C₁₈ tR: 13.79 min. C₈ tR: 12.86 min. Phenylíc tR: 13.42 min.

Determination of Aqueous Solubility

The aqueous solubility of the compounds was determined using an approach based on the saturation
shake-flask method. Approximately 2 mg of each compound was suspended in 0.3 mL pH 7.0 buffer in a glass vial and shaken 72 h at room temperature. The resulting mixture was filtrated over a 22 µm filter to discard the insoluble residues and the concentration in the supernatant was measured by UV absorbance in a Helios Alfa Spectrophotometer. Previously, for each compound the maximum wavelength of absorbance is determined and a calibration line is performed.

**Inhibition of tubulin polymerization**

Bovine brain tubulin was isolated as previously described. The assays were carried out at pH 6.7 with 1.5 mg/mL protein and the measured ligand concentration in 0.1 M MES buffer, 1 mM EGTA, 1 mM MgCl₂, 1 mM β-ME, 1.5 mM GTP. The samples were incubated at 20 °C for 30 min and subsequently cooled on ice for 10 min. Tubulin polymerization was monitored by measuring the absorbance increase in the UV at 450 nm caused by a temperature shift from 4 °C to 37 °C. After reaching a stable plateau, temperature was switched back to 0 °C and return to the initial absorption values was ascertained, in order to confirm the reversible nature of the monitored process. The difference in amplitude between the stable plateau and the initial baseline of the curves was taken as the degree of tubulin assembly for each experiment. Comparison with control curves with identical conditions but without ligands yielded tubulin polymerization inhibition as a percentage value. Initially, all compounds were assayed at a concentration of 5 µM in at least two independent measurements, and those displaying a TPI higher than 40% were selected for further studies. The tubulin polymerization inhibitory activity of the selected compounds was measured at different ligand concentrations and, as expected, the extent of inhibition by all compounds increased monotonically with the mole ratio of the total ligand to total tubulin in the solution. The obtained values were fitted to monoexponential curves and the IC₅₀ values of tubulin polymerization inhibition were calculated from the best fitting curves.

**Cell culture.** HL-60 (human acute myeloid leukemia) cell line was grown in RPMI-1640 culture
medium containing 10% (v/v) heat-inactivated fetal bovine serum (FBS), 2 mM L-glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin at 37 °C in humidified 95% air and 5% CO2. A549 (human non-small lung carcinoma), HeLa (human cervical carcinoma), and HT-29 (human colon carcinoma) cell lines were grown in DMEM culture medium containing 10% (v/v) heat-inactivated fetal bovine serum (FBS), 2 mM L-glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin at 37 °C in humidified 95% air and 5% CO2. Cells were periodically tested for Mycoplasma infection and found to be negative.

**Cell Growth Inhibition Assay.** The effect of the different compounds on the proliferation of human tumor cell lines (cytostatic activity) was determined as previously described\(^1\) using the XTT (sodium 3′-[1-(phenylaminocarbonyl)-3,4-tetrazolium]-bis(4-methoxy-6-nitro)-benzenesulfonic acid hydrate) cell proliferation kit (Roche Molecular Biochemicals, Mannheim, Germany) according to the manufacturer’s instructions. Cells (1.5 × 10³ A549, HeLa, HT-29, and 3 × 10³ HL-60 in 100 μL) were incubated in culture medium containing 10% heat-inactivated FBS in the absence and in the presence of the indicated compounds at different concentration ranges from 10⁻⁵ to 10⁻¹³ M, in 96-well flat-bottomed microtiter plates, and following 72 h of incubation at 37 °C in a humidified atmosphere of air/CO2 (19/1), the XTT assay was performed. Measurements were performed in triplicate, and each experiment was repeated three times. The IC₅₀ (50% inhibitory concentration) value, defined as the drug concentration required to cause 50% inhibition in cellular proliferation with respect to the untreated controls, was determined for each compound. Nonlinear curves fitting the experimental data were carried out for each compound.

**Cell Cycle Analysis.** For cell cycle analyses, untreated and drug-treated HeLa cells ((2–4) × 10⁵) were centrifuged and fixed overnight in 70% ethanol at 4 °C. Then cells were washed three times with PBS, incubated for 1 h with 1 mg/mL RNase A and 20 μg/mL propidium iodide at room temperature, and
analyzed with a Becton Dickinson fluorescence-activated cell sorter (FACSCalibur) flow cytometer (San Jose, CA) as described previously.\textsuperscript{27, 28} Quantification of apoptotic cells was calculated as the percentage of cells in the sub-G\textsubscript{0}/G\textsubscript{1} peak in cell cycle analysis.\textsuperscript{27, 28}

**Confocal Microscopy.** HeLa cells were grown on 0.01% poly-L-lysine coated coverslips, and after drug treatment, the coverslips were washed three times with HPEM buffer (25 mM HEPES, 60 mM PIPES, 10 mM EGTA, 3 mM MgCl\textsubscript{2}, pH 6.6), fixed with 4% paraformaldehyde in HPEM buffer for 15 min, and permeabilized with 0.5% Triton X-100 as previously described.\textsuperscript{14} Coverslips were incubated with a specific Ab-1 anti-α-tubulin mouse monoclonal antibody (diluted 1:150 in PBS) (Calbiochem, San Diego, CA) for 1 h, washed four times with PBS, and then incubated with CY3-conjugated sheep antimouse IgG (diluted 1:100 in PBS) (Jackson ImmunoResearch, West Grove, PA) for 1 h at 4 °C. After being washed four times with PBS, cell nuclei were stained with DAPI (Sigma, St. Louis, MO) for 5–10 min and washed with PBS, and then the samples were analyzed by confocal microscopy using a ZeissLSM 310 laser scan confocal microscope. A drop of SlowFade light antifading reagent (Molecular Probes, Eugene, OR) was added to preserve fluorescence. Negative controls, lacking the primary antibody or using an irrelevant antibody, showed no staining.

**Western Blot.** About 5 × 10\textsuperscript{6} cells were pelleted by centrifugation, washed with PBS, lysed, and subjected to Western blot analysis as described previously.\textsuperscript{27} Proteins were separated through SDS polyacrylamide gels under reducing conditions, transferred to nitrocellulose filters, blocked with 5% defatted milk powder, and incubated overnight with anti-activated caspase-3 (1:1000 dilution) rabbit monoclonal antibody, which detects the full-length (35 kDa) and large fragment (17/19 kDa) of caspase-3 (Cell Signaling Technology, Danvers, MA), or anti-poly(ADP-ribose) polymerase (PARP) (1:1000 dilution) rabbit monoclonal antibody that detects the full-length PARP-1 and the large fragment (89 kDa) produced by casopase cleavage (Cell Signaling Technology). Signals were developed using an
enhanced chemiluminescence detection kit (Amersham Biosciences, Aylesbury, UK). Immunoblotting with AC-15 anti-42 kDa β-actin (1:5000 dilution) mouse monoclonal antibody (Sigma) was used as an internal loading control, revealing equivalent amounts of protein in each lane of the gel. Prestained protein molecular mass standards (Bio-Rad) were run in parallel.

**Chemical Structure.** Calculations were performed at the molecular mechanics level (MMFF94s), semiempirical level (AM1), and HF and B3LYP 6-31+G* DFT levels using the Spartan 08 and Gaussian G03 software packages. Conformational analyses with the MMFF94s forcefield were performed by systematically rotating in 18° steps the bonds between the bridges and the pending aromatic rings. The phenyl rings of the trimethoxybenzene rings obtained were superimposed over one another and over the trimethoxyphenyl ring of combretastatin A4 extracted from the X-ray crystal structure with tubulin (pdb: 5LYJ), and the conformations for which the phenyl ring of the indoles covered a region closer to the 3-hydroxy-4-methoxyphenyl ring of combretastatin A4 were subjected to further unrestrained energy minimization steps at the semiempirical (AM1), 6-31G** HF, and then B3LYP/6-31+G* DFT levels of theory. The conformations were then superimposed again onto combretastatin A4 by means of the Spartan 08 pharmacophore option, by selecting as pharmacophoric references the two aromatic rings of combretastatin A4 (hydrophobic), the two external oxygens of the trimethoxyphenyl moiety (hydrogen-bond acceptors), and the excluded volume, as calculated from the complex of combretastatin A4 with tubulin.

**Docking Experiments.** The pdb structure data of the tubulin – combretastatin A4 complex (5LYJ.pdb) and 15 more complexes of colchicine site ligands with tubulin (1SA1.pdb, 3HKC.pdb, 3HKD.pdb, 3HKE.pdb, 3N2G.pdb, 3N2K.pdb, 3UT5.pdb, 4O2A.pdb, 4O2B.pdb, 4YJ2.pdb, 4YJ3.pdb, 5C8y.pdb, 5CA0.pdb, 5CA1.pdb, 5GON.pdb, 5CB4.pdb) were retrieved and chains C–E were removed. Five additional tubulin models were built from 1SA1.pdb after energy minimization and
molecular dynamics simulations at 300 K, initially with a restrained backbone and then with an unrestrained one using AMBER14\textsuperscript{30} and representative structures were selected as previously described.\textsuperscript{31} The ligands were built with Spartan 08, prepared with AutodockTools and docked with AutoDock 4.2,\textsuperscript{32, 33} by running the Lamarckian genetic algorithm (LGA) 100–300 times with a maximum of $2.5 \times 10^6$ energy evaluations, 150 individuals in the population, and a maximum of 27000 generations, PLANTS\textsuperscript{34} and DOCK3.7\textsuperscript{35}. The results were analyzed with AutoDockTools,\textsuperscript{32, 33} with Marvin,\textsuperscript{36} OpenEye\textsuperscript{37} and with JADOPPT.\textsuperscript{38} Docking poses were selected if they were found by at least two docking programs using different scoring functions and based on the scoring.

**FIGURE LEGENDS**

**Figure 1.** Representative combretastatin type ligands

**Figure 2.** Effects of compounds 17, 21 and 23 on the microtubule network of Hela cells. Cells were incubated in the absence (Control) or in the presence of 10 or 100 nM of compounds 17, 21 and 23 for 15 h and then fixed and processed to analyze microtubules (red fluorescence) and nuclei (blue fluorescence) as described in the Experimental Section. Bar: 25 µm. The photomicrographs shown are representative of at least three independent experiments performed.

**Figure 3.** Effect of different compounds on the distinct cell cycle phases in HeLa cells. Compounds 16E, 17, 21, 23, 25, 27 and 30 were incubated for 15 h and then their DNA content was analyzed by flow cytometry as described in the Experimental Section. The different cell cycle phases were quantified and represented in pie charts to easily visualize the changes in G2/M arrest. Untreated control cells were run in parallel. Data shown are representative of at least three independent experiments performed.
Figure 4. Time-course of the effects of compounds \textbf{21} and \textbf{23} on cell cycle in HeLa cells. Cells were incubated with 1 or 10 nM of \textbf{21} and \textbf{23} for the indicated times, and their DNA content was analyzed by fluorescence flow cytometry. The positions of the G0/G1 and G2/M peaks are indicated by arrows, and the proportion of cells in each phase of the cell cycle was quantified by flow cytometry. The cell population in the sub-G0/G1 region represents cells with hypodiploidal DNA, an indicator of apoptosis, and the percentages of cells at this region are indicated in each histogram. Untreated control cells were run in parallel. The experiments shown were representative of three performed.

Figure 5. Effect of verapamil on the sensitivities of A549 cancer cells to \textbf{21} and \textbf{23}. Cells were co-incubated with verapamil and different concentrations of \textbf{21} and \textbf{23}. The dose–response curves were drawn using the GraphPad Prism 5 software. Results are mean values ± S.D. of a representative experiment carried out in triplicate, out of three performed.

Figure 6 Best superpositions of the X-ray structure of combretastatin A4 (in cyan) in the complex with tubulin and the conformations of representative compounds showing the effects of the structural modifications and the corresponding docking results (the protein is shown in grey): a) with \textbf{15}; b) with \textbf{23}; c) with \textbf{24}; d) with the 11,diarylethene isomer (isocombretastatin) of \textbf{15} and e) with the 11,diarylethene isomer (isocombretastatin) of \textbf{24} and f) a combination of b,c and e.

Scheme 1: Synthesis of 3-indolecarbaldehyde combretastatin derivatives 13-16 and bromoderivatives 35 and 36.\textsuperscript{a}

(a) (i) (3,4,5-(MeO)\textsubscript{3}PhCH\textsubscript{2})Ph\textsubscript{3}PBr (11), THF, BuLi (1.6M), THF, -40°C, 1h; (ii) 1-ethyl-1H-indole-5-carbaldehyde (1), 1-methyl-1H-indole-5-carbaldehyde (2), 1-ethyl-1H-indole-3,5-dicarbaldehyde (3), 1-methyl-3-bromo-1H-indole-5-carbaldehyde (4) or 1-methyl-2,3-dibromo -1H-indole -5-carbaldehyde
(5), THF, -40 °C to rt, 24 h. (b) (i) (2,3,4-(MeO)₃PhCH₂)Ph₃PBr (12), THF, BuLi (1.6M), THF, -40°C, 1h; (ii) 1-methyl-1H-indole-5-carbaldehyde (2), THF, -40 °C to rt, 24 h.

**Scheme 2: Preparation of benzimidazole combretastatin derivatives 37+38.**

Reagents and conditions: (a) (i) Ph₃P(3,4,5-MeO₃PhCH₂)I, THF, nBuLi (1.6M), THF, - 40 ºC, 1h; (ii) 1-methyl-1H-benzimidazole-5(6)-carbaldehydes, THF, -40 °C to rt, 24 h.

**Scheme 3: Preparation of 3-substituted indole-combretastatin derivatives 17-34.**

Reagents and conditions: (a) (i) POCl₃, DMF, 0 ºC, 1 h; (ii) 15 or 16, 60 ºC, 2 h. (b) NaBH₄, MeOH, rt, 30 min. (c) NH₂OH·HCl, MeOH, pyr (2 drops), rt, 24 h. (d) Ac₂O, pyr, rt, 4 h. (e) 17 or 18 in tBuOH, 2-methyl-2-butene, THF; add NaClO₂, NaH₂PO₄, H₂O, rt, 12 h. (f) TMSCHN₂, MeOH, 15 min. (g) (i) NH₂CH₂COOEt·HCl; EDCI; 4-DMAP; rt, 48 h, inert atmosphere. (h) CH₂NHCH₂COOMe·HCl; EDCI; 4-DMAP; rt, 48 h, inert atmosphere. (i) NH₂CH₂CH₂COOEt; EDCI; 4-DMAP; rt, 48 h, inert atmosphere. (j) (COOMe)₂CHNH₂·HCl; EDCI; 4-DMAP; rt, 48 h, inert atmosphere. (k) KOH/MeOH, rt, 30 min.

**Table 1.** Aqueous solubility of representative compounds in aqueous buffer pH 7.0

**Table 2.** Tubulin Polymerization Inhibitory Activity, Cytotoxic Activity against Human Cancer Cell Lines, and Results of the Docking Studies.

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REFERENCES

12. Quan, H.; Liu, H.; Li, C.; Lou, L. 1,4-Diamino-2,3-dicyano-1,4-bis(methylthio)butadiene (U0126) enhances the cytotoxicity of combretastatin A4 independently of mitogen-activated protein kinase kinase. J Pharmacol Exp Ther 2009, 330, 326-33.


37. [https://www.eyesopen.com/](https://www.eyesopen.com/)