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Synthesis and biological evaluation of polymethoxylated 4-heteroarylcoumarins as tubulin assembly inhibitor

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ABSTRACT

A series of *syn*-restricted polymethoxylated 4-heteroarylcoumarins—the isostuctural analogs of combretastatin A-4—was synthesized by Suzuki–Miyaura cross-coupling reaction and evaluated for antiproliferative activity. The 4-(1-methyl-1*H*-indol-5-yl)chromen-2-ones exhibit a potent cytotoxicity against HBL100 epithelial cell line with an IC_{50} value amounting to 0.098 and 0.078 μM, respectively. The two compounds, having an indolyl moiety, potent inhibit in vitro microtubule assembly with a substoichiometric mode of action. A structure–activity relationship was discussed and the indolyl moiety was proved to be a good surrogate for the 3-hydroxy-4-methoxyphenyl ring of CA-4.

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1. Introduction

Tubulin protein is a major target for anticancer drugs and, as a result, there has been in recent years an intense effort to discover and develop antimitotic agent that inhibit tubulin assembly for treatment of cancer.[1] The class of combretastatins was shown to be particulary effective for that perspective. Isolated from the African willow tree *Combretum caffrum*, combretastatin A-4 (CA-4 **2**, Fig. 1) binds to tubulin at the colchicines binding site, and it disrupts normal mitotic spindle functions.[2] Its simple molecular structure, along with its ability to selectively damage tumor neovasculature, make CA-4 of great interest for medicinal chemistry. The limitation of the natural product **2** due to the poor bioavailability and solubility in biological media led to the development of structural analogs, such as the water-soluble disodium phosphate prodrug CA-4P,[3] which has shown promising results as a tumor vascular targeting agent in clinical trials.[4] The simple structure, bringing only two aromatic rings linked by a double bond in the cis configuration limits the number of parent analogs. Research to develop new analogs with a more favorable therapeutic window is ongoing.

4-Arylcoumarins are naturally occurring neoflavonoids found in numerous plant species, bacteria and fungi with low toxicity in human.[5] The presence of two non-coplanar *syn*-aryl fragments, which

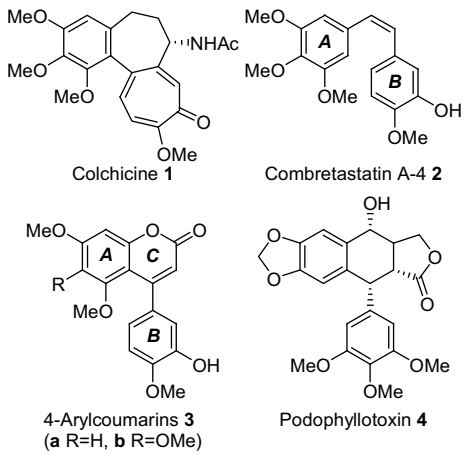

Figure 1. Colchicine-site ligands.

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displays a 1,1-diarylmethane scaffold, results in structural analogies with combretastatin **2**.[6] A number of substituted 4-arylcoumarins were synthesized within the author's laboratories to study the effects of various functional groups on antiproliferative activity. The neoflavonoids (**3a** and **b**, Fig. 1), which carry a substitution pattern of A and B rings similar to that of combretastatin A-4, have been found to possess a promising antitubulin activity.[7] The superior antimitotic activity of CA-4 **2** in comparison to **3a**,**b** analogs was explained assuming that hydrogen bonds between the Thr179 of the tubulin peptide chain and combretastatin cycle B are stronger than with the neoflavonoid ring.[8] Recently, in a search for antitumor agents, 4-arylcoumarins from endophytic *Streptomyces aureofaciens* have been found to inhibit cell proliferation and act on oncoprotein expression.[9]

Taking into account that a search for new microtubule-damaging agents is an important therapeutic issue, this work is devoted to the synthesis and biological evaluation of new polyoxygenated coumarins, containing heteroaromatic moiety in place of the B ring. It is important to note that these neoflavonoid derivatives are more stable in terms of isomerization compared to their naturally occurring analog **2**. For example, the cis double bond in CA-4 is prompt to evolve to the more stable trans isomer during storage or delivering, resulting in dramatic loss of antitumor activity.[10]

2. Chemistry

4-Heteroarylcoumarins can be obtained by treatment of 4-trifluoromethylsulfonyloxycoumarins with either heteroarylboronic acids or their pinacolate esters under Suzuki–Miyaura cross-coupling conditions. During the last decade the Suzuki reaction has become a commonly used method for arylation of coumarins.[7,11] Functional group tolerance, selectivity and low toxicity of the reagents, thermal, air and moisture stability of arylboronic acids made this catalytic approach one of the most useful for C–C connection.[12] However, synthesis of 4-heteroarylcoumarins by combination of highly donor polyoxygenated 2*H*-1-benzopyranone and strongly electron-withdrawing heteroaryl subunits can seriously hamper both the oxidative addition and the transmetallation steps in the catalytic cycle.[12] Indeed, application of catalytic system used for the synthesis of 4-arylcoumarins—$Pd(PPh_3)_4/Na_2CO_3/CuI$ (0.04:7.0:1.1 equiv, respectively) in a toluene–ethanol mixture—did not afford the desired product in the case of reaction of triflate **5a** with benzofuranylboronic acid **6**. Optimization of the reaction conditions established that application of $Pd(dppf)Cl_2$ (0.05 equiv)–K_3PO_4 (3.0 equiv)–Bu_4NBr (0.1 equiv) system in acetonitrile yielded 4-heteroarylcoumarin **14a** in a quantitative yield (Scheme 1, Table 1).

Apart from CA-4 **2**, many natural products possessing a trimethoxybenzene ring, for example, colchicine **1**, podophyllotoxin **4**, and steganacin, were found to be potent cytotoxic agents and exert their antitumor property by their antitubulin character.[13] Therefore, we have focused our main synthetic investigations on the functionalization of triflate **5a** belonging to the same structural prototype (Table 1). A range of heteroarylboronic acids **6**–**13** was used in the cross-coupling reactions (Fig. 2). The diversity of heteroaromatic fragments to be transferred was chosen to improve the understanding of the binding interactions at the colchicine site.

Table 1
Synthesis of 4-heteroarylcoumarins **14**–**21a**–**d** using $Pd(dppf)Cl_2$ (0.05 equiv)–K_3PO_4 (3.0 equiv)–Bu_4NBr (0.1 equiv) catalytic system

Substrate	$HetB(OR)_2$	Product	Yield (%)
5,6,7-OMe substituted			
5a	**6**	**14a**	98
5a	**7**	**15a**	73
5a	**8**	**16a**	92
5a	**9**	**17a**	79
5a	**10**	**18a**	75
5a	**11**	**19a**	87
5a	**12**	**20a**	87
5a	**13**	**21a**	84
5,7-OMe substituted			
5b	**9**	**17b**	71
5b	**10**	**18b**	79
5b	**11**	**19b**	88
5b	**12**	**20b**	93
5b	**13**	**21b**	96
6,7-OMe substituted			
5c	**11**	**19c**	81
5c	**12**	**20c**	85
5c	**13**	**21c**	86
Unsubstituted			
5d	**12**	**20d**	87
5d	**13**	**21d**	88

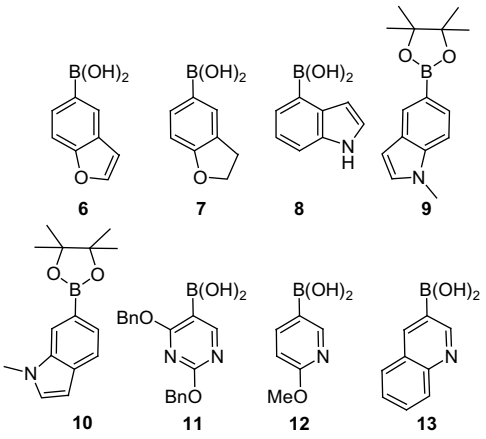
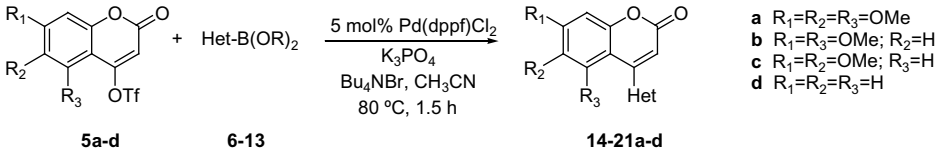


Figure 2. Structures of heteroarylboronic acids and esters **6**–**13**.

It is a significant challenge to establish the essential structural features for antitumor activity. Up to now, no common pharmacophore for colchicines site inhibitors has been clearly identified.

Among heteroarylboronic acids listed in Figure 2, indolyl species **8**–**10** are of particular interest since insertion of *N*-methylindolyl moiety to the α-position of 3,4,5-trimethoxyphenylimidazole, led to an antitubulin agent isostructural with combretastatin A-4.[14]

Proposed catalytic system based on $Pd(dppf)Cl_2$ has shown to be equally suitable for cross-coupling reactions implying application of heteroarylboronic acids and their less reactive pinacolate



Scheme 1. Palladium-catalyzed coupling between triflates **5a**–**d** and heteroarylboronic reagents **6**–**13**.

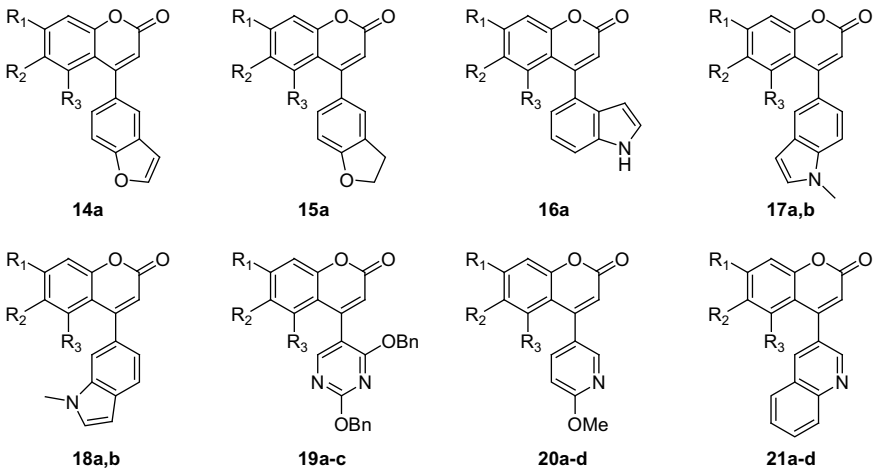


Figure 3. Structures of 4-heteroarylcoumarins **14**–**21a**–**d**.

analogs. All 4-heteroarylcoumarins synthesized using the catalytic system were isolated in good to nearly quantitative yields (71–98%), whatever the electron-donating properties of coumarin fragment and the nature of heteroaromatic group (Fig. 3, Table 1).

3. Biology

In vitro cytotoxicity of the synthesized compounds was investigated toward HBL100 human epithelial mammary cell line. A tetrazolium-based assay was used for determination of the drug concentration required to inhibit cell growth by 50% after incubation in the culture medium for 72 h. The calculated IC_{50} values are summarized in Table 2. Compounds **14a**–**16a**, **18a**–**21a**, and **20b**–**c** were found to be inactive and a modest cytotoxic effect was observed with **21d**. In contrast, compounds **17a** and **17b** exhibited a potent antiproliferative activity with an IC_{50} value amounting to 98 and 73 nM, respectively. This result implies that the indolyl ring is a good surrogate for the 3-hydroxy-4-methoxyphenyl ring of combretastatin A-4.

In order to confirm that the activity of these compounds are due to interaction with tubulin binding site, in vitro studies on their effect on microtubules assembly were performed. Figure 4A and B shows the effects of **17a** and **17b** on the 4′,6-diamidino-2-phenylindole (DAPI) fluorimetric time course of in vitro microtubule assembly from pure tubulin. A clear inhibition was noted, and the rate of assembly as well as the final amount of microtubules was lower in the presence of **17a** and **17b** than in the control experiment.

The insets of Figure 4A and B show that the extent of inhibition by **17a** and **17b**, respectively, increased monotonically with the mole ratio of the total ligand to total tubulin in the solution (R). In these figures, 50% inhibition occurred at a mole ratio of 0.55 mol of **17a** per mol of tubulin and at a mole ratio of 0.22 mol of **17b** per mol of tubulin. Previously an experiment performed with combretastatine A-4 showed that a mole ratio of 0.07 mol of drug per mol of tubulin was necessary to halve the microtubule formation.[8] Although less potent in tubulin polymerization inhibition, **17a** and **17b** show like combretastatin A-4, a substoichiometric mode of inhibition.

The molecular structure of 4-arylcoumarin **17b** was determined by single-crystal X-ray crystallography. The ORTEP diagram is presented in Figure 5, and selected parameters are listed in Table 3.

The structure revealed a conformation in which the two aromatic rings, A and B, are not coplanar (Fig. 6). Thus, these hydrophobic planar groups form the basis of a diaryl system, and serve as the rigid moiety of the molecular scaffold that should satisfy the overall geometric and steric requirements of binding with tubulin. The different potential pharmacophoric groups can be divided among these two planes and match the shape of the colchicine site.[15]

The neoflavonoid **17b** is structurally similar to colchicine and combretastatin,[16] possessing the common structural features namely a polyoxygenated moiety and a non-coplanar diaryl system in the appropriate chiral torsion angle (51° compared to 55° and 53°, respectively). Moreover the narrow range of distances between the centroid rings, as well as the two sets of ring substituents is the same (Table 3). These similarities are a good explanation for the potent cytotoxicity of indolyl derivatives, acting on the colchicine site of tubulin as assembly inhibitor.

Table 2
Cytotoxic potencies of compounds synthesized

Compound	**CA–4**	**14a**	**15a**	**16a**	**17a**	**17b**
IC_{50}[a] (μM)	0.003	49	>50	53	0.098	0.073
Compound	**18a**	**19a**	**20a**	**20b**	**20c**	**21a**	**21d**
IC_{50}[a] (μM)	>50	>50	>20[b]	>50	47	>50	13.4

[a] Drug concentration that inhibits the growth of HBL100 by 50% after incubation in liquid medium for 72 h. Each drug concentration was tested in triplicate, and the SE of each point is <10%.
[b] Crystallization at 20 μM.

4. Conclusion

Suzuki–Miyaura reaction has proven to be an effective approach to the synthesis of various A and B rings substituted 4-heteroarylcoumarins. Indolyl compound **17a** and **17b** showed promising cytotoxic activity toward human epithelial mammary HBL100 cell line. The two compounds potently inhibit microtubules assembly and therefore can be considered as functional analogs of CA-4. Application of these *syn*-locked neoflavonoid derivatives can be advantageous thanks to increasing specificity of drugs compared to the easily isomerized combretastatin analogs. The design strategy and the strong antimitotic activity exhibited by indolyl derivatives are promising for the development of new anticancer compounds.

5. Experimental

5.1. Chemistry

Melting points were taken on a Büchi capillary apparatus and are uncorrected. NMR spectra were obtained on a Brucker AC

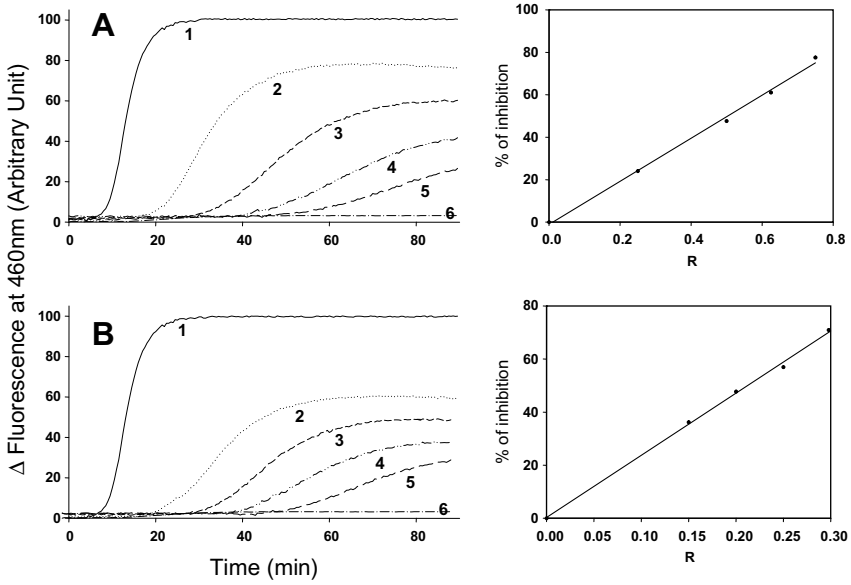


Figure 4. Effects of **17a** and **17b** on DAPI fluorimetric time course of in vitro microtubule assembly. The reaction was started by warming the solution from 4 to 37 °C. Panel A shows (1) tubulin at 20 μM and (2–5) aliquots of the same solution with 5, 10, 12.5 and 15 μM of **17a**. Panel B shows (1) tubulin at 20 μM and (2–5) aliquots of the same solution with 3, 4, 5 and 6 μM of **17b**. As a positive control inhibitor, (6) shows 20 μM of tubulin in the presence of 4 μM of combretastatin A-4. The insets show the percentage of fluorescence inhibition as a function of the mole ratio of total ligand to tubulin in the solution (R).

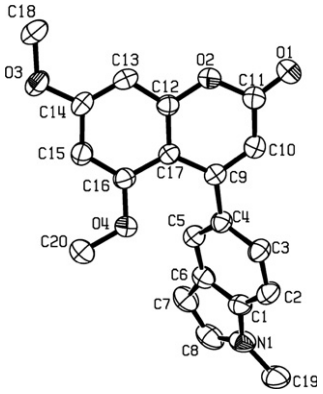


Figure 5. ORTEP drawing of 4-arylcoumarin **17b** (all hydrogen atoms are omitted for clarity).

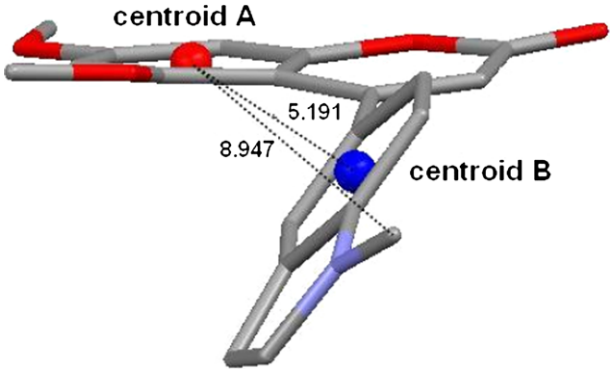


Figure 6. Twisted conformation of 4-arylcoumarin **17b**.

Table 3
X-ray crystallographic data of **17b**

Torsion angle	Degrees	Distance	Å
Ring (A)–ring (B)	51.2	Centroid (A)–N1	7.725
Ring (A)–O3–C18	2.8	Centroid (A)–C19	8.947[a]
Ring (A)–O4–C20	8.1	Centroid (A)–(B)	5.191[b]

[a] CA-4: 8.586 Å.
[b] CA-4: 5.245 Å.

300 spectrometer. Chemical shifts (δ) are reported in ppm for a solution of the compound in $CDCl_3$ with internal reference Me_4Si, and J values are in Hertz. Elemental analysis were performed at the "Laboratoire de Microanalyse de l'Universite d'Aix-Marseille 3". Mass-spectra were recorded using Bruker Daltonics Proflex III Maldi spectrometer. Separation by column chromatography was performed using Merck Kieselgel 60 (70–230 mesh). Petroleum ether refers to the fraction with distillation range 40–65 °C. All solvents were purified by standard techniques. Heteroarylboronic acids and their esters **6**–**13** were obtained from Khimmed (**6**–**8**, **11**), Aldrich (**9**), Acros (**10**) and Lancaster (**12**, **13**) and used as received. 4-Trifluoromethylsulfonyloxycoumarins **5a**–**d** were prepared as previously reported.[7]

5.1.1. Synthesis of 4-heteroarylcoumarins—general procedure

A mixture of 4-trifluoromethylsulfonyloxycoumarin (0.15–0.4 mmol, 1 equiv), heteroarylboronic acid or ester (1.3 equiv), potassium phosphate (3.0 equiv), tetrabutylammonium bromide (0.1 equiv) and 1,1′-bis(diphenylphosphino)ferrocene-palladium(II)dichloride dichloromethane complex (0.05 equiv) in dry acetonitrile (1–2 mL), was refluxed under argon until the substrate was completely consumed (0.5–3 h, monitored by TLC). The reaction mixture was diluted with water and extracted with dichloromethane. The combined organic layers were dried over Na_2SO_4, and the solvent was distilled under reduced pressure. The residue was purified by column chromatography, eluent EtOAc/petroleum ether (3:2), to afford the neoflavonoid derivative.

5.1.1.1. 4-(Benzofuran-5′-yl)-5,6,7-trimethoxycoumarin (14a).

Colorless plates, 98%, mp 137–139 °C. ^{1}H NMR: δ_H 3.21 (s, 3H, OMe), 3.80 (s, 3H, OMe), 3.95 (s, 3H, OMe), 6.10 (s, 1H, 3-H), 6.74 (s, 1H, 8-H), 6.82 (d, J = 1.2 Hz, 1H, 3′-H), 7.27 (d, J = 8.5 Hz, 1H, 6′-H), 7.53 (d, J = 8.5 Hz, 1H, 7′-H), 7.57 (s, 1 H, 4′-H) and 7.69 (d,

J = 1.2 Hz, 1H, 2′-H); ^{13}C NMR: δ_C 56.3 (OMe), 61.0 (OMe), 61.1 (OMe), 96.3 (C-8), 106.7 (C-3′), 107.5 (C-10), 110.4 (C-7′), 114.5 (C-3), 119.8 (C-4′), 124.2 (C-6′), 126.7 (C-9′), 133.8 (C-5′), 139.5 (C-6), 145.7 (C-2′), 151.2 (C-9), 151.7 (C-7), 154.6, 155.7, 156.9 (C-5) and 160.7 (C-2) (Found C, 67.96; H, 4.62. $C_{20}H_{16}O_6$ requires: C, 68.18; H, 4.58%).

5.1.1.2. 4-(2′,3′-Dihydrobenzofuran-5′-yl)-5,6,7-trimethoxycoumarin (15a). Colorless plates, 73%, mp 140–143 °C. ^{1}H NMR: δ 3.28 (t, J = 8.7 Hz, 2H, 3′-H), 3.33 (s, 3H, OMe), 3.82 (s, 3H, OMe), 3.95 (s, 3H, OMe), 4.65 (t, J = 8.7 Hz, 2H, 2′-H), 6.06 (s, 1H, 3-H), 6.73 (s, 1H, 8-H), 6.81 (d, J = 8.1 Hz, 1H, 7′-H), 7.10 (dd, J = 8.1 and 1.6 Hz, 1H, 6′-H), 7.19 (d, J = 1.6 Hz 1H, 4′-H); ^{13}C NMR: δ 29.5 (C-3′), 56.2 (OMe), 61.1 (2× OMe), 71.5 (C-2′), 96.3 (C-8), 107.4 (C-10), 108.2 (C-7′), 113.9 (C-3), 124.4, 126.2, 127.6, 131.0 (C-5′), 139.5 (C-6), 151.2 (C-9), 151.7 (C-7), 155.5 (C-4), 156.7 (C-5), 160.3 (C-8′), and 160.7 (C-2) (Found C, 67.61; H, 5.37. $C_{20}H_{16}O_6$ requires: C, 67.79; H, 5.12%).

5.1.1.3. 4-(1*H*-Indol-4′-yl)-5,6,7-trimethoxycoumarin (16a). Colorless plates, 92%, mp 169–172 °C. ^{1}H NMR: δ 3.04 (s, 3H, OMe), 3.74 (s, 3H, OMe), 3.96 (s, 3H, OMe), 6.25 (s, 1H, 3-H), 6.26 (m, 1H, 3′-H), 6.77 (s, 1H, 8-H), 7.05 (dd, J = 7.2 and 0.7 Hz, 1H, 5′-H), 7.20 (dd, J = 8.3 and 7.2 Hz, 1H, 6′-H), 7.26 (m, 1H, 2′-H), 7.46 (d, J = 8.3 Hz, 1H, 7′-H), 8.58 (br s, 1H, NH); ^{13}C NMR: δ 56.3 (OMe), 61.0 (OMe), 61.2 (OMe), 96.1 (C-8), 101.3 (C-3′), 107.8 (C-10), 111.1 (C-7′), 114.0 (C-3), 117.9 (C-5′ or C-6′), 121.4 (C-6′ or C-5′), 124.6 (C-2′), 126.1 (C9′), 131.6 (C-4′), 136.3 (C-8′), 139.3 (C-6), 151.4 (C-9), 151.7 (C-7), 154.8 (C-4), 156.8 (C-5) and 161.3 (C-2) (Found C, 68.14; H, 5.01; N, 3.81. $C_{20}H_{17}NO_5$ requires: C, 68.37; H, 4.88; N, 3.99%).

5.1.1.4. 5,6,7-Trimethoxy-4-(1′-methyl-1*H*-indol-5′-yl)coumarin (17a). Light yellow plates, 79%, mp 165–168 °C. ^{1}H NMR: δ 3.18 (s, 3H, OMe), 3.79 (s, 3H, OMe), 3.85 (s, 3H, Me), 3.94 (s, 3H, OMe), 6.13 (s, 1H, 3-H), 6.52 (d, J = 3.1 Hz, 1H, 3′-H), 6.74 (s, 1H, 8-H), 7.11 (d, J = 3.1 Hz, 1H, 2′-H), 7.20 (dd, J = 8.5 and 1.6 Hz, 1H, 6′-H), 7.35 (d, J = 8.5 Hz, 1H, 7′-H) and 7.60 (d, J = 1.6 Hz, 1H, 4′-H); ^{13}C NMR: δ 33.0 (Me), 56.3 (OMe), 61.1 (OMe), 61.2 (OMe), 96.2 (C-8), 101.1 (C-3′), 107.7 (C-10), 108.1 (C-7′) 114.3 (C-3), 119.5 (C-4′), 121.8 (C-6′), 127.6 (C-9′), 129.5 (C-2′) 130.2 (C-5′), 136.4 (C-8′), 139.5 (C-6), 151.4 (C-9), 151.8 (C-7), 156.6 (C-4), 156.8 (C-5), 160.9 (C-2) (Found C, 68.88; H, 5.41; N, 3.76. $C_{21}H_{19}NO_5$ requires: C, 69.03; H, 5.24; N, 3.83%).

5.1.1.5. 5,7-Dimethoxy-4-(1′-methyl-1*H*-indol-5′-yl)coumarin (17b). White polycrystalline solid, 71%, mp 190–191 °C. ^{1}H NMR: δ 3.40 (s, 3H, OMe), 3.84 (s, 3H, Me), 3.87 (s, 3H, OMe), 6.06 (s, 1H, 3-H), 6.24 (d, J = 2.5 Hz, 1H, 6-H), 6.51 (dd, J = 3.1 and 0.6 Hz, 1H, 3′-H), 6.53 (d, J = 2.5 Hz, 1H, 8-H), 7.09 (d, J = 3.1 Hz, 1H, 2′-H), 7.13 (dd, J = 8.5 and 1.7 Hz, 1H, 6′-H), 7.29 (d, J = 8.5 Hz, 1H, 7′-H) and 7.55 (dd, J = 1.7 and 0.6 Hz, 1H, 4′-H); ^{13}C NMR: δ 32.8 (Me), 55.4 (OMe), 55.6 (OMe), 93.5 (C-8), 95.7 (C-6), 101.3 (C-3′), 104.0 (C-10), 107.8 (C-7′) 112.6 (C-3), 119.4 (C-4′), 121.7 (C-6′), 127.5 (C-9′), 129.5 (C-2′), 130.9 (C-5′), 136.3 (C-8′), 157.0 (C-4 or C-9), 157.1 (C-9 or C-4), 158.4 (C-5), 161.1 (C-2) and 163.0 (C-7) (Found C, 71.49; H, 5.14; N, 4.16. $C_{20}H_{17}NO_4$ requires: C, 71.63; H, 5.11; N, 4.18%).

5.1.1.6. 5,6,7-Trimethoxy-4-(1′-methyl-1*H*-indol-6′-yl)coumarin (18a). Light yellow plates, 75%, mp 173–175 °C. ^{1}H NMR: δ 3.23 (s, 3H, OMe), 3.79 (s, 3H, OMe), 3.81 (s, 3H, Me), 3.95 (s, 3H, OMe), 6.15 (s, 1H, 3-H), 6.53 (dd, J = 3.1 and 0.8 Hz, 1H, 3′-H), 6.74 (s, 1H, 8-H), 7.08 (dd, J = 8.1 and 1.3 Hz, 1H, 5′-H), 7.12 (d, J = 3.1 Hz, 1H, 2′-H), 7.29–7.31 (m, 1H, 7′-H) and 7.62 (dd, J = 8.1 and 0.8 Hz, 1H, 4′-H); ^{13}C NMR δ 33.0 (Me), 56.3 (OMe), 61.1 (OMe), 61.2 (OMe), 96.2 (C-8), 101.1 (C-3′), 107.7 (C-10), 108.1 (C-7′), 114.3 (C-3), 119.5 (C-4′ or C-5′), 119.7 (C-5′ or C-4′), 128.3 (C-9′), 129.8 (C-2′), 132.6 (C-6′), 135.9 (C-8′), 139.5 (C-6), 151.4 (C-9), 151.8 (C-7), 156.6 (C-4), 156.8 (C-5) and 160.9 (C-2) (m/z found 366.279. $C_{21}H_{20}NO_5$ (M+H$^+$) requires: 366.387).

5.1.1.7. 5,7-Dimethoxy-4-(1′-methyl-1*H*-indol-6′-yl)coumarin (18b). Light yellow polycrystalline solid, 79%, mp 201–203 °C. ^{1}H NMR: δ 3.40 (s, 3H, OMe), 3.82 (s, 3H, Me), 3.88 (s, 3H, OMe), 6.10 (s, 1H, 3-H), 6.25 (d, J = 2.4 Hz, 1H, 6-H), 6.52 (dd, J = 3.1 and 0.8 Hz, 1H, 3′-H), 6.54 (d, J = 2.4 Hz, 1H, 8-H), 7.01 (dd, J = 8.2 and 1.5 Hz, 1H, 5′-H), 7.11 (d, J = 3.1 Hz, 1H, 2′-H), 7.26–7.29 (m, 1H, 7′-H) and 7.58 (dd, J = 8.2 and 0.8 Hz, 1H, 4′-H); ^{13}C NMR δ 32.8 (Me), 55.2 (OMe), 55.6 (OMe), 93.5 (C-8), 95.7 (C-6), 100.8 (C-3′), 103.8 (C-10), 107.8 (C-7′), 112.6 (C-3), 119.2 (C-4′ or C-5′), 119.6 (C-5′ or C-4′), 128.0 (C-9′), 129.6 (C-2′), 133.2 (C-6′), 135.8 (C-8′), 157.0 (C-4 or C-9), 157.1 (C-9 or C-4), 158.3 (C-5) 161.0 (C-2) and 163.1 (C-7) (Found C, 71.58; H, 5.16; N, 4.06. $C_{20}H_{17}NO_4$ requires: C, 71.63; H, 5.11; N, 4.18%).

5.1.1.8. 5,6,7-Trimethoxy-4-(2′,4′-dibenzyloxypyrimidin-5′-yl)coumarin (19a). White polycrystalline solid, 87%, mp 139 °C. ^{1}H NMR: δ 3.31 (s, 3H, OMe), 3.63 (s, 3H, OMe), 3.89 (s, 3H, OMe), 5.40 (s, 2H, CH_2), 5.49 (s, 2H, CH_2), 6.06 (s, 1H, 3-H), 6.66 (s, 1H, 8-H), 7.11–7.49 (m, 10H, Ph), 8.15 (s, 1H, 6′-H); ^{13}C NMR: δ 56.3 (OMe), 60.8 (OMe), 61.1 (OMe), 68.4 (CH_2), 69.4 (CH_2), 96.2 (C-8), 107.1 (C-10), 114.4 (C-3), 115.3 (C-5′), 127.2 (2× C-Ph), 127.9 (C-Ph), 128.0 (2× C-Ph), 128.1 (C-Ph), 128.3 (2× C-Ph), 128.4 (2× C-Ph), 135.6 (Cq-Ph), 136.3 (Cq-Ph), 139.0 (C-6), 147.3 (C-4), 150.8 (C-9), 151.2 (C-7), 154.4 (C-6′), 156.8 (C-5), 160.4 (C-2), 164.8 (C-2′ or C-4′) and 167.9 (C-4′ or C-2′) (Found C, 68.52; H, 5.15; N, 5.26. $C_{30}H_{26}N_2O_7$ requires: C, 68.43; H, 4.98; N, 5.32%).

5.1.1.9. 5,7-Dimethoxy-4-(2′,4′-dibenzyloxypyrimidin-5′-yl)coumarin (19b). White polycrystalline solid, 88%, mp 136 °C. ^{1}H NMR: δ 3.24 (s, 3H, IMe), 3.86 (s, 3H, IMe), 5.46 (s, 2H, CH_2), 5.49 (s, 2H, CH_2), 6.01 (s, 1H, 3-H), 6.10 (d, J = 2.1 Hz, 1H, 6-H), 6.50 (d, J = 2.1 Hz, 1H, 8-H), 7.13–7.52 (m, 10H, Ph), 8.10 (s, 1H, 6′-H); ^{13}C NMR: δ = 55.3 (OMe), 55.8 (OMe), 68.0 (CH_2), 69.4 (CH_2), 93.4 (C-8), 95.4 (C-6), 103.7 (C-10), 113.0 (C-3), 116.1 (C-5′), 127.7 (2× C-Ph), 128.0 (4× C-Ph), 128.1 (C-Ph), 128.4 (C-Ph), 128.5(2× C-Ph), 135.7 (Cq-Ph), 136.5 (Cq-Ph), 147.7 (C-4), 154.7 (C-6′), 156.8 (C-9), 158.0 (C-5), 160.6 (C-2), 163.2 (C-7), 164.6 (C-2′ or C-4′) and 167.5 (C-4′ or C-2′) (Found C, 70.54; H, 5.11; N, 5.41. $C_{29}H_{24}N_2O_6$ requires: C, 70.15; H, 4.87; N, 5.64%).

5.1.1.10. 6,7-Dimethoxy-4-(2′,4′-dibenzyloxypyrimidin-5′-yl)coumarin (19c). White polycrystalline solid, 81%, mp 133 °C. ^{1}H NMR: δ 3.55 (s, 3H, OMe), 3.95 (s, 3H, OMe), 5.46 (s, 2H, CH_2), 5.51 (s, 2H, CH_2), 6.24 (s, 1H, 3-H), 6.48 (s, 1H, 8-H), 6.88 (s, 1H, 5-H), 7.28–7.54 (m, 10H, Ph), 8.28 (s, 1H, 6′-H); ^{13}C NMR: δ 56.2 (OMe), 56.4 (OMe), 68.5 (CH_2), 69.7 (CH_2), 100.1 (C-8), 106.8 (C-5), 110.9 (C-3), 111.2 (C-10), 114.0 (C-5′), 128.0 (2× C-Ph), 128.2 (2× C-Ph), 128.3 (C-Ph), 128.4 (C-Ph), 128.5 (2× C-Ph), 128.6 (2× C-Ph), 135.3 (Cq-Ph), 135.9 (Cq-Ph), 146.0 (C-6), 148.3 (C-9), 149.6 (C-7), 153.0 (C-6′), 158.5 (C-4), 160.9 (C-2), 165.3 (C-2′ or C-4′) and 167.5 (C-4′ or C-2′) (Found C, 70.32; H, 5.03; N, 5.45. $C_{29}H_{24}N_2O_6$ requires: C, 70.15; H, 4.87; N, 5.64%).

5.1.1.11. 5,6,7-Trimethoxy-4-(6′-methoxypyridin-3′-yl)coumarin (20a). White polycrystalline solid, 87%, mp 164 °C. ^{1}H NMR: δ 3.31 (s, 3H, OMe), 3.80 (s, 3H, OMe), 3.93 (s, 3H, OMe); 3.98 (s, 3H, OMe), 6.05 (s, 1H, 3-H), 6.72 (s, 1H, 8-H), 7.77 (d, J = 8.7 Hz, 1H, 5′-H), 7.59 (dd, J = 8.7 and 2.5 Hz, 1H, 4′-H), 8.14 (d, J = 2.5 Hz, 1H, 2′-H); ^{13}C NMR: δ 53.5 (OMe), 56.2 (OMe), 60.8 (OMe). 61.1 (OMe), 96.4 (C-8), 107.6 (C-10), 109.3 (C-5′), 114.3 (C-3), 127.7 (C-3′),

138.6 (C-4′), 139.4 (C-6), 144.5 (C-2′), 150.8 (C-9), 151.7 (C-4 and C-7), 156.9 (C-5), 160.2 (C-2) and 164.0 (C-6′) (Found C, 62.71; H, 5.31; N, 3.96. $C_{18}H_{17}NO_6$ requires: C, 62.97; H, 4.99; N, 4.08%).

5.1.1.12. 5,7-Dimethoxy-4-(6′-methoxypyridin-3′-yl)coumarin (20b). White polycrystalline solid, 93%, mp 148 °C. ^{1}H NMR: δ 3.5 (s, 3H, OMe), 3.85 (s, 3H, OMe), 3.97 (s, 3H, OMe), 5.97 (s, 1H, 3-H), 6.24 (d, J = 2.3 Hz, 1H, 6-H), 6.50 (d, J = 2.3 Hz, 1H, 8-H,), 6.74 (d, J = 8.5 Hz, 1H, 5′-H), 7.50 (dd, J = 8.5 and 2.3 Hz, 1H, 4′-H) and 8.08 (d, J = 2.3 Hz, 1H, 2′-H); ^{13}C NMR: δ 53.6 (OMe), 55.4 (OMe), 55.9 (OMe), 93.6 (C-8), 95.7 (C-6), 103.1 (C-10), 109.1 (C-5′), 112.9 (C-3), 128.7 (C-3′), 138.3 (C-4′), 144.5 (C-2′), 152.1 (C-4), 157.2 (C-9), 158.0 (C-5), 160.5 (C-2), 163.4 (C-7), 163.9 (C-6′) (Found C, 65.02; H, 5.00; N, 4.25. $C_{17}H_{15}NO_5$ requires: C, 65.17; H, 4.83; N, 4.47%).

5.1.1.13. 6,7-Dimethoxy-4-(6′-methoxypyridin-3′-yl)coumarin (20c). White polycrystalline solid, 85%, mp 186 °C. ^{1}H NMR: δ 3.79 (s, 3H, OMe), 3.96 (s, 3H, OMe), 4.01 (s, 3H, OMe), 6.21 (s, 1H, 3-H), 6.82 (s, 1H, 8-H), 6.91 (d, J = 8.5 Hz, 1H, 5′-H), 6.91 (s, 1H, 5-H), 7.69 (dd, J = 8.5 and 2.3 Hz, 1H, 4′-H), 8.30 (d, J = 2.3 Hz, 1H, 2′-H); ^{13}C NMR: δ 53.8 (OMe), 56.3 (OMe), 56.4 (OMe), 100.4 (C-8), 106.7 (C-5), 111.1 (C-10), 111.2 (C-5′), 112.4 (C-3) 124.7 (C-3′), 138.4 (C-4′), 146.2 (C-6), 146.3 (C-2′), 150.1 (C-9), 152.2 (C-7), 153.1 (C-4), 161.1 (C-2) and 165.0 (C-6′) (Found C, 65.01; H, 5.11; N, 4.21. $C_{17}H_{15}NO_5$ requires: C, 65.17; H, 4.83; N, 4.47%).

5.1.1.14. 4-(6′-Methoxypyridin-3′-yl)coumarin (20d). White polycrystalline solid, 87%, mp 125 °C. ^{1}H NMR: δ 4.02 (s, 3H, OMe), 6.37 (s, 1H, 3-H), 6.90 (d, J = 8.5 Hz, 1H, 5′-H), 7.25 (ddd, J = 8.1, 7.8 and 1.2 Hz, 1H, 6-H), 7.40 (dd, J = 8.1 and 1.5 Hz, 1H, 5-H), 7.47 (dd, J = 8.1 and 1.2 Hz, 1H, 8-H), 7.56 (ddd, J = 8.1, 7.8 and 1.5 Hz, 1H, 7-H), 7.67 (dd, J = 8.5 and 2.6 Hz, 1H, 4′-H), 8.29 (d, J = 2.6 Hz, 1H, 2′-H); ^{13}C NMR: δ 53.8 (OMe), 111.2 (C-5′), 115.2 (C-3), 117.5 (C-8), 118.7 (C-10), 124.2 (C-3′), 124.3 (C-6), 126.4 (C-5), 132.2 (C-7), 138.6 (C-4′), 146.5 (C-2′), 152.3 (C-9), 154.2 (C-4), 160.4 (C-2) and 165.0 (C-6′) (Found C, 71.11; H, 4.29; N, 5.61. $C_{15}H_{11}NO_3$ requires: C, 71.14; H, 4.38; N, 5.53%).

5.1.1.15. 5,6,7-Trimethoxy-4-(quinol-3′-yl)coumarin (21a). White polycrystalline solid, 84%, mp 155 °C. ^{1}H NMR: δ 3.23 (s, 3H, OMe), 3.77 (s, 3H, OMe), 3.95 (s, 3H, OMe), 6.16 (s, 1H, 3-H), 6.75 (s, 1H, 8-H), 7.62 (ddd, J = 8.3, 7.4 and 1.1 Hz, 1H, 7′-H), 7.78 (ddd, J = 8.3, 7.4 and 1.5 Hz, 1H, 6′-H), 7.88 (dd, J = 8.3 and 1.1 Hz, 1H, 5′-H), 8.13 (d, J = 2.2 Hz, 1H, 4′-H), 8.18 (dd, J = 8.3 and 1.5 Hz, 1H, 8′-H), 8.89 (d, J = 2.2 Hz, 1H, 2′-H); ^{13}C NMR: δ 56.4 (OMe), 60.8 (OMe), 61.1 (OMe), 96.4 (C-8), 106.7 (C-10), 115.0 (C-3), 127.0 (C-10′), 127.3, 128.0, 129.1, 130.1, 132.3 (C-3′), 133.2 (C-4′), 139.2 (C-6), 147.0 (C-9′), 149.4 (C-2′), 150.8 (C-9), 151.7 (C-7), 151.9 (C-4), 157.4 (C-5), 160.2 (C-2) (Found C, 69.23; H, 4.97; N, 3.66. $C_{21}H_{17}NO_5$ requires: C, 69.41; H, 4.72; N, 3.85%).

5.1.1.16. 5,7-Dimethoxy-4-(quinol-3′-yl)coumarin (21b). White polycrystalline solid, 96%, mp 182 °C. ^{1}H NMR: δ 3.40 (s, 3H, OMe), 3.88 (s, 3H, OMe), 6.10 (s, 1H, 3-H), 6.24 (d, J = 2.4 Hz, 1H, 6-H), 6.56 (d, J = 2.4 Hz, 1H, 8-H), 7.60 (ddd, J = 8.5, 7.6 and 1.1 Hz, 1H, 7′-H), 7.77 (ddd, J = 8.4, 7.6 and 1.3 Hz, 1H, 6′-H), 7.87 (dd, J = 8.4 and 1.1 Hz, 1H, 5′-H), 8.08 (d, J = 1.8 Hz, 1H, 4′-H), 8.14 (dd, J = 8.5 and 1.3 Hz, 1H, 8′-H), 8.81 (d, J = 1.8 Hz, 1H, 2′-H); ^{13}C NMR: δ 55.5 (OMe), 55.9 (OMe), 93.8 (C-8), 95.7 (C-6), 102.9 (C-10), 113.6 (C-3), 127.2, 128.0, 129.0, 130.1, 133.2 (C-4′), 133.1 (C-3′), 146.8 (C-9′), 149.5 (C-2′), 152.0 (C-4), 157.2 (C-9), 157.9 (C-5), 160.8 (C-2) and 163.8 (C-7) (Found C, 71.84; H, 4.81; N, 4.14. $C_{20}H_{15}NO_4$ requires: C, 72.06; H, 4.54; N, 4.20%).

5.1.1.17. 6,7-Dimethoxy-4-(quinol-3′-yl)coumarin (21c). White polycrystalline solid, 86%, mp 177 °C. ^{1}H NMR: δ 3.72 (s, 3H, OMe), 3.98 (s, 3H, OMe), 6.36 (s, 1H, 3-H), 6.79 (s, 1H, 8-H), 6.95 (s, 1H, 5-H), 7.68 (ddd, J = 8.5, 7.9 and 1.1 Hz, 1H, 7′-H), 7.85 (ddd, J = 8.2, 7.9 and 1.4 Hz, 1H, 6′-H), 7.94 (dd, J = 8.2 and 1.1 Hz, 1H, 5′-H), 8.22 (dd, J = 8.5 and 1.4 Hz, 1H, 8′-H), 8.30 (d, J = 2.1 Hz, 1H, 4′-H), 9.02 (d, J = 2.1 Hz, 1H, 2′-H); ^{13}C NMR: δ 56.4 (OMe), 56.5 (OMe), 100.5 (C-8), 106.6 (C-5), 111.0 (C-10), 113.6 (C-3), 121.8 (C-10′), 127.5, 128.0, 128.2, 130.0 (C-3′), 131.2, 136.3 (C-4′), 146.5 (C-6), 147.2 (C-9′), 148.6 (C-2′), 150.3 (C-9), 152.0 (C-7), 153.4 (C-4), 160.8 (C-2) (Found C, 71.93; H, 4.67; N, 4.19. $C_{20}H_{15}NO_4$ requires: C, 72.06; H, 4.54; N, 4.20%).

5.1.1.18. 4-(Quinol-3′-yl)coumarin (21d). White polycrystalline solid, 88%, mp 174 °C. ^{1}H NMR: δ 6.50 (s, 1H, 3-H), 7.29 (td, J = 7.8 and 0.9 Hz, 1H, 6-H), 7.44–7.47 (m, 2H, 7- and 8-H), 7.59 (td, J = 7.8 and 1.5 Hz, 1H, 5-H), 7.68 (ddd, J = 8.3, 7.1 and 1.0 Hz, 1H, 7′-H), 7.84 (ddd, J = 8.1, 7.1 and 1.5 Hz, 1H, 6′-H), 7.93 (d, J = 8.1, and 1.0 Hz, 1H, 5′-H), 8.14 (dd, J = 8.3 and 1.5 Hz, 1H, 8′-H), 8.29 (d, J = 2.2, 1H, 4′-H), 8.81 (d, J = 2.2, 1H, 2′-H); ^{13}C NMR: δ 116.4 (C-3), 117.6 (C-8), 118.7 (C-10), 124.5 (C-6), 126.4 (C-5), 126.5 (C-10′), 127.8, 128.2, 128.3 (C-3′), 129.4, 131.0, 132.4 (C-7), 136.0 (C-4′), 148.2 (C-9′), 149.0 (C-2′), 152.3 (C-9), 154.2(C-4), 160.2 (C-2) (Found C, 78.89; H, 4.31; N, 5.03. $C_{18}H_{11}NO_2$ requires: C, 79.11; H, 4.06; N, 5.13%).

5.2. Cell cultures and survival assay

Human epithelial mammary HBL100 cells were grown in Dulbecco/Vogt modified Eagle's medium (DMEM, Gibco) supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, and 1% penicillin/streptomycin (Gibco) and maintained in a humidified incubator at 37 °C with 5% CO_2. For experiments, exponentially growing cells (2.6×10^4 cells/cm^2) were trypsinized with 0.25% trypsin/2 mM EDTA and seeded 24 h before drug treatment.

Cells were seeded in 96-well plates to be treated during 72 h. Compounds were dissolved in DMSO at a concentration of 10 mM, and diluted in culture medium before use. The numbers of viable cells were estimated by using the colorimetric 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma) assay, and absorbance was measured at 550 nm with a Dynatech MR 7-000 plate reader. At least three independent experiments were performed, and the IC_{50} values (i.e., concentration half-inhibiting cell proliferation) were graphically determined.

5.3. Preparation of lamb brain tubulin

Tubulin was purified from lamb brain by ammonium sulfate fractionation and ion-exchange chromatography. The protein was stored in liquid nitrogen and prepared as described.[17] Protein concentrations were determined spectrophotometrically with a Perkin Elmer spectrophotometer Lambda 800 and an extinction coefficient at 275 nm of 1.07 L g^{-1} cm^{-1} in neutral aqueous buffer or 1.09 L g^{-1} cm^{-1} in 6 M guanidine hydrochloride.

5.4. Microtubules assembly monitored by fluorescence

Microtubules assembly was performed on a Fluoroscan Ascent FL spectrofluorometer (Labsystems) using a 96-well plate. The excitation wavelength was set at 355 nm and the emission wavelength was set at 460 nm. Experiments were carried out at 37 °C and performed with 10 μM Dapi, 20 μM tubulin in 20 mM sodium phosphate buffer, 1 mM EGTA, 10 mM $MgCl_2$ and 3.4 M glycerol, pH 6.5. Under these conditions, the Dapi fluorescence enhancement is directly proportional to the concentration of polymerized

tubulin[18] and was monitored as a function of time. DMSO concentration was maintained below 4% in all samples and controls. Experiments were done in triplicate.

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