Conformational mimetics of the α -methyl chalcone TUB091

binding tubulin: Design, synthesis and antiproliferative activity

Oskía Bueno,^a Gloria Tobajas,^a Ernesto Quesada,^a Juan Estévez-Gallego,^b Sam Noppen,^c María-José Camarasa,^a José-Fernando Díaz,^b Sandra Liekens,^c Eva-María Priego^{a*} and María-Jesús Pérez-Pérez^{a*}

^a Instituto de Química Médica (IQM, CSIC), Juan de la Cierva 3, E-28006 Madrid, Spain

^b Centro de Investigaciones Biológicas (CIB, CSIC), Ramiro de Maeztu 9, 28040 Madrid, Spain

^c Rega Institute for Medical Research, KU Leuven, B-3000 Leuven, Belgium.

* Corresponding author: Dr. María-Jesús Pérez-Pérez

Instituto de Química Médica (CSIC)

c/ Juan de la Cierva 3

E-28006 Madrid (Spain)

Phone number: 34 91 258 75 16

Fax number: 34 91 5644853

e-mail: mjperez@iqm.csic.es

Dr. Eva-Maria Priego

Instituto de Química Médica (CSIC)

c/ Juan de la Cierva 3

E-28006 Madrid (Spain)

Phone number: 34 91

Fax number: 34 91 5644853

e-mail: empriego@iqm.csic.es

Highlights

- Novel 1,2,3,4-tetrahydronaphthalen-2-yl aryl ketones have been synthesized.
- These derivatives are proposed to mimic the conformation of α -methyl chalcones.
- The most potent derivative has antiproliferative activity around 20 nM.
- The binding mode of CA4 and the α -methyl chalcone to tubulin has been compared.

Abbreviations : CA1 : combretastatin A1; CA2: combretastatin A2; CA4: combretastatin A4; CA4P: combretastatin A4 phosphate; R-PT: (*R*)-(+)-ethyl 5-amino 2-methyl-1,2-dihydro-3-phenylpyrido[3,4-*b*]pyrazin-7-yl carbamate; VDAs : vascular disrupting agents

Abstract

Based on the conformation of the α -methyl chalcone TUB091 in its complex with tubulin, a series of conformational mimetics have been designed and synthesized where the methyl group of the chalcone has been fused to phenyl ring B resulting in 1,2,3,4-tetrahydronaphthalen-2-yl aryl ketones. Among the synthesized compounds, the 5-amino-6-methoxy derivative, with a similar substitution pattern to that of TUB091, showed antiproliferative activity around 20 nM against tumor and endothelial cells. Tubulin binding experiments confirmed its binding to tubulin at the colchicine site with a K_b of $2.4 \times 10^6 \text{ M}^{-1}$ resulting in the inhibition of the in vitro assembly of purified tubulin. Moreover, based on the recently reported complex of combretastatin A4 (CA4) with tubulin, a comparative analysis of the binding mode of CA4 and the α -methyl chalcone to tubulin has been performed.

Keywords: tubulin; chalcones; antiproliferative activity; molecular modeling

1. – Introduction

Combretastatins are a group of naturally occurring *cis* stilbenes isolated from the bark of *Combretum caffrum*. Several members of this family of compounds, including combretastatin A4 (CA4, **1**) [1, 2], combretastatin A1 (CA1, **2**) [1] and to a lesser extend combretastatin A2 (CA2, **3**) [3], have been deeply investigated due to their capacity to inhibit tubulin polymerization by binding at the colchicine site (Figure 1). These compounds show antimitotic properties against cancer cells but in addition they are able to disrupt vascular irrigation in the tumor environment, thus CA4 represents the prototype compound of the so called vascular disrupting agents (VDAs) acting on tubulin [4-6]. Despite the interesting properties of these compounds, combretastatins suffer from two significant drawbacks: poor aqueous solubility and easy isomerization towards the *trans* stilbenes, that are significantly less active [7]. The low solubility issue has been addressed through the synthesis of prodrugs, of which the phosphate of CA-4 (fosbretabulin, **4**) [8] or the diphosphate of CA-1 (OXi4503, **5**) [9] are being tested in clinical trials. Concerning the stability of the *cis* stilbene, many efforts have been performed to fix the *cis* conformation by incorporation of the stilbene into a restricted bridge or by replacing it by other linkers, as recently reviewed [10-13].

Among skeletons abundantly present in compounds inhibiting tubulin polymerization of natural or synthetic origin, chalcones and chalconoids occupy an important position [14]. In 2009, a series of chalcones (compounds **6-8**) (Figure 1) were described as CA4 surrogates, among which the α -methyl chalcone **7** showed antiproliferative activity at the nM or subnM range [15, 16]. Moreover, based on docking studies, it was proposed that the chalcones adopt an orientation similar to that of podophyllotoxin (**9**), so that the α -phenyl chalcone **8** could be seen as a podophyllotoxin mimetic [16]. More recently, other α -methyl chalcones with an indole as ring B, have been described as potent antiproliferative agents, also binding at the colchicine domain of tubulin [17]. We have also reported the α -methyl chalcone TUB091 (**10**), whose A ring corresponds to that of CA-2, as a potent tubulin depolymerizing agent, inhibiting cancer and endothelial cell growth at IC₅₀ values of

1-10 nM [18]. In addition, the Lys-Pro prodrug of TUB091 (**11**), that strongly enhanced the aqueous solubility, showed potent antitumor activity in melanoma and cancer xenograft models as well as antimetastatic activity [18]. The active conformation of these chalcones when binding at the colchicine site is the *s*-trans conformer, as initially propose by Ducki [16] and unequivocally confirmed by the resolution of the complex of the hydroxyl derivative TUB092 (**12**) with tubulin [18].

Despite their interesting biological properties, chalcones are Michael acceptors due to their $\alpha\beta$ -unsaturated ketone structure and this feature may compromise their pharmacological development [19, 20]. Moreover, we [18] and others [21] have described that chalcones are prone to photoisomerization while the biological activity is due to one isomer, in our case the *E*-isomer. We have now used the conformation of the *E*-isomer of the α -methyl chalcone **12** bound to tubulin to design a conformational constrained mimetic through the incorporation of a tetrahydronaphthalene scaffold as ring B. With the incorporation of such a scaffold and the conformational restrictions that this implies, it is intended to surmount some of the general drawbacks associated to chalcones, such as possible isomerization to the *Z*-isomer or potential Michael additions.

Moreover, since the X-Ray structure of CA-4 binding to tubulin has been recently determined [22], we have compared the binding mode of chalcone **12** with that of CA4, to determine which common residues are crucial in the interaction of these ligands.

2.- Results and discussion

2.1. Structural information and conformational mimetic proposal.

As already mentioned, the X-Ray structure of the T₂R-TTL-TUB092 complex has recently been solved at 2.4 Å resolution (PDB ID: 5JVD) [18], where the ligand **12** was soaked into crystals formed by a protein complex composed of two bovine brain $\alpha\beta$ -tubulin heterodimers, the rat stathmin-like protein RB3 and chicken tubulin tyrosine ligase (T₂R-TTL). The binding site is formed by residues of strands S8 and S9, loop T7 and helices H7 and H8 of the β -tubulin, while

loop T5 of the α -tubulin is also involved. Detailed interactions between the ligand and the protein have been reported [18], and some of the key interactions have been taken into consideration for the design of the new compounds. Thus, the carbonyl group of the chalcone is at hydrogen-bond distance to the backbone amide of Asp β 251, while the OH at ring B establishes an hydrogen bond interaction with the main-chain of Thr179 (Figure 2). It is expected that the NH₂ of its analogue (**11**) establishes a similar interaction with this residue. Rings A and B of chalcone **12** are stacked between the side chains of Cys241 and Leu255, and between Asn258 and Lys352, respectively. Regarding the bioactive conformation of the ligand, chalcone **12** binds as the *s*-trans conformer of the double bond, and rings A and B are not coplanar. Indeed, the deviation of ring B with respect to the plane containing ring A is approximately 45°. By analyzing this binding mode, it was hypothesized that a similar conformation might be accomplished by bridging the methyl group at position α of the chalcone and position 2 of ring B. This closing should be performed by using a saturated ring to accomplish the deviation from the plane above mentioned (Figure 3). Thus, compound **13** was constructed in its two enantiomeric forms and the geometries of both isomers were optimized with MOPAC2012 [23]. This was followed by a manual superposition of the geometry-optimized structures with the conformation of **12** in its complex with tubulin. As shown in Figure 4, the 3D structure of the *R*-enantiomer of **13** very nicely overlaps with that of the chalcone **12**. Thus, the synthesis of a series of 1,2,3,4-tetrahydronaphthalen-2-yl arylketones was addressed.

2.2. Chemistry

The 6-methoxy-1,2,3,4-tetrahydronaphthalene-2-carboxylic acid (**14**) was chosen as the starting material. This acid was converted to the aldehyde **15** by treatment BH₃·Me₂S followed by Dess-Martin oxidation of the intermediate alcohol (Scheme 1). Reaction of the 6-bromo-4-methoxybezo[*d*][1,3]dioxole in the presence of *n*-BuLi with the aldehyde **15** did not lead to the expected alcohol, and only the debrominated derivative 4-methoxy[*d*][1,3]dioxole was detected. As an alternative pathway, a Friedel-Crafts acylation between the acid chloride **16**, prepared by

treatment of the acid **14** with SOCl_2 in toluene at reflux, and 4-methoxy[*d*][1,3]dioxole (**17**) [24] was assayed (Scheme 1). When the reaction was performed using AlCl_3 as catalyst at rt or heating at 60 °C, no acylation products were detected. By using ZnCl_2 and performing the reaction at 60 °C in anhydrous 1,2-dichloroethane, two new isomeric compounds were obtained (**18** and **19**, in low yield) whose structures were determined by NMR experiments. Confirmation of the correct assignment of compound **19** was performed based on NOESY experiments where a correlation peak is observed between the OCH_3 signal of ring A (δ 3.90 ppm) and an aromatic proton of this same ring (δ 6.82 ppm) (see spectra in the Supplementary data). In order to avoid the formation of regioisomers, the symmetrical 1,2,3-trimethoxybenzene (**20**) was used as the arene (Scheme 2). In this case, the Friedel-Crafts acylation between **20** and the acid chloride **16** afforded two compounds, that were characterized as **21** (24% yield) and **22** (7% yield) (Scheme 2). In the ^1H NMR spectrum of **22**, a phenolic OH was detected at 12.46 ppm, suggesting that this OH could be involved in an internal hydrogen bond with the carbonyl through a pseudo 6-membered ring. Thus, the phenolic OH should be at position 2 in ring A. This demethylation reaction vicinal to the ketone during Friedel-Crafts acylations has been described by other authors [25-27]. By changing the catalyst, the reaction could be selectively directed towards each of these two compounds. Thus, when the reaction between **16** and **20** was performed in the presence of TfOH [28], compound **21** was isolated in 64% yield. On the other hand, by employing $\text{BF}_3 \cdot \text{OEt}_2$ [29] and extending the reaction time to 16 h, the phenolic derivative **22** was obtained as the major compound in 46% yield. When the arene used was 1,2-dimethoxybenzene (**23**), and using TfOH as the catalyst, the ketone **24** was obtained as the only regioisomer in 76% yield, containing the methoxy groups at positions 3 and 4, according to previous reports [30, 31].

Compounds **21** and **24** were nitrated by treatment with HNO_3 in Ac_2O at -40 °C for 20-30 min (Scheme 3). As expected, a mixture of the corresponding 5- and 7- NO_2 derivatives was obtained. It should be highlighted that the control of the temperature is crucial to avoid double nitration [32]. The assignment of the nitration position in **25** and **26** was performed based on NMR experiments

(COSY, HBMC and HSQC). Catalytic hydrogenation of **25** in the presence of Pt/S afforded the 7-amino derivative **27** in 50% yield. Interestingly, when these reaction conditions were applied to the 5-nitro isomer **26**, no reduction was observed, probably due to steric hindrance. Alternatively, treatment of **26** with Fe in the presence of HCl in a mixture EtOH/H₂O [33] afforded the 5-amino derivative **28** in moderate yield. In the case of compound **24**, the nitration reaction led to a mixture of the 5- and 7-NO₂ isomers that could not be separated by chromatography. Thus, the mixture was reduced by treatment with Fe in acidic media. In this way, the 7-amino and 5-amino derivatives **31** and **32** were isolated in 16 and 10% overall yield for the two steps. Indeed, the different behavior of the 5- and 7-NO₂ isomers when subjected to catalytic hydrogenation may be exploited to selectively reduce the 7-isomer in a mixture.

2.3 Biological Evaluation.

2.3.1 Antiproliferative activity

The synthesized compounds were evaluated for their antiproliferative activity (Table 1) in four different cell lines: three tumor cell lines (L1210, CEM and HeLa) and one endothelial cell line (HMEC-1). Data are expressed as the 50% inhibitory concentration (IC₅₀), defined as the concentration at which the compounds reduced cell proliferation by 50%. Colchicine and CA4P were included as reference compounds. The IC₅₀ values of the chalcone **10** [18] are also included for comparative purposes.

It is interesting to highlight the strict structural requirements in this series of tetrahydronaphthalene derivatives for antiproliferative activity. The most potent compound is the 5-NH₂-6-OMe derivative (**28**) with IC₅₀ values around 20-50 nM in all the cell lines tested. Its regioisomer, the 7-NH₂-6-OMe derivative (**27**) is between 10-15 fold less active, with IC₅₀ values similar to those of the 6-OMe derivative **21**, all of them in the subμM range. The substitution pattern on ring A is also crucial for activity. Thus, the 2,3,4-trimethoxy derivatives (**21**, **26**, **27** and **28**) are the ones that provide better antiproliferative activity. If the OCH₃ at position 2 is absent (as in **32** compared to **28**) or if the

substituent at position 2 is an OH instead of an OCH₃ (compare **22** versus **21**), the antiproliferative activity is significantly reduced.

2.3.2 Tubulin binding and assembly inhibition

In order to determine if the antiproliferative activity of the here reported compounds was related to their capacity to destabilize tubulin, representative compounds were tested in a tubulin polymerization assay including podophyllotoxin and the chalcone **10** for comparative purposes. As shown in Figure 5, compound **28** display at the tested concentration a destabilizing effect similar to those of podophyllotoxin and the chalcone **10**. For compound **21**, a similar effect was observed although some aggregation seemed to occur at the end of the reaction. Compound **26** showed a weaker effect on tubulin polymerization, while compound **24** was inactive in this assay. These data are in agreement with the antiproliferative activity in cell culture.

In addition, for compound **28**, a competition experiment with R-PT was performed, as previously described [34], to determine its capacity to bind at the colchicine site and its binding potency. The K_b for R-PT was $3.2 \times 10^6 \text{ M}^{-1}$ [35], while the K_b value determined for **28** was $2.4 \pm 0.1 \times 10^6 \text{ M}^{-1}$. This K_b, as well as all other data for these compounds, corresponds to the racemic mixtures.

2.3.3 Inhibition of mitotic spindle formation and cell cycle experiments

Tubulin-binding agents typically interfere with DNA division during mitosis. Immunofluorescence assays in HeLa cells were performed for compound **28** at two different concentrations (0.3 and 0.1 μM). As shown in Figure 6, highly aberrant multipolar spindles were observed in cells treated with compound **28** compared to control cells.

In addition, we assessed the effect of compound **28** on cell cycle progression of HeLa cells. As shown in Figure 7, control cells show a typical pattern of cells in G1, S and G2/M phase of the cell cycle. In contrast, compound **28**, at concentrations of 0.1 μM and higher caused almost a complete blockage of cells in G2/M phase, indicating that the compound halts cell mitosis.

2.4. Docking studies

Using the crystalline complex of the chalcone **12** binding tubulin (PDB ID: 5JVD) and Autdock 4.2 [36], docking studies of the R isomer of **28** were performed. The best solution obtained was subjected to molecular dynamic simulations. As shown in Figure 8, the tetrahydronaphthalene derivative is bound almost in the same location as the chalcone **12**, with the trimethoxyphenyl ring located towards the β -subunit and surrounded by residues Tyr β 202, Gly β 237, Val β 238, Thr β 239, Cys β 241, Leu β 242, Ala β 250, Asp β 251, Leu β 252, Lys β 254, Leu β 255, Ile β 318 and Ile β 358. However, there is no hydrogen bond between the ketone of **28** and the NH of Asp β 251. The tetrahydronaphthalene ring is located close to the $\alpha\beta$ -interface, so that not only residues from the β -subunit but also residues from loop 5 of α -tubulin are involved. It should be stressed that the NH₂ of **28** is in hydrogen bond distance (1.8 Å) to the backbone carbonyl of Thr α 179, mimicking the interaction of the OH of chalcone **12** with this same residue.

2.5. Comparison of the complexes CA-4 and chalcone TUB092 with tubulin

Next, we compared the binding mode of CA-4 (PDB id: 5LJD) [22] and chalcone **12** (PDB id: 5JVD) [18] with tubulin. Thus, superimposition of the tubulin-CA4 complex onto the tubulin-**12** structure revealed that rings B from both compounds occupy the same space in the binding site (Figure 9A). In fact, the hydrogen bond between the OH group in B-ring and the backbone carbonyl of Thr α 179 is present in both complexes. However, the 1,3-benzodioxole ring of chalcone **12** is located deeper in the binding site compared with the 3,4,5-methoxybenzene ring of CA-4. As a consequence, the side chain of Ile318 in the **12**-tubulin structure changes its position to accommodate the dioxole ring and allow the interaction with Cys241 and Gly237.

Overall, from the comparison of CA-4- and **12**-tubulin complexes, no significant differences in the binding site were observed. Only very few and punctual differences were found, such as polar interactions (hydrogen bonds), rearrangements in the side chain of some residues (Ile 318) and energy contribution of the residues involved in the interaction, as shown in Figure 9B.

Taking together, the α -methyl chalcone **12** can be considered as a synthetic mimetic of the natural product CA-4.

3. Conclusions

Chalcones are interesting chemical entities from a medicinal chemistry perspective with a wide variety of applications although their ease of isomerization and Michael-acceptor potential may hamper some of these applications. We have used the bioactive conformation of the α -methylchalcone TUB092 in its complex with $\alpha\beta$ tubulin to search for conformational mimetics that surmount the above refereed limitations. Thus, tetrahydronaphthalene derivatives were proposed as restricted ring B mimetics, and their synthesis was performed. From the synthesized compounds, compound **28** showed potent antiproliferative activity with IC_{50} values around 20 nM, and complete blockage of cells in G2/M phase at a concentration of 0.1 μ M or higher. Its binding to tubulin (Kb) at the colchicine site was also determined by displacement of R-PT, thus confirming that our working hypothesis was correct. Moreover, a comparison of the complexes of combretastatin A4 and chalcone **12** with tubulin, both recently published, led to the conclusion that the α -methyl chalcone binds to tubulin using almost the same interactions as CA4, thus indicating that the chalcone could be considered a good mimetic of the natural combretastatin.

4. Experimental

4.1. Synthesis

Melting points were obtained on a Mettler Toledo M170 apparatus and are uncorrected. The elemental analysis was performed with a Heraeus CHN-O-RAPID instrument. Analyses indicated by the symbols of the elements or functions were within ± 0.4 % of the theoretical values. For the tested compounds, satisfactory elemental analysis or HPLC purity greater than 95% was obtained. For compounds **18** and **31**, HPLC purity is indicated and the compounds were not submitted to biological evaluation. Electrospray mass spectra were measured on a quadruple mass spectrometer equipped with an electrospray source (Hewlett-Packard, LC/MS HP 1100). 1H and ^{13}C NMR spectra were recorded on a Varian INNOVA-300 operating at 299 MHz (1H) and 75 MHz (^{13}C),

respectively, a Varian INNOVA-400 operating at 399 MHz (^1H) and 99 MHz (^{13}C), respectively, and a VARIAN SYSTEM-500 operating at 499 and 125 MHz, respectively.

Analytical TLC was performed on silica gel 60 F₂₅₄ (Merck) precoated plates (0.2 mm). Spots were detected under UV light (254 nm) and/or charring with ninhydrin or phosphomolybdic acid. Separations on silica gel were performed by preparative centrifugal circular thin-layer chromatography (CCTLC) on a Chromatotron^R (Kieselgel 60 PF₂₅₄ gipshaltig (Merck)), with layer thickness of 1 and 2 mm and flow rate of 4 or 8 mL/min, respectively. Flash column chromatography (HPFC) was performed in a Biotage Horizon instrument.

HPLC analysis was performed in Agilent 1120 compact LC, column ACE 5 C18-300 (15 cm x 4.6 mm), UV detection, and flow rate 1 mL/min, using as mobile phase A H₂O (containing 0.1% TFA) and as mobile phase B acetonitrile.

Microwave reactions were performed using the Biotage Initiator 2.0 single-mode cavity instrument from Biotage (Uppsala). Experiments were carried out in sealed microwave process vials utilizing the standard absorbance level (400 W maximum power). The temperature was measured with an IR sensor on the outside of the reaction vessel.

(±)-6-Methoxy-1,2,3,4-tetrahydronaphthalene-2-carbaldehyde (15)

To a solution of 6-methoxy-1,2,3,4-tetrahydronaphthalene-2-carboxylic acid (**14**) (150 mg, 0.73 mmol) in anhydrous THF (6.5 mL), 2 M BH₃·Me₂S in THF (0.5 mL, 0.89 mmol) was added at rt under argon. After stirring at rt for 1 h, ethyl acetate was added and the solution was successively washed with saturated aqueous NaHCO₃, water and brine. The combined organic layers were dried over Na₂SO₄, filtered and evaporated to dryness to provide a yellow oil which was used in the next step without further purification. To a solution of this alcohol in CH₂Cl₂ (7.2 mL) Dess–Martin periodinane (370 mg, 0.88 mmol) was added at rt. The mixture was stirred for 2 h at rt, washed with saturated aqueous NaHCO₃ and extracted with ethyl acetate. The combined organic layers were dried over Na₂SO₄, filtered and evaporated under reduced pressure. The crude residue was purified

by CCTLC (dichloromethane/methanol, 10:0.5) to yield 68 mg (49%) of **15** as colorless oil. ¹H NMR (DMSO-*d*₆, 400 MHz) δ: 1.68 (m, 1H, H-3), 2.08 (m, 1H, H-3), 2.70–2.84 (m, 5H, H-1, H-4, H-2), 3.69 (s, 3H, OCH₃), 6.64 (d, *J* = 2.7 Hz, 1H, Ar), 6.69 (dd, *J* = 8.4, 2.8 Hz, 1H, Ar), 7.03 (d, *J* = 8.4 Hz, 1H, Ar), 9.70 (s, 1H, COH).

(±)-6-Methoxy-1,2,3,4-tetrahydronaphthalene-2-carbonyl chloride (16)

To a solution of 6-methoxy-1,2,3,4-tetrahydronaphthalene-2-carboxylic acid (**14**) (260 mg, 1.26 mmol) in anhydrous toluene (12 mL), distillate thionyl chloride (1.48 mL, 12.6 mmol) was added at room temperature under argon. The solution was heated at reflux for 3h, cooled to room temperature and concentrated under reduced pressure to provide a tan solid. The acid halide was used in the next step without further purification.

4-Methoxybenzo[*d*][1,3]dioxole (17)[24]

To a mixture of 3-methoxybenzene-1,2-diol (500 mg, 3.56 mmol) and K₂CO₃ (785 mg, 4.28 mmol) in anhydrous DMF (18 mL) bromochloromethane (374 μL, 5.35 mmol) was added. The mixture was heated at 100 °C for 4 h. The solution was allowed to reach room temperature and volatiles were evaporated under reduced pressure. The residue was diluted with dichloromethane and neutralized with 1N HCl. The aqueous phase was further extracted with dichloromethane (3 x 20 mL). The combined organic layers were washed with brine, dried over Na₂SO₄, filtered and evaporated to dryness. The crude was purified by flash chromatography (hexane/ethyl acetate, 10:1) to yield 255 mg (47%) of **17** as colorless oil. ¹H NMR (DMSO-*d*₆, 400 MHz) δ: 3.81 (s, 3H, OCH₃), 5.96 (s, 2H, OCH₂O), 6.58 (d, *J* = 7.8 Hz, 1H, Ar), 6.63 (d, *J* = 8.1 Hz, 1H, Ar), 6.79 (t, *J* = 8.2 Hz, 1H, Ar).

(±)-(6-Methoxy-1,2,3,4-tetrahydronaphthalen-2-yl)(4-methoxybenzo[d][1,3]dioxol-5-yl)methanone (18) and **(±)-(6-Methoxy-1,2,3,4-tetrahydronaphthalen-2-yl)(7-methoxybenzo[d][1,3]dioxol-4-yl)methanone (19)**

To a solution of 4-methoxybenzo[d][1,3]dioxole (**17**) (18 mg, 0.12 mmol) in anhydrous 1,2-dichloroethane (1.0 mL), ZnCl₂ (16 mg, 0.12 mmol) was added under argon, followed by the addition of **16** (55 mg, 0.24 mmol). The resultant mixture was stirred at 60 °C for 4h, quenched with ice water and extracted with dichloromethane (3 x 15 mL). The combined organic layers were dried over Na₂SO₄, filtered and evaporated to dryness. The crude was purified by CCTLC (hexane/ethyl acetate, 2:1) to yield 7 mg of **18** (68% purity), as a white amorphous solid and 6 mg (16% isolated yield) of **19** as a white amorphous solid.

Experimental data for **18**: MS (ES, positive mode): *m/z* 341 (M+H)⁺. Analytical HPLC (gradient 10% to 100% phase B in 10 min): T_R: 9.36; area: 68 %. ¹H NMR (DMSO-*d*₆, 500 MHz) δ: 1.59 (m, 1H, H-3), 2.03 (m, 1H, H-3), 2.71–2.86 (m, 4H, H-1, H-4), 3.49 (m, 1H, H-2), 3.69 (s, 3H, OCH₃), 3.98 (s, 3H, OCH₃), 6.12 (m, 2H, OCH₂O), 6.64 (d, *J* = 2.7 Hz, 1H, Ar), 6.67 (dd, *J* = 8.3, 2.7 Hz, 2H, Ar), 6.74 (d, *J* = 8.2 Hz, 1H, Ar), 7.00 (d, *J* = 8.4 Hz, 1H, Ar), 7.14 (d, *J* = 8.2 Hz, 1H, Ar).

Experimental data for **19**: MS (ES, positive mode): *m/z* 341 (M+H)⁺. Analytical HPLC (gradient 10% to 100% phase B in 10 min): T_R: 9.16; area: 98 %. (gradient 60 to 80% phase B in 10 min): T_R: 3.62; area: 98 %. ¹H NMR (DMSO-*d*₆, 500 MHz) δ: 1.63 (m, 1H, H-3), 2.06 (m, 1H, H-3), 2.70 – 2.91 (m, 4H, H-1, H-4), 3.54 (m, 1H, H-2), 3.70 (s, 3H, OCH₃), 3.90 (s, 3H, OCH₃), 6.13 (m, 2H, OCH₂O), 6.66 (d, *J* = 2.7 Hz, 1H, Ar), 6.68 (dd, *J* = 8.3, 2.8 Hz, 1H, Ar), 6.82 (d, *J* = 9.1 Hz, 1H, Ar), 7.01 (d, *J* = 8.3 Hz, 1H, Ar), 7.39 (d, *J* = 9.0 Hz, 1H, Ar). ¹³C NMR (DMSO-*d*₆, 125 MHz) δ: 25.6 (C-3), 28.7 (C-4), 30.1 (C-1), 44.5 (C-2), 55.0 (OCH₃), 56.4 (OCH₃), 102.1 (OCH₂O), 108.1, 112.1, 113.1, 113.2, 122.8, 127.3, 129.8, 135.1, 136.8, 147.2, 148.6, 157.3 (Ar), 198.3 (CO).

(±)-(6-Methoxy-1,2,3,4-tetrahydronaphthalen-2-yl)(2,3,4-trimethoxyphenyl)methanone (21)

To a solution of 1,2,3-trimethoxybenzene (**20**) (346 mg, 2.06 mmol) in anhydrous 1,2-dichloroethane (8.2 mL), **16** (692 mg, 3.09 mmol) was added under argon, followed by the addition of TfOH (183 μ L, 2.06 mmol). The resultant mixture was stirred at 60 °C for 1h. The mixture was quenched by the addition of saturated aqueous solution of NaHCO₃ and ice water and extracted with dichloromethane (3 x 20 mL). The combined organic layers were washed with brine, dried over Na₂SO₄, filtered and evaporated to dryness. The crude was purified by flash chromatography (hexane/ethyl acetate, 2:1) to yield 355 mg (64%) of **21** as a white solid. Mp 66-67 °C. MS (ES, positive mode): m/z 357 (M+H)⁺. ¹H NMR (DMSO-*d*₆, 500 MHz) δ : 1.61 (m, 1H, H-3), 2.03 (m, 1H, H-3), 2.71 – 2.86 (m, 4H, H-1, H-4), 3.51 (m, 1H, H-2), 3.69 (s, 3H, OCH₃), 3.77 (s, 3H, OCH₃), 3.85 (s, 3H, OCH₃), 3.86 (s, 3H, OCH₃), 6.65 (d, J = 2.7 Hz, 1H, Ar), 6.67 (dd, J = 8.3, 2.7 Hz, 1H, Ar), 6.91 (d, J = 8.8 Hz, 1H, Ar), 7.00 (d, J = 8.3 Hz, 1H, Ar), 7.33 (d, J = 8.8 Hz, 1H, Ar). ¹³C NMR (DMSO-*d*₆, 100 MHz) δ : 25.7 (C-3), 28.6 (C-4), 30.4 (C-1), 45.4 (C-2), 54.9 (OCH₃), 56.1 (OCH₃), 60.4 (OCH₃), 61.6 (OCH₃), 107.8, 112.1, 113.1, 124.8, 125.3, 127.3, 129.8, 136.8, 141.5, 152.5, 156.6, 157.2 (Ar), 203.2 (CO). Anal. calc. for (C₂₁H₂₄O₅): C, 70.77; H, 6.79. Found: C, 70.38; H, 6.84.

(±)-(2-Hydroxy-3,4-dimethoxyphenyl)(6-methoxy-1,2,3,4-tetrahydronaphthalen-2-yl)methanone (22)

To a solution of 1,2,3-trimethoxybenzene (**20**) (82 mg, 0.49 mmol) in anhydrous 1,2-dichloroethane (2 mL), BF₃·OEt₂ (62 μ L, 0.49 mmol) was added under argon, followed by the addition of **20** (163 mg, 0.73 mmol). The resultant mixture was stirred at 60 °C overnight. The mixture was quenched by the addition of ice water and extracted with dichloromethane (3 x 20 mL). The combined organic layers were washed with brine, dried over Na₂SO₄, filtered and evaporated to dryness. The crude was purified by flash chromatography (hexane/ethyl acetate, 2:1) to yield 76 mg (46%) of **22** as a white solid. Mp 78-80 °C. MS (ES, positive mode): m/z 343 (M+H)⁺. ¹H NMR (DMSO-*d*₆, 500 MHz) δ : 1.73 (m, 1H, H-3), 2.03 (m, 1H, H-3), 2.77 – 2.86 (m, 3H, H-1, H-4), 2.92 (m, 1H, H-1),

3.71 (s, 6H, OCH₃), 3.82 (m, 1H, H-2), 3.89 (s, 3H, OCH₃), 6.66 – 6.72 (m, 3H, Ar), 7.02 (d, *J* = 7.9 Hz, 1H, Ar), 7.84 (d, *J* = 9.1 Hz, 1H, Ar), 12.46 (br s, 1H, OH). ¹³C NMR (DMSO-*d*₆, 125 MHz) δ: 26.4 (C-3), 28.4 (C-4), 31.0 (C-1), 41.3 (C-2), 55.0 (OCH₃), 56.1 (OCH₃), 59.9 (OCH₃), 103.8, 112.1, 113.1, 113.7, 127.0, 127.2, 129.7, 135.9, 136.8, 156.3, 157.3, 158.3 (Ar), 208.1 (CO). Anal. calc. for (C₂₀H₂₂O₅): C, 70.16; H, 6.48. Found: C, 69.78; H, 6.62.

(±)-(3,4-Dimethoxyphenyl)(6-methoxy-1,2,3,4-tetrahydronaphthalen-2-yl)methanone (24)

To a solution of 1,2-dimethoxybenzene (**23**) (95 μL, 0.74 mmol) in anhydrous 1,2-dichloroethane (3 mL), TfOH (65 μL, 0.74 mmol) was added under argon, followed by the addition of **16** (228 mg, 1.02 mmol). The resultant mixture was stirred at 60 °C for 2h. The mixture was quenched by the addition of saturated aqueous solution NaHCO₃ and ice water and extracted with dichloromethane (3 x 15 mL). The combined organic layers were dried over Na₂SO₄, filtered and evaporated to dryness. The crude was purified by flash chromatography (hexane/ethyl acetate, 2:1) to yield 184 mg (76%) of **24** as a white solid. Mp 98-100 °C. MS (ES, positive mode): *m/z* 327 (M+H)⁺. ¹H NMR (DMSO-*d*₆, 500 MHz) δ: 1.67 (m, 1H, H-3), 2.01 (m, 1H, H-3), 2.74 – 2.86 (m, 3H, H-1, H-4), 2.92 (m, 1H, H-1/ H-4), 3.71 (s, 3H, OCH₃), 3.78 (m, 1H, H-2), 3.82 (s, 3H, OCH₃), 3.85 (s, 3H, OCH₃), 6.67 (s, 1H, Ar), 6.68 (d, *J* = 8.1 Hz, 1H, Ar), 7.01 (d, *J* = 8.0 Hz, 1H, Ar), 7.08 (d, *J* = 8.5 Hz, 1H, Ar), 7.49 (s, 1H, Ar), 7.72 (d, *J* = 8.5 Hz, 1H, Ar). ¹³C NMR (DMSO-*d*₆, 125 MHz) δ: 26.4 (C-3), 28.5 (C-4), 31.0 (C-1), 40.8 (C-2), 55.0 (OCH₃), 55.5 (OCH₃), 55.8 (OCH₃), 110.5, 111.0, 112.0, 113.1, 122.9, 127.3, 128.6, 129.7, 136.9, 148.7, 153.0, 157.3 (Ar), 201.1 (CO). Anal. calc. for (C₂₀H₂₂NO₄): C, 73.60; H, 6.79. Found: C, 73.21; H, 6.64.

(±)-(6-Methoxy-7-nitro-1,2,3,4-tetrahydronaphthalen-2-yl)(2,3,4-trimethoxyphenyl)

methanone (25) and (±)-(6-Methoxy-5-nitro-1,2,3,4-tetrahydronaphthalen-2-yl)(2,3,4-trimethoxyphenyl)methanone (26)

To a solution of **21** (233 mg, 0.65 mmol) in acetic anhydride (2 mL) at -40 °C, 65% HNO₃ (100 μL) was slowly added. The reaction was allowed to reach -14 °C and stirred 20 min at this temperature. The reaction mixture was diluted with ethyl acetate and ice water was added. The crude product

was washed with brine and extracted with ethyl acetate (3 x 15 mL). The combined organic layers were dried over Na₂SO₄, filtered and evaporated to dryness. The crude was purified by CCTLC (dichloromethane/ethyl acetate, 10:0.2) to yield 121 mg (46%) of **25** as a yellow solid and 100 mg (38%) of **26** as a yellow solid.

Experimental data for **25**: Mp 120-122 °C. MS (ES, positive mode): *m/z* 401 (M+H)⁺. ¹H NMR (DMSO-*d*₆, 400 MHz) δ: 1.67 (m, 1H, H-3), 2.06 (m, 1H, H-3), 2.79 – 2.95 (m, 4H, H-1, H-4), 3.58 (m, 1H, H-2), 3.77 (s, 3H, OCH₃), 3.87 (s, 6H, OCH₃), 3.89 (s, 3H, OCH₃), 6.91 (d, *J* = 8.8 Hz, 1H, Ar), 7.08 (s, 1H, Ar), 7.35 (d, *J* = 8.8 Hz, 1H, Ar), 7.68 (s, 1H, Ar). ¹³C NMR (DMSO-*d*₆, 100 MHz) δ: 25.0 (C-3), 28.5 (C-4), 29.7 (C-1), 44.5 (C-2), 56.1 (OCH₃), 56.5 (OCH₃), 60.4 (OCH₃), 61.6 (OCH₃), 107.8, 113.8, 124.8, 125.0, 125.1, 128.0, 137.1, 141.5, 143.6, 150.0, 152.5, 156.7 (Ar), 202.6 (CO). Anal. calc. for (C₂₁H₂₃NO₇·0.5H₂O): C, 61.46; H, 5.89; N, 3.41. Found: C, 61.61; H, 5.91; N, 3.56.

Experimental data for **26**: Mp 93-95 °C. MS (ES, positive mode): *m/z* 401 (M+H)⁺. ¹H NMR (DMSO-*d*₆, 400 MHz) δ: 1.64 (m, 1H, H-3), 2.06 (m, 1H, H-3), 2.53 – 2.72 (m, 2H, H-4), 2.77 – 2.98 (m, 2H, H-1), 3.55 (m, 1H, H-2), 3.77 (s, 3H, OCH₃), 3.83 (s, 3H, OCH₃), 3.86 (s, 3H, OCH₃), 3.87 (s, 3H, OCH₃), 6.91 (d, *J* = 8.8 Hz, 1H, Ar), 7.12 (d, *J* = 8.6 Hz, 1H, Ar), 7.33 (d, *J* = 8.6 Hz, 1H, Ar), 7.35 (d, *J* = 8.8 Hz, 1H, Ar). ¹³C NMR (DMSO-*d*₆, 100 MHz) δ: 23.1 (C-4), 24.4 (C-3), 30.2 (C-1), 44.2 (C-2), 56.1 (OCH₃), 56.5 (OCH₃), 60.4 (OCH₃), 61.6 (OCH₃), 107.8, 111.1, 124.8, 124.9, 127.7, 129.0, 131.7, 140.6, 141.5, 148.1, 152.6, 156.8 (Ar), 202.5 (CO). Anal. calc. for (C₂₁H₂₃NO₇): C, 62.84; H, 5.78; N, 3.49. Found: C, 62.80; H, 5.96; N, 3.58.

(±)-(7-Amino-6-methoxy-1,2,3,4-tetrahydronaphthalen-2-yl)(2,3,4-trimethoxyphenyl)methanone (27)

Compound **25** (30 mg, 0.07 mmol) was dissolved in ethyl acetate (12 mL) in a pressure vessel and then 5% Pt/S (catalytic amount) was added. The mixture was hydrogenated at 30 psi for 3 h at 30 °C. Then, the reaction mixture was filtered and the solvent was evaporated. The crude was purified by flash chromatography (dichloromethane/ethyl acetate, 10:0.5) to yield 14 mg (54%) of **27** as

colorless oil. MS (ES, positive mode): m/z 372 ($M+H$)⁺. ¹H NMR (DMSO-*d*₆, 500 MHz) δ : 1.55 (m, 1H, H-3), 2.00 (m, 1H, H-3), 2.61 – 2.72 (m, 4H, H-1, H-4), 3.45 (m, 1H, H-2), 3.70 (s, 3H, OCH₃), 3.77 (s, 3H, OCH₃), 3.84 (s, 3H, OCH₃), 3.86 (s, 3H, OCH₃), 4.43 (br s, 2H, NH₂), 6.34 (s, 1H, Ar), 6.48 (s, 1H, Ar), 6.90 (d, J = 8.8 Hz, 1H, Ar), 7.32 (d, J = 8.8 Hz, 1H, Ar). Anal. calc. for (C₂₁H₂₅NO₅·H₂O): C, 64.77; H, 6.99; N, 3.60. Found: C, 64.66; H, 7.23; N, 3.89.

(±)-(5-Amino-6-methoxy-1,2,3,4-tetrahydronaphthalen-2-yl)(2,3,4-trimethoxyphenyl)methanone (28)

Iron powder (125 mg, 2.2 mmol) and 37% HCl (1 drop) were added to a solution of **26** (90 mg, 0.22 mmol) in EtOH (2.2 mL) and water (1 mL). The mixture was heated at reflux for 1h. Ethyl acetate (10 mL) was added to the mixture, washed with water and extracted with ethyl acetate (3 x 15 mL). The combined organic layers were dried over Na₂SO₄, filtered and evaporated to dryness. The crude was purified by CCTLC (dichloromethane/ethyl acetate, 10:1) to yield 34 mg (40%) of **28** as a white solid. Mp 133-135 °C. MS (ES, positive mode): m/z 372 ($M+H$)⁺. ¹H NMR (DMSO-*d*₆, 500 MHz) δ : 1.59 (m, 1H, H-3), 2.09 (m, 1H, H-3), 2.43 (m, 1H, H-4), 2.59 (m, 1H, H-4), 2.67 – 2.79 (m, 2H, H-1), 3.41 (m, 1H, H-2), 3.72 (s, 3H, OCH₃), 3.77 (s, 3H, OCH₃), 3.84 (s, 3H, OCH₃), 3.86 (s, 3H, OCH₃), 4.35 (br s, 2H, NH₂), 6.34 (d, J = 8.1 Hz, 1H, Ar), 6.62 (d, J = 8.1 Hz, 1H, Ar), 6.91 (d, J = 8.8 Hz, 1H, Ar), 7.33 (d, J = 8.8 Hz, 1H, Ar). ¹³C NMR (DMSO-*d*₆, 125 MHz) δ : 23.9 (C-4), 25.7 (C-3), 31.6 (C-1), 45.2 (C-2), 55.6 (OCH₃), 56.1 (OCH₃), 60.4 (OCH₃), 61.6 (OCH₃), 107.8, 108.4, 116.2, 120.0, 124.8, 125.4, 127.9, 134.4, 141.5, 144.2, 152.5, 156.6 (Ar), 203.5 (CO). Anal. calc. for (C₂₁H₂₅NO₅·H₂O): C, 64.77; H, 6.99; N, 3.60. Found: C, 64.39; H, 6.85; N, 4.00.

(±)-(7-Amino-6-methoxy-1,2,3,4-tetrahydronaphthalen-2-yl)(3,4-dimethoxyphenyl)methanone (31) and (±)-(5-Amino-6-methoxy-1,2,3,4-tetrahydronaphthalen-2-yl)(3,4-dimethoxyphenyl)methanone (32)

To a solution of **24** (170 mg, 0.49 mmol) in acetic anhydride (2 mL) at -40 °C, 65% HNO₃ (100 μ L) was slowly added. The reaction was allowed to reach -10 °C and stirred for 20 min at this temperature. The reaction mixture was diluted with ethyl acetate and ice water was added. The

aqueous layer was extracted with ethyl acetate (3x15 mL). The combined organic layers were washed with brine, dried over Na₂SO₄, filtered and evaporated to dryness. The residue was purified by CCTLC (dichloromethane/ethyl acetate, 10:0.1) to yield 95 mg of a mixture of the 5/7 nitro isomers. Analytic HPLC (gradient 15-95% acetonitrile in 10 min): T_R: 7.48; area: 60 % and T_R: 7.78; area: 40 %. MS (ES, positive mode): m/z 372 (M+H)⁺. The mixture of the two isomers was dissolved in EtOH (2.7 mL) and water (1 mL), and iron powder (140 mg, 2.5 mmol) and HCl 37% (2 drops) were added to the solution. The mixture was heated to reflux for 3h. Ethyl acetate (6 mL) was added to the mixture, washed with water and extracted with ethyl acetate (3 x 15 mL). The combined organic layers were dried over Na₂SO₄, filtered and evaporated to dryness. The crude was purified by CCTLC (dichloromethane/ethyl acetate, 10:2) to yield 27 mg (16%, area 75%) of **31** as a colorless oil and 17 mg (10%) of **32** as a white solid.

Experimental data for **31**: Analytical HPLC (gradient from 10% to 100% of mobile phase B in 10 min): T_R: 5.96; area: 75%. MS (ES, positive mode): m/z 342 (M+H)⁺. ¹H NMR (DMSO-*d*₆, 400 MHz) δ: 1.62 (m, 1H, H-3), 1.97 (m, 1H, H-3), 2.61 – 2.85 (m, 4H, H-1, H-4), 3.71 (s, 3H, OCH₃), 3.75 (m, 1H, H-2), 3.82 (s, 3H, OCH₃), 3.85 (s, 3H, OCH₃), 4.43 (br s, 2H, NH₂), 6.35 (s, 1H, Ar), 6.51 (s, 1H, Ar), 7.07 (d, *J* = 8.4 Hz, 1H, Ar), 7.48 (d, *J* = 2.0 Hz, 1H, Ar), 7.71 (dd, *J* = 8.5, 2.0 Hz, 1H, Ar).

Experimental data for **32**: Mp 125-127 °C. MS (ES, positive mode): m/z 342 (M+H)⁺. ¹H NMR (DMSO-*d*₆, 400 MHz) δ: 1.66 (m, 1H, H-3), 2.07 (m, 1H, H-3), 2.52 – 2.70 (m, 2H, H-1, H-4), 2.71 – 2.81 (m, 2H, H-1, H-4), 3.68 (m, 1H, H-2), 3.73 (s, 3H, OCH₃), 3.82 (s, 3H, OCH₃), 3.85 (s, 3H, OCH₃), 4.37 (br s, 2H, NH₂), 6.35 (d, *J* = 8.2 Hz, 1H, Ar), 6.64 (d, *J* = 8.2 Hz, 1H, Ar), 7.07 (d, *J* = 8.4 Hz, 1H, Ar), 7.49 (d, *J* = 2.0 Hz, 1H, Ar), 7.71 (dd, *J* = 8.5, 2.0 Hz, 1H, Ar). ¹³C NMR (DMSO-*d*₆, 125 MHz) δ: 23.8 (C-4), 26.3 (C-3), 32.2 (C-1), 40.6 (C-2), 55.6 (OCH₃), 55.7 (OCH₃), 108.4, 110.4, 111.0, 116.2, 120.1, 122.8, 127.9, 128.7, 134.4, 144.2, 148.7, 153.0 (Ar), 201.4 (CO). Anal. calc. for (C₂₀H₂₃NO₄·H₂O): C, 66.84; H, 7.01; N, 3.90. Found: C, 66.94; H, 6.94; N, 4.29.

4.2 Biological Methods

4.2.1. Cell proliferation.

Human microvascular endothelial (HMEC-1) cells were seeded in 48-well plates at 20,000 cells/well. After 24 h, 5-fold dilutions of the compounds were added. The cells were allowed to proliferate 4 days in the presence of the compounds, trypsinized, and counted by means of a Coulter counter (Analisis, Belgium). Human cervical carcinoma (HeLa) cells were seeded in 48-well plates at 10,000 cells/well. After 24h, different concentrations of the compounds were added. After 3 days of incubation, the cells were trypsinized and counted in a Coulter counter. Suspension cells (human T-cell leukemia Cem and mouse B-cell leukemia L1210 cells) were seeded in 96-well plates at 60,000 cells/well in the presence of different concentrations of the compounds, allowed to proliferate for 96 h, and counted in a Coulter counter. The 50% inhibitory concentration (IC_{50}) was defined as the compound concentration required to reduce cell proliferation by 50%.

4.2.2. Tubulin binding

Calf brain tubulin was purified as described [37]. Aliquots of frozen tubulin (10 mg) were clarified by exclusion chromatography in Sephadex G-25 and equilibrated in GAB buffer (10 mM sodium phosphate, 1 mM EGTA (ethylene glycol-bis(β -aminoethyl ether)-N,N,N',N'-tetraacetic acid), 25% v/v Glycerol, 0.1 mM GTP pH= 7.0) to a final concentration of 25 μ M of soluble protein. Protein concentrations were determined by spectrophotometric absorbance at $\lambda=295$ nm ($\epsilon= 107000$ M⁻¹ cm⁻¹).

4.2.2.1. Tubulin assembly time-course.

100 μ L of Tubulin prepared as described was vacuum-degassed and dispensed in a 96-well microplate and 30 μ M of the tested compounds were added. Assembly at 37 °C was spectrophotometrically monitored in a Varioskan Flash (Thermo Fisher Scientific) at $\lambda = 350$ nm up to 6000 s. Negative controls (DMSO) and positive controls (podophyllotoxin) for polymerization inhibition were included to validate the analysis. Figures represent normalized absorbance at 350 nm.

4.2.2.2. Tubulin binding constant

(R)-(+)-ethyl 5-amino 2-methyl-1,2-dihydro-3-phenylpyrido[3,4-b]pyrazin-7-yl carbamate (R-PT) was a kind gift of Prof. G.A. Rener, Organic Chemistry Research Department, Southern Research Institute, Birmingham, Alabama (Temple 1989). The binding constant of **28** to tubulin was determined by competition with a well characterized colchicine binding site ligand (R-PT) as described.[38, 39] For that purpose, the fluorescence emission of a previous mixed sample of 0.2 μ M of R-PT and 0.2 μ M of tubulin was evaluated in presence of increasing concentrations of studied ligand in a black 96-well plate (0; 0.05; 0.2; 0.5; 2; 5; 10; 30; 50; 70 μ M). The samples were incubated 30 minutes at 25 °C in a Varioskan plate reader (Thermo Scientific Waltham, Massachusetts, USA) before the fluorescence emission intensity at 456 nm (excitation 374 nm) was measured. The data were analyzed and the binding constants determined using Equigra V5.0 [40]

4.2.3. Immunocytochemistry.

HeLa cells were seeded overnight on poly-L-Lysine coated 8-well Millicell slides (Millipore). Next, 0.1% DMSO (control cells) or compound **28** (0.3 and 0.1 μ M) were added for 24 h. The cells were fixed, permeabilized and incubated with a monoclonal anti- β -tubulin antibody (2 μ g/ml, Sigma-Aldrich). After washing, cells were incubated with goat anti-mouse Alexa Fluor 555 antibody (4 μ g/ml; Molecular Probes, Invitrogen). Nuclei were stained with 2 μ g/ml Hoechst33342 (Sigma-Aldrich). Fluorescence microscopy was done using an Axiovert 200 M inverted microscope (Zeiss, Göttingen, Germany).

4.2.4. Cell cycle analysis.

HeLa cells were seeded in 6-well plates at 125,000 cells/well in DMEM with 10 % FBS. After 24 h, the cells were exposed to different concentrations of the compound. After 24 h, the DNA of the cells was stained with propidium iodide using the CycleTEST PLUS DNA Reagent Kit (BD Biosciences, San Jose, CA). The DNA content of the stained cells was assessed by flow cytometry on a FACSCalibur flow cytometer and analyzed with CellQuest software (BD Biosciences) within

3h after staining. Cell debris and clumps were excluded from the analysis by appropriate dot plot gating. Percentages of sub-G1, G1, S, and G₂/M cells were estimated using appropriate region markers.

4.3. Computational methods.

4.3.1. Docking of compound 28

3D structure of compound **28** was generated through conversion from their SMILES strings to 3D pdb format using CORINA (Molecular Networks GmbH, Germany and Altamira, LLC, USA). The geometry of **28** were first optimized using the semiempirical quantum chemistry program MOPAC2012 (AM1 method) [23]. Partial atomic charges were then obtained using the RESP [41] methodology with the 6-31G(d) basis set as implemented in the ab initio quantum chemistry program Gaussian09 [42].

Compound **28** was used as ligand for the automated docking experiments using TUB092-tubulin complex (PDB ID: 5JVD) [18]. The Lamarckian genetic algorithm implemented in AutoDock 4.2 [36] as used to generate the docked conformations within the putative binding cavity by randomly changing the overall orientation of the molecule as well as the torsion angles of all rotatable bonds. Default settings were used except for the number of runs, population size, and maximum number of energy evaluations, which were fixed at 250, 100 and 250.000, respectively. Rapid intra- and intermolecular energy evaluations of each configuration was achieved by having the receptor's atomic affinity potentials for aliphatic and aromatic carbon, oxygen, nitrogen and hydrogen atoms pre-calculated in a three-dimensional grid with a spacing of 0.375 Å. A distance-dependent dielectric function was used in the computation of electrostatic interactions.

4.3.2. Molecular dynamics simulations

The MD simulations were carried out using the AMBER 12 suite of programs [43]. The bonded and non-bonded parameters for **28**, GTP⁴⁻, GDP³⁻ and Mg²⁺ were assigned, by analogy or through

interpolation from those already present in the AMBER database, in a way consistent with the general AMBER force field (GAFF) for organic molecules [44].

The molecular system consisting of bovine α,β -tubulin, **28**, GDP³⁻, GTP⁴⁻ and 2 Mg²⁺ ions was neutralized by the addition of 36 Na⁺ ions [45] and immersed in a truncated octahedron of ~29500 TIP3P water molecules [46]. Periodic boundary conditions were applied and electrostatic interactions were treated using the smooth particle mesh Ewald (PME) method [47] with a grid spacing of 1 Å. The cutoff distance for the non-bonded interactions was 9 Å. The SHAKE algorithm [48] was applied to all bonds and an integration step of 2.0 fs was used throughout. Solvent molecules and counterions were relaxed by energy minimization and allowed to redistribute around the positionally restrained solute (25kcal·mol⁻¹·Å⁻²) during 50 ps of MD at constant temperature (300 K) and pressure (1 atm). These initial harmonic restraints were gradually reduced in a series of progressive energy minimizations until they were completely removed. The resulting systems were heated again from 100 to 300 K during 20 ps and allowed to equilibrate in the absence of any restraints for 10.0 ns during which system coordinates were collected every 2 ps for further analysis.

Three-dimensional complexes structures and trajectories were visually inspected using the computer graphics program PyMOL (The PyMOL Molecular Graphics System, Version 1.5.0.5. Schrödinger, LLC). Interatomic distances and angles, as well as root-mean-square deviations (rmsd) from a given structure, were monitored using the Ptraj module in AMBER.

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Conflict of interests

The authors declare no conflict of interests

Appendix A. Supplementary data

The following is the supplementary data related to this article

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Legends for Figures and Schemes

Figure 1. Chemical structure of combretastatins, podophylotoxin and selected chalcones binding to the colchicine-domain in tubulin.

Figure 2. Close-up view of **12** (magenta) bound to the colchicine-domain in tubulin (α -tubulin in pink cartoon and β -tubulin in cyan cartoon) (PDB id: 5JVD). Selected interacting residues are shown in sticks and labeled. Hydrogen bonds are represented in dashed lines.

Figure 3. Proposed conformational mimetic of compound **12**

Figure 4. Overlap of the 3D structures of chalcone **12** (magenta) and the R isomer of compound **13** (yellow). White arrows indicate the atom pairs used for the superimposition.

Figure 5. Time course of tubulin polymerization at 37 °C measured by 350 nm turbidimetry for DMSO vehicle (control, black line), podophyllotoxin (red line), **10** (light green line), **21** (dark green line), **24** (dark blue line), **26** (pink line), **28** (light blue line). Tubulin concentration: 25 μ M in 10 mM sodium Phosphate, 1 mM EDTA and 4 mM MgCl_2 ; GTP: 1 mM; compounds: 30 μ M in DMSO (0.6% final concentration).

Figure 6. Inhibition of mitotic spindle formation. HeLa cells were treated for 24 h with 0.1% DMSO [control (line A)] or compound **28** [at 0.1 μ M (line B) or 0.3 μ M (line C)], fixed and stained with anti- β -tubulin antibody to visualize the microtubules (orange) and Hoechst33342 for DNA (blue). Column 1, overlay of double staining; column 2, DNA staining; column 3, β -tubulin staining

Figure 7. Inhibition of cell cycle progression. HeLa cells were treated with DMSO (control) or compound **28** for 24 h. Next, the cells were harvested, stained with propidium iodide (PI), and cell cycle distribution was evaluated by flow cytometry. Percentages of cells in the different phases of the cell cycle are indicated.

Figure 8. Docking and molecular dynamics simulations of the binding of **28** in the colchicine domain.

Figure 9. Comparison of the binding modes of CA-4 and chalcone **12**.

Scheme 1. *Reagents and conditions:* (a) (i) $\text{BH}_3 \cdot \text{Me}_2\text{S}$, THF anh., rt, 1 h; DMP; (ii) DMP, CH_2Cl_2 , rt, 2 h; (b) $n\text{-BuLi}$, TBME anh., $-78\text{ }^\circ\text{C}$, 20 h; (c) SOCl_2 , toluene, reflux, 3 h; (d) ZnCl_2 , $\text{ClCH}_2\text{CH}_2\text{Cl}$ anh., $60\text{ }^\circ\text{C}$, 4 h.

Scheme 2. *Reagents and conditions:* (a) ZnCl_2 , $\text{ClCH}_2\text{CH}_2\text{Cl}$ anh., $60\text{ }^\circ\text{C}$, 1 h, yield: **21** (24%) and **22** (7%); (b) TfOH , $\text{ClCH}_2\text{CH}_2\text{Cl}$ anh., $60\text{ }^\circ\text{C}$, 1 h, yield: **21** (64%); (c) $\text{BF}_3 \cdot \text{OEt}_2$, $\text{ClCH}_2\text{CH}_2\text{Cl}$ anh., $60\text{ }^\circ\text{C}$, 16 h, yield: **22** (46%); (d) TfOH , $\text{ClCH}_2\text{CH}_2\text{Cl}$ anh., $60\text{ }^\circ\text{C}$, 2 h.

Scheme 3. *Reagents and conditions:* (a) HNO_3 , Ac_2O , $-40\text{ }^\circ\text{C}$ to $-10\text{ }^\circ\text{C}$, 20-30 min.; (b) H_2 , Pt(S) 5% wt, EtOAc , 30 psi, $30\text{ }^\circ\text{C}$, 3 h.; (c) Fe powder, HCl cat., EtOH , H_2O , reflux, 1-3 h.

Figure 1. Chemical structure of combretastatins, podophyllotoxin and selected chalcones binding to the colchicine-domain in tubulin.

1 R=H; Combretastatin A-4 (CA-4)
2 R=OH Combretastatin A-1 (CA-1)

3 Combretastatin A-2 (CA-2)

4 R¹=H; R²=OPO(ONa)₂ (CA-4P)
5 R¹= R²=OPO(ONa)₂ (CA-1P)

6 R=H
7 R=CH₃
8 R=Ph

9 Podophyllotoxin

10 R= NH₂ (TUB091)
11 R= NH-Pro-Lys
12 R= OH (TUB092)

Figure 2. Close-up view of **12** (magenta) bound to the colchicine-domain in tubulin (α -tubulin in pink cartoon and β -tubulin in cyan cartoon) (PDB id: 5JVD). Selected interacting residues are shown in sticks and labeled. Hydrogen bonds are represented in dashed lines.

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Figure 8. Docking and molecular dynamics simulations of the binding of **28** in the colchicine domain.

Predicted binding mode of **28** (green sticks) inside the colchicine-domain in $\alpha\beta$ -tubulin (α -tubulin in pink, β -tubulin in cyan). Hydrogen bonds are represented as black dashed lines. Residues within 4 Å to **28** are represented in lines and labeled.

Figure 9. Comparison of the binding modes of CA-4 and chalcone **12**.

(A) Superimposition of CA4-tubulin (α -tubulin in green, β -tubulin in cyan) and **12**-tubulin (α -tubulin in green forest, β -tubulin blue slate) complexes. CA4 is shown in salmon sticks and **12** in magenta sticks. Selected residues are shown in sticks and labelled. Hydrogen bonds are represented in dashed lines (yellow for CA4 interactions and black for chalcone **12** interactions). (B) Energy contribution per residue (calculate with MM-ISMA program [49]) involved in the binding mode of CA-4 (salmon) and chalcone **12** (magenta).

Scheme 1.

Reagents and conditions: (a) (i) $\text{BH}_3 \cdot \text{Me}_2\text{S}$, THF anh., rt, 1 h; DMP; (ii) DMP, CH_2Cl_2 , rt, 2 h; (b) $n\text{-BuLi}$, TBME anh., -78°C , 20 h; (c) SOCl_2 , toluene, reflux, 3 h; (d) ZnCl_2 , $\text{ClCH}_2\text{CH}_2\text{Cl}$ anh., 60°C , 4 h.

Scheme 2.

Reagents and conditions: (a) ZnCl_2 , $\text{ClCH}_2\text{CH}_2\text{Cl}$ anh., 60 °C, 1 h, yield: **21** (24%) and **22** (7%); (b) TfOH , $\text{ClCH}_2\text{CH}_2\text{Cl}$ anh., 60 °C, 1 h, yield: **21** (64%); (c) $\text{BF}_3 \cdot \text{OEt}_2$, $\text{ClCH}_2\text{CH}_2\text{Cl}$ anh., 60 °C, 16 h, yield: **22** (46%); (d) TfOH , $\text{ClCH}_2\text{CH}_2\text{Cl}$ anh., 60 °C, 2 h.

Scheme 3.

Reagents and conditions: (a) HNO_3 , Ac_2O , $-40\text{ }^\circ\text{C}$ to $-10\text{ }^\circ\text{C}$, 20-30 min.; (b) H_2 , $\text{Pt(S)}\ 5\%$ wt, EtOAc , 30 psi, $30\text{ }^\circ\text{C}$, 3 h.; (c) Fe powder, HCl cat., EtOH , H_2O , reflux, 1-3 h.

Table 1. Anti-proliferative activity of the tetrahydronaphthalene derivatives in endothelial and tumor cell lines.

Comp.	Tumor cells			Endothelial cells
	L1210	CEM	HeLa	HMEC-1
	IC ₅₀ (μM) ^a			IC ₅₀ (μM) ^a
10	0.0010 ± 0.0007	0.0010 ± 0.0007 ^b	0.0039 ± 0.0033 ^b	0.0023 ± 0.0005 ^b
19	160 ± 20	39 ± 5	88 ± 5	38 ± 3
21	0.24 ± 0.01	0.17 ± 0.07	0.40 ± 0.15	1.2 ± 0.1
22	75 ± 36	38 ± 3	40 ± 11	≥250
24	>250	221 ± 40	>250	>250
25	>250	>250	>250	199 ± 41
26	4.2 ± 0.0	0.86 ± 0.54	0.81 ± 0.06	5.3 ± 0.8
27	0.86 ± 0.02	0.48 ± 0.03	0.74 ± 0.01	1.3 ± 0.0
28	0.027 ± 0.000	0.022 ± 0.003	0.030 ± 0.002	0.047 ± 0.004
32	52 ± 4	6.3 ± 2.6	23 ± 1	122 ± 70
Colchicine	0.007 ± 0.003	0.013 ± 0.0004	0.0087 ± 0.0001	0.0038 ± 0.0011
CA-4P	0.0021 ± 0.0003	0.011 ± 0.001	0.013 ± 0.001	0.0029 ± 0.0001

^aIC₅₀ (50 % inhibitory concentration) is given as the mean ± SD of three independent experiments.

^b Data as reported in ref [18]

Graphical Abstract