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Original article

Synthesis, antiproliferative activity and tubulin targeting effect of acridinone and dioxophenothiazine derivatives

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ABSTRACT

The synthesis of new acridinone and dioxophenothiazine derivatives along with their tubulin polymerization inhibitory and antiproliferative activities is reported. The analysis of correlation for cytotoxic and antitubulin potential of tested compounds showed that 4-methoxyphenylethyl derivatives **18a** and **19a** were highly cytotoxic but were regarded to have no significant antitubulin activity. However, the introduction of a 3-hydroxy substituent leading to compounds **18e** and **19e**, strongly increased the antitubulin potential but was associated with a loss of the antiproliferative activity. Modeling studies, topoisomerase inhibition assays and cell cycle analysis have been performed to better investigate the mechanism of action of such compounds.

1. Introduction

Antimitotic agents are one of the major classes of cytotoxic drugs for cancer treatment, and tubulin is the target for numerous small natural and synthetic molecules that inhibit the formation of the mitotic spindle [1]. Microtubules play an important role in the formation and function of the spindle apparatus, which realizes the controlled segregation of the chromosomes in dividing cells. Microtubules are also directly involved in a variety of other cellular functions, such as cell movement, maintenance of the cell shape, as well as the transport of organelles inside the cell. Interfering with the dynamic instability of microtubules, spindle poisons arrest dividing cells in G2/M phases of the cell cycle, causing mitotic catastrophy and finally apoptotic cell death [2]. There are two major groups of these antitumor agents, microtubule stabilizers such as paclitaxel [3] and microtubule destabilizers such as vinca alkaloids [4], colchicine [5], combretastatin A-4 [6], and sulfonamide E-7010 [7]. In recent years, much effort has been devoted to the identification of ligands binding at the colchicine site of tubulin derived not only from natural sources but also by screening compound libraries in combination with traditional medicinal chemistry [8–10]. However, no representatives of this class have yet been approved for use in cancer chemotherapy.

We have earlier described a series of benzopyridooxathiazepine-derived tubulin polymerization inhibitors, that could be considered as constraint analogs of E-7010, with potent in vitro and moderately in vivo antitumor activity (Fig. 1) [11,12]. The relatively low in vivo antitumor activity of compound **1**, has been explained by chemical stability study [13]. Results from this study showed that compound **1** suffered from a lack of stability of the oxathiazepine ring which could have some consequences on the storage conditions. Continuing our search strategy for novel potent tubulin-targeting compounds, we extended our studies to tricyclic heterocycles, with improved chemical stability, which were important scaffolds in medicinal chemistry and were in agreement with the structure–activity relationships previously established [12]. 10*H*-Acridin-9-one and its sulfonyl analog, 10*H*-phenothiazine 5,5-dioxide, have shown diverse biological activities including antimalarial [14], antineoplastic [15], antiasthmatic [16,17] and antidiabetic properties [18]. In addition,

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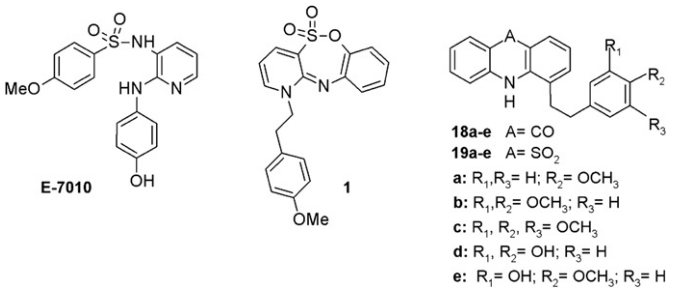


Fig. 1. Structures of reference and synthesized compounds.

N-benzoylated phenothiazines have recently been described as potent inhibitors of tubulin polymerization. The antitubulin activity of the most active compounds was comparable or superior to those of the reference compounds, such as nocodazole, podophyllotoxin, and colchicine [19].

This article reported the synthesis and biological evaluation of 10*H*-acridin-9-one **18a**–**e** and 10*H*-phenothiazine 5,5-dioxide **19a**–**e** derivatives bearing a phenyl-substituted-ethyl side chain on the benzenic part of the tricycle as potential antitumor agents (Fig. 1). The newly synthesized compounds have been tested together with their tubulin polymerization inhibitory activities and their cytotoxicities toward the human colon adenocarcinoma HT29 cell line.

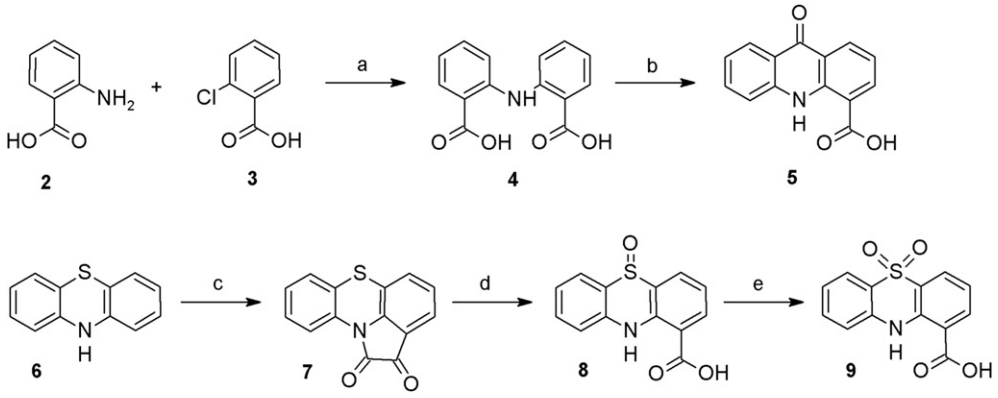
2. Results and discussion

2.1. Chemistry

10*H*-Acridin-9-one-4-carboxylic acid **5** and 10*H*-phenothiazine-1-carboxylic acid 5,5-dioxide **9** were synthesized according to the general methods described in Scheme 1. The N-phenylanthranilic acid **4** was prepared by the Jourdan–Ullmann copper-catalyzed condensation of 2-chlorobenzoic acid **3** and 2-aminobenzoic acid **2** in 82% yield [20]. Cyclization into the corresponding acridinone **5** was performed by acid catalyzed reaction with concentrated H_2SO_4 [21]. The 10*H*-phenothiazine-1-carboxylic acid 5,5-dioxide **9** was synthesized using phenothiazine **6** as starting material. The construction of the tetracyclic isatin intermediate **7** consisted in treatment of **6** with oxalyl chloride and aluminum chloride, following an 'one-pot' two steps procedure previously described [22]. Basic hydrolysis of amide bond of **7** with a 30% aqueous solution of sodium hydroxide followed by oxidative cleavage of the α-ketoacid intermediate mediated by addition of 35% aqueous hydrogen peroxide solution did not provide the desired 10*H*-phenothiazine-1-carboxylic acid as described in the literature [23] but led to the formation of its 5-oxide analog **8**. Additional oxidation into sulfone **9** was realized by refluxing sulfoxide **8** in acetic acid with an excess of 35% aqueous hydrogen peroxide solution in 67% yield. The final target compounds **18a**–**e** and **19a**–**e** were synthesized following the same procedure described in Scheme 2. Carboxylic acids **5** and **9** were first reacted with thionyl chloride in refluxing toluene followed by a palladium-catalyzed reduction of the formed acyls chlorides **10** and **11** with tributyltin hydride to provide exclusively the corresponding aldehydes **12** and **13** under very mild conditions and quantitative yields [24]. The systematic investigation of substituted acridinone or dioxo-phenothiazine derivatives bearing various arylethyl substituents was realized by condensation of aldehydes **12** and **13** under Wittig conditions with several phosphonium salt intermediates **15a**–**e** giving a *Z*/*E*-isomeric mixture of the corresponding olefins which were not separated. The phosphonium salts were prepared by refluxing in toluene the corresponding commercially available **14a**–**d** or described **14e** [25] benzyl chlorides with triphenylphosphine. Finally, catalytic hydrogenation allowed reduction of the double bond and hydrogenolysis of benzyloxy group for compounds **16e** and **17e**.

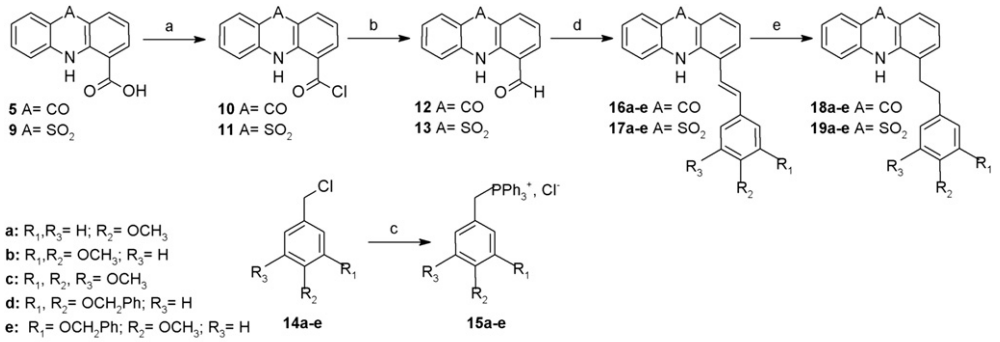
2.2. In vitro antiproliferative and tubulin polymerization assays

Antiproliferative effects of 10*H*-acridin-9-one and 10*H*-phenothiazine 5,5-dioxide derivatives **18** and **19** and the reference compounds (E-7010 and **1**) were evaluated on human colon adenocarcinoma HT29 cell line. The results presented in Table 1 showed that the nature and the location of the substituents at the phenylethyl side chain played a critical role for antiproliferative potency. Only 4-methoxy-substituted derivatives **18a** and **19a** showed IC_{50} values in the submicromolar range with respectively 0.051 and 0.87 μM. Interestingly, introduction of a 3,4,5-trimethoxyphenyl group in **18c** and **19c**, a typical pharmacophore found in many inhibitors of tubulin polymerization [26] led to a total loss of antiproliferative activity as documented by our earlier work [12,27] and as confirmed herein. Introduction of a 3-hydroxy



Reagents and conditions: a) Cu, K_2CO_3, DMF, reflux, 2 h; b) H_2SO_4, 100 °C, 4 h; c) i: $(COCl_2)_2$, THF, reflux, 4 h; ii: $AlCl_3$, CS_2, reflux, 12 h; d) i: 30% NaOH aqueous solution, r.t., 1 h; ii: 35% H_2O_2 solution, r.t., 2 h; d) 35% H_2O_2, AcOH, 70 °C, 3 h.

Scheme 1. Synthesis of 10*H*-Acridin-9-one-4-carboxylic acid **5** and 10*H*-phenothiazine-1-carboxylic acid 5,5-dioxide **9**.



Reagents and conditions: a) $SOCl_2$, toluene, 80 °C, 12 h; b) Bu_3SnH, $Pd(PPh_3)_4$, benzene, r.t., 2 h; c) PPh_3, toluene, reflux, 24 h; d) DBU, CH_3CN, reflux, 3 h; e) H_2, Pd/C, EtOH, r.t., 12 h.

Scheme 2. Synthesis of target compounds **18a**–**e** and **19a**–**e**.

substituent at the aryethyl side chain of **18a** or **19a** led to weaker cytotoxicities in the micromolar range (IC_{50} of respectively 12.0 and 7.2 μM for compounds **18e** and **19e**). Furthermore, quite other variations of substituents within the aryl moiety of **18a** or **19a** resulted either to reduced activities (**18b**, **18d**, **19b**) or made new compounds almost inactives (**18c**, **19c**, **19d**) at concentrations as high as 100 μM.

To characterize the possible interaction with the microtubule system of this novel series of 10*H*-acridin-9-one and 10*H*-phenothiazine 5,5-dioxide derivatives, compounds **18a**–**e** and **19a**–**e** were evaluated for their in vitro inhibition of tubulin polymerization. For comparison, E-7010 and compound **1** were examined in contemporaneous experiments (Table 1). Namely, some recent reports suggest that nonlinear relationship between antiproliferative activity and the effect on tubulin polymerization of described inhibitors, as combretastatin A-4, may occur, where highly cytotoxic compounds are not necessarily potent inhibitors of tubulin polymerization and vice versa [6,28,29]. In this assembly assay, only compounds **18e** and **19e** displayed potent inhibitory activities and were more actives than the reference compound E-7010 and compound **1**, with IC_{50} values of 0.51, 1.25, 2.2 and 2.4 μM, respectively. Compounds **18a**–**d** and **19a**–**d** seemed to be inactive as inhibitors of tubulin polymerization and did not inhibit tubulin assembly at concentrations as high as 10 μM. These findings indicated that the 3-hydroxy-4-methoxyphenyl was crucial moiety for inhibition of tubulin polymerization and was preferential partial structure seen with many colchicine site inhibitors. The analysis of correlation for cytotoxic and antitubulin potential of tested compounds showed that 4-methoxyphenylethyl derivatives **18a** and **19a** were highly cytotoxic but were regarded to have no significant antitubulin activity. However, the introduction of a 3-hydroxy substituent strongly increased the antitubulin potential but was associated with a loss of the antiproliferative activity. A possible explanation for the significantly lower cytotoxicities of **18e** and **19e** in respect to E7010 and compound **1** might be the bad physicochemical properties (such as solubility, membrane permeability, log *P*) that influence pharmacokinetic parameters in part did not allow compounds to reach tubulin itself within cells.

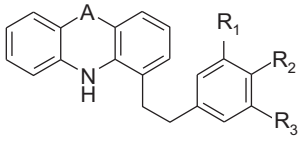
Next, to further investigate the antiproliferative potential of tumor cells, the effects of the highly active 4-methoxyphenylethyl analogs **18a** and **19a** as well as the negative control **18c** were investigated against four other cell lines derived from head and neck carcinoma (HNSCC), leukemia (HL60) and prostate tumors (PC3, DU145) (Table 2). Acridinone **18a** showed potent nanomolar cytotoxic effects on HNSCC and DU145 cell lines with IC_{50} of respectively 37 and 50 nM and was found less active on HL60 and PC3 cell lines with IC_{50} values in the submicromolar range (0.1 and 0.75 μM respectively). Dioxophenothiazine **19a** displayed the same cytotoxic profile but was found less potent than its carbonyl counterpart.

2.3. Topoisomerase I and II inhibition

Numerous acridinone and phenothiazine derivatives were described in the literature to act as DNA binders and topoisomerase (Topo) inhibitors [30]. For these reasons, compounds **18a**, **18c** and **19a** were tested for their topoisomerase I and II inhibition. As shown in Fig. 2A, supercoiled plasmid DNA was treated with human Topo I in the presence of the reference camptothecin (CPT) or synthesized compounds at 10 and 20 μM. The DNA relaxation/cleavage products were resolved by electrophoresis. Inhibition of Topo I was clearly specifically detected with the reference CPT, which produced a marked level of DNA double stranded breaks while no increase in the nicked band or modification of the relaxation of the DNA was observed with the tested compounds.

The same tests, with Topo I being replaced with Topo II, were performed to evaluate effects on the catalytic activity of Topo II. In these experiments supercoiled plasmid DNA was treated with Topo II in the presence of 50 μM of the tested drugs or 20 and 50 μM of the reference etoposide (ETO) which produced a marked level of

Table 1
Structures, inhibition of tubulin polymerization and in vitro antiproliferative activities against human colon adenocarcinoma HT29 cell line of compounds **18a**–**e** and **19a**–**e**.



Compd	A	R_1	R_2	R_3	IC_{50} (μM)[a]	IC_{50} TPI (μM)[b]
E-7010	–	–	–	–	0.21	2.2
Cpd 1	–	–	–	–	0.024	2.4
18a	CO	H	OCH_3	H	0.051	>10
18b	CO	OCH_3	OCH_3	H	>50	>10
18c	CO	OCH_3	OCH_3	OCH_3	>100	>10
18d	CO	OH	OH	H	>50	>10
18e	CO	OH	OCH_3	H	12.0	0.51
19a	SO_2	H	OCH_3	H	0.87	>10
19b	SO_2	OCH_3	OCH_3	H	>50	>10
19c	SO_2	OCH_3	OCH_3	OCH_3	>100	>10
19d	SO_2	OH	OH	H	>100	>10
19e	SO_2	OH	OCH_3	H	7.2	1.25

[a] % of inhibition at 10 μM or concentration inhibiting HT29 cell proliferation by 50% relative to untreated controls after 72 h of drug exposure.
[b] Inhibition of tubulin polymerization.

Table 2
Antiproliferative activities (IC_{50} μM) of compounds **18a**, **18c** and **19a** on different tumor cell lines.

Cell type	IC_{50}[a] (μM)			
	HNSCC[b]	Leukemia	Prostate	Prostate
Cell line	CAL33	HL60	PC3	DU145
Cpd 1	N.t.[c]	N.t.	0.475 ± 0.05	0.017 ± 0.02
18a	0.037 ± 0	0.1 ± 0.01	0.75 ± 0.1	0.05 ± 0
18c	>100	>50	>100	>100
19a	0.627 ± 0.02	1.65 ± 0.07	>50	0.81 ± 0.05

[a] Concentration inhibiting cell proliferation by 50% relative to untreated controls after 48 h of drug exposure.
[b] Head and neck squamous cell carcinoma.
[c] Not tested.

DNA double stranded breaks, corresponding to linear DNA (Fig. 2B). No inhibition of Topo II was also detected with our new derivatives. The results from this Topo I or II assay formally established that compounds **18a**, **18c** and **19a** were not topoisomerase poisons and did not interact with DNA.

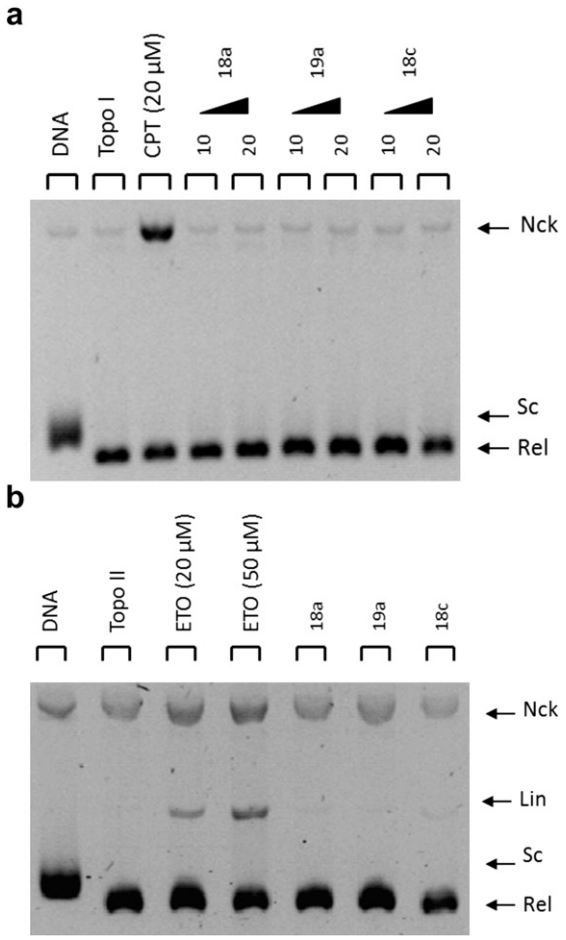


Fig. 2. (A) Effects of compounds **18a** and **19a** on the relaxation of plasmid DNA by human topoisomerase I. Native supercoiled PUC18 (130 ng, lane DNA) was incubated with 4 units topo I in the absence (lane Topo I) or presence of tested compounds at the indicated concentration (10 or 20 μM). Camptothecin (CPT) was used as reference compound. DNA samples were separated by electrophoresis on a 1% agarose gel containing 1 μg/mL ethidium bromide. (B) Effects of compounds **18a** and **19a** on the relaxation of plasmid DNA by human topoisomerase II. Native supercoiled pUC19 (350 ng, lane DNA) was incubated with 8 units topo II in the absence (lane Topo II) or presence of tested compounds at the indicated concentration 50 μM. Etoposide (ETO) was used at 20 and 50 μM. DNA samples were separated by electrophoresis on a 1% agarose gel containing 1 μg/mL ethidium bromide. Gels were photographed under UV light. Nck, nicked; Sc, supercoiled; Lin: linear; Rel, relaxed.

2.4. Cell cycle analysis and apoptosis (flow cytometry)

The effects of the compounds **18a**, **18c** and **19a** and reference antimitotic compounds (vinorelbine, vincristine, docetaxel) on cell cycle progression of HNSSC CAL33, colon HT29 and leukemia HL60 cells lines were investigated by flow cytometric analysis and the data are disclosed in Table 3 which shows the percentage of cells in each phase of the cell cycle. Compound **18a** allowed a large increase of cells in the sub-G1 and G2 phases at concentrations of 0.5 μM and 5 μM after 24 h of exposure and it was accompanied by a corresponding reduction of cells in the G1 phase. The marked accumulation of cells in sub-G1 phase indicated the induction of cellular apoptosis as for the reference compounds. Compound **19a** displayed the same cell cycle profile but only at a concentration of 5 μM, while compound **18c** had no effects compared to the controls. These results indicated that the induction of cellular apoptosis by **18a** and **19a** could be by virtue of their antimitotic activities as there was an increase in cell cycle arrest at G2 phase with parallel increase in apoptotic cells at sub-G1 phase.

2.5. Modeling studies

To explain marked difference between cytotoxicity and antitubulin potential of compounds **18a**, **18e**, **19a** and **19e**, docking studies have been performed on the colchicine binding site of tubulin to explore the interaction mechanism and understand the structural features responsible for their activity (Fig. 3). Compounds **18e** and **19e** only showed a single conformation occupying the binding site and engaging in the same hydrogen bonds with Ser 178, Thr 179 and Ala 250. The tricyclic core of **18e** and **19e** was oriented near the residues Ala 250 and Lys 254 and interacted *via* hydrogen bonding with the carbonyl or sulfonyl moieties while the 2-(3-hydroxy-4-methoxyphenyl)ethyl adopted a folded up conformation stabilized by hydrogen bonds formed between the hydroxy and methoxy groups and Ser 178 and Thr 179. Docking studies performed with others compounds, as **18a** and **19a**, revealed that the scoring functions employed were not able to clearly indicate which would be the preferred conformations. These compounds could not be docked successfully in the colchicine binding site and the molecular modeling studies were in good agreement with the biological data. These results suggested that 3-hydroxy-4-methoxyphenyl substitution might be crucial for binding interactions and provided a possible explanation why compounds **18e** and **19e** displayed potent tubulin inhibitory activities with regard to their counterparts **18a** and **19a**.

3. Conclusion

We have designed and synthesized acridinone and dioxophenothiazine analogs of the oxathiazepine **1**, bearing various substituted arylethyl chains. The 3-hydroxy-4-methoxyphenylethyl derivatives **18e** and **19e** were potent in vitro tubulin polymerization inhibitors but with weak cytotoxicities toward the human colon adenocarcinoma HT29 cell line, in the micromolar range. This antitubulin activity could be explained by modeling studies which have shown that the aryethyl chain adopted a folded up conformation stabilized by hydrogen bonds which were not recovered with the others analogs. Determination of the physicochemical properties as solubility, membrane permeability or log *P* is necessary to understand the bad cellular activity compared to the potent in vitro tubulin polymerization inhibition. The 4-methoxyphenylethyl counterparts **18a** and **19a** seemed to be inactive as inhibitors of tubulin polymerization and did not inhibit tubulin assembly at concentrations as high as 10 μM. Nevertheless, compound **18a** displayed very interesting antiproliferative activities over a panel of five tumor cell lines

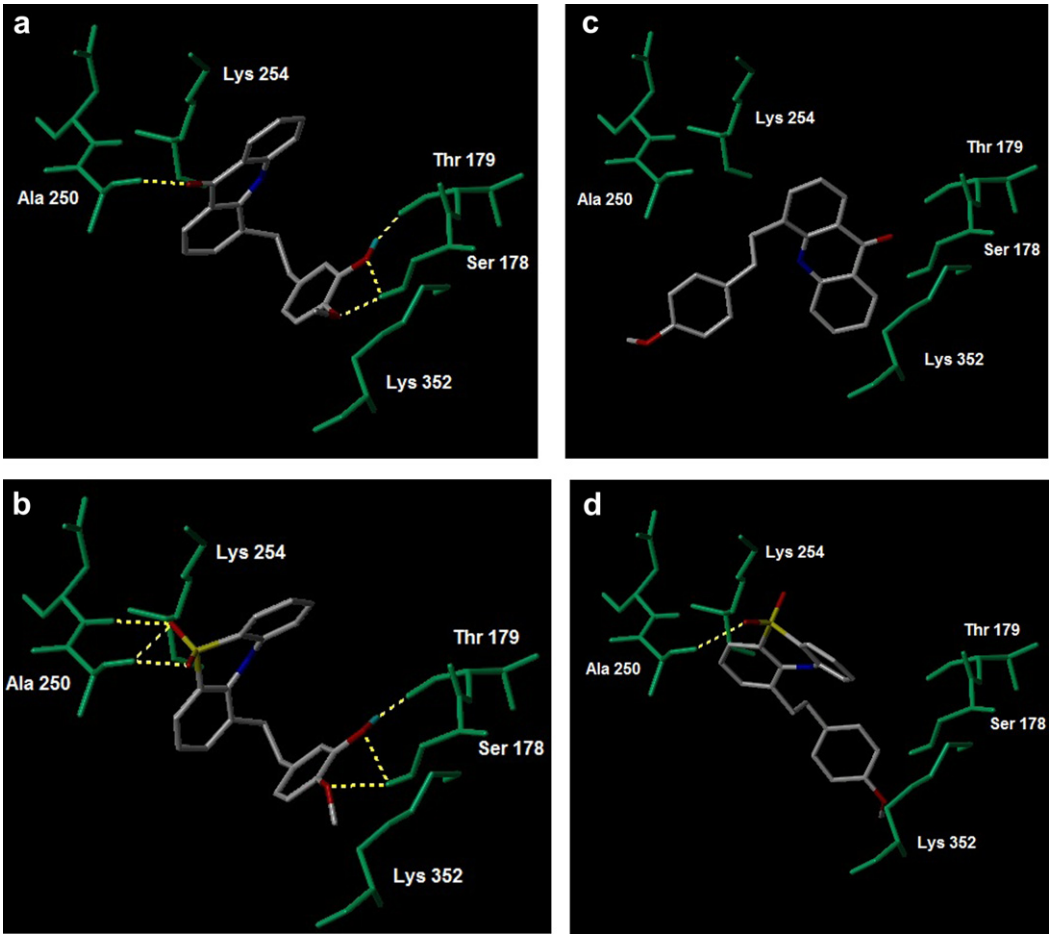


Fig. 3. Docking studies into the colchicine binding site of tubulin of (a) compound **18e**, (b) compound **19e**, (c) compound **18a** and (d) compound **19a**.

in the submicromolar range for all of the histological types tested and flow cytometric studies performed on selected tumor cells showed an accumulation of the cells in the sub-G1 and G2 phases indicating induction of cellular apoptosis. Further experiments are therefore ongoing to better investigate the mechanism of action of such compounds and confirm the improvement of pharmacokinetic properties.

4. Experimental protocols

4.1. Chemistry and chemical methods

General methods: All reagents and solvents were purchased and used without further purification Melting points were determined on a BÜCHI B-540 apparatus and are uncorrected. Mass

Table 3
Cell cycle analysis of selected tumor cell lines exposed to compounds **18a**, **18c** and **19a**.

Phase of cell cycle	Cell type	Cell line	Control cell	Vino[a] 0.5 μM	Vinc[b] 0.5 μM	Doce[c] 0.5 μM	18a		18c	19a	
							0.5 μM	5 μM	5 μM	0.5 μM	5 μM
Sub G1 (%)	HNSCC[d]	CAL33	0.92	24.44	20.5	17.11	9.84	22.73	1.43	N.t.[e]	N.t.
	Colon	HT29	0.81	1.39	5.97	0.86	0.65	0.83	1.58	1.63	0.47
	Leukemia	HL60	6.84	N.t.	N.t.	N.t.	37.16	34.34	8.46	8.22	34.12
G1 (%)	HNSCC	CAL33	68.89	9.96	14.29	4.99	4	8.35	64.33	N.t.	N.t.
	Colon	HT29	78.2	21.42	16.43	7.85	14.55	21.11	74.22	73.56	8.63
	Leukemia	HL60	56.81	N.t.	N.t.	N.t.	25.15	24.4	58.11	57.66	26.83
S (%)	HNSCC	CAL33	16.01	17.62	17.13	15.41	28.46	33.12	16.17	n.t.	n.t.
	Colon	HT29	8.67	3.41	4.65	2.76	2.75	4.6	10.72	8.63	2.46
	Leukemia	HL60	21.94	N.t.	N.t.	N.t.	18.5	26.55	21.55	21.83	24.77
G2 (%)	HNSCC	CAL33	14.21	49.16	49.2	64.1	57.42	35.16	21.55	n.t.	n.t.
	Colon	HT29	12.2	72.85	72.53	88.24	81.81	73.1	13.43	15.95	87.92
	Leukemia	HL60	14.54	N.t.	N.t.	N.t.	19.58	15.2	11.87	12.4	14.77

[a] Vino: vinorelbine.
[b] Vinc: vincristine.
[c] Doce: docetaxel.
[d] Head and neck squamous cell carcinoma.
[e] Not tested.

spectra were performed on a Finnigan MAT SSQ 710 Advantage spectrometer and were recorded in the ESþ mode. ^{1}H NMR (300 MHz) and ^{13}C NMR (75 MHz) spectra were recorded on a Bruker AC 300 P (chemical shifts δ in parts per million, the following abbreviations are used: singlet (s), broad singlet (br s), doublet (d), doubled doublet (dd), triplet (t)). Elemental analyses were performed by C.N.R.S-Vernaison, and were in agreement with the calculated values within ±0.2%.

*4.1.1. 2-[(2-Carboxyphenyl)amino]benzoic acid (**4**)*

A mixture of 2-chlorobenzoic acid (10.0 g, 64 mmol), 2-aminobenzoic acid (17.5 g, 130 mmol), potassium carbonate (8.8 g, 64 mmol) and copper powder (30 mg) were refluxed for 2 h in DMF (100 mL). After cooling, 6 N HCl aqueous solution (200 mL) was added and the mixture was stirred for 12 h and filtered. The resulting solid was washed with water and methanol, then dried to afford 13.5 g (82%) of a gray solid: mp 317–319 °C; ^{1}H NMR (DMSO) δ: 6.94 (m, 2H), 7.40–7.55 (m, 4H), 7.90 (m, 2H), 10.80 (br s, 1H), 13.00 (br s, 2H).

*4.1.2. 10H-Acridin-9-one-4-carboxylic acid (**5**)*

A solution of diacid **4** (6.0 g, 23 mmol) in concentrated H_2SO_4 (50 mL) was stirred at 100 °C for 4 h. The reaction mixture was cooled, poured into ice-cold water and the resulting precipitate was filtered and washed with water. The solid was dissolved in 2 N aqueous NaOH solution (100 mL) and the solution was filtered. The filtrate was then acidified with glacial acetic acid until precipitation. The precipitated product was collected and washed with EtOH to give 3.95 g (72%) of yellow solid; mp 338–340 °C; ^{1}H NMR (DMSO) δ: 7.27–7.37 (m, 2H), 7.76 (m, 2H), 8.23 (d, 1H, ^3J = 8.0 Hz), 8.44 (dd, 1H, ^3J = 7.6 Hz, ^4J = 1.75 Hz), 8.50 (dd, 1H, ^3J = 8.0 Hz, ^4J = 1.75 Hz), 11.1 (br s, 1H), 12.3 (br s, 1H).

*4.1.3. 1,2-Dihydropyrrolo[3,2,1-kl]-phenothiazin-1,2-dione (**7**)*

To a boiling solution of oxalyl chloride (5.1 g, 40 mmol) in THF (20 mL) was added dropwise over a period of 1 h a solution of phenothiazine (4.0 g, 20 mmol) in THF (30 mL) and the reaction mixture was boiled under reflux an additional 4 h and then concentrated under reduced pressure. The resulting green solid was dissolved in CS_2 (100 mL) and $AlCl_3$ (5.3 g, 40 mmol) was added portion wise over a period of 30 min. When addition was complete, the reaction mixture was boiled under reflux an additional 12 h. After cooling and decantation of the CS_2 solution, 0.6 N HCl aqueous solution was added at 0 °C to the residue and the mixture was stirred until precipitation. The solid was filtered and washed respectively with water, EtOH and Et_2O to afford 4.8 g (95%) of purple powder; mp 199–200 °C; ^{1}H NMR (DMSO) δ: 6.95 (dd, 1H, ^3J = 8.1 Hz, ^4J = 1.5 Hz), 7.0–7.20 (m, 4H), 7.33 (dd, 1H, ^3J = 7.3 Hz, ^4J = 1.3 Hz), 8.65 (dd, 1H, ^3J = 8.4 Hz, ^4J = 1.5 Hz).

*4.1.4. 10H-Phenothiazine-1-carboxylic acid 5-oxide (**8**)*

A 30% NaOH aqueous solution (30 mL) was added at 30 °C to a suspension of isatin **7** (2.2 g, 8.7 mmol) and the mixture was stirred at room temperature for 1 h. A 35% H_2O_2 solution (5 mL, 34 mmol) in water (50 mL) was added and the reaction mixture was stirred for 2 h at room temperature before acidification with concentrated HCl until precipitation. The solid was collected by filtration, washed with water, EtOH and dried to provide 2.1 g (93%) of pale brown solid; mp 159–162 °C; ^{1}H NMR (DMSO) δ: 7.30 (m, 2H), 7.60 (dd, 1H, ^3J = 8.2 Hz, ^4J = 1.4 Hz), 7.67 (td, 1H, ^3J = 8.2 Hz, ^4J = 1.4 Hz), 8.00 (dd, 1H, ^3J = 7.85 Hz, ^4J = 1.4 Hz), 8.25 (dd, 1H, ^3J = 7.7 Hz, ^4J = 1.7 Hz), 8.32 (dd, 1H, ^3J = 7.8 Hz, ^4J = 1.7 Hz), 11.7 (br s, 1H); Anal. Calcd for $C_{13}H_9NO_3S$: C, 60.22; H, 3.50; N, 5.40. Found: C, 60.35; H, 3.41; N, 5.47.

*4.1.5. 10H-Phenothiazine-1-carboxylic acid 5,5-dioxide (**9**)*

To a suspension of sulfoxide **8** (2.0 g, 7.7 mmol) in anhydrous acetic acid (40 mL) was added a 35% H_2O_2 solution (2 mL, 13.6 mmol) and the mixture was stirred at 70 °C for 3 h. After cooling, the precipitate was filtered, washed with water, EtOH and dried to afford 1.6 g (67%) of pale pink solid; ^{1}H NMR (DMSO) δ: 7.35 (m, 2H), 7.56 (dd, 1H, ^3J = 8.4 Hz, ^4J = 1.4 Hz), 7.70 (td, 1H, ^3J = 8.4 Hz, ^4J = 1.4 Hz), 7.98 (dd, 1H, ^3J = 7.95 Hz, ^4J = 1.4 Hz), 8.25 (dd, 1H, ^3J = 7.85 Hz, ^4J = 1.4 Hz), 8.35 (dd, 1H, ^3J = 7.8 Hz, ^4J = 1.4 Hz), 11.55 (br s, 1H); Anal. Calcd for $C_{13}H_9NO_4S$: C, 56.72; H, 3.30; N, 5.09. Found: C, 56.83; H, 3.19; N, 5.14.

*4.1.6. General procedure for synthesis of acyl chlorides **10** and **11***

$SOCl_2$ (2.35 mL, 30 mmol) was added dropwise to a suspension of carboxylic acid **5** or **9** (6 mmol) in toluene (60 mL) and the mixture was stirred at 80 °C for 12 h. After cooling, the solution was filtered and the filtrate evaporated under reduced pressure. The residue was taken up into Et_2O and the resulting precipitate collected by filtration to give the corresponding acyl chlorides **10** and **11**.

*4.1.6.1. 10H-Acridin-9-one-4-carbonyl chloride (**10**).* Yellow solid; 80% yield; mp 224–226 °C; ^{1}H NMR (DMSO) δ: 7.30–7.37 (m, 2H), 7.75–7.79 (m, 2H), 8.23 (dd, 1H, ^3J = 8.2 Hz, ^4J = 1.75 Hz), 8.43 (dd, 1H, ^3J = 7.6 Hz, ^4J = 1.75 Hz), 8.52 (dd, 1H, ^3J = 7.95 Hz, ^4J = 1.75 Hz), 11.95 (br s, 1H); Anal. Calcd for $C_{14}H_8ClNO_2$: C, 65.26; H, 3.13; N, 5.44. Found: C, 65.38; H, 3.04; N, 5.50.

*4.1.6.2. 10H-Phenothiazine-1-carbonyl chloride 5,5-dioxide (**11**).* Red solid; 91% yield; mp 233–236 °C; ^{1}H NMR (DMSO) δ: 7.25 (dd, 1H, ^3J = 8.2 Hz, ^4J = 1.4 Hz), 7.37 (m, 2H), 7.65 (td, 1H, ^3J = 8.2 Hz, ^4J = 1.65 Hz), 8.10 (dd, 1H, ^3J = 8.15 Hz, ^4J = 1.4 Hz), 8.44 (dd, 1H, ^3J = 7.9 Hz, ^4J = 1.4 Hz), 8.62 (dd, 1H, ^3J = 8.0 Hz, ^4J = 1.65 Hz), 10.60 (br s, 1H); Anal. Calcd for $C_{13}H_8ClNO_3S$: C, 53.16; H, 2.75; N, 4.77. Found: C, 53.25; H, 2.68; N, 4.84.

*4.1.7. General procedure for synthesis of carboxaldehydes **12** and **13***

A solution of acyl chloride **10** or **11** (5 mmol) and $Pd(PPh_3)_4$ (57.7 mg, 0.05 mmol) in deoxygenated benzene (30 mL) was stirred under argon in a Schlenck tube. Bu_3SnH (1.06 mL, 5.4 mmol) was then added in three portions at intervals of 10 min. After completion of the reaction (monitored by TLC), the solvent was evaporated, and petroleum ether was added to the residue until precipitation. The precipitate was collected by filtration and washed with Et_2O to afford the corresponding carboxaldehydes **12** and **13**.

*4.1.7.1. 10H-Acridin-9-one-4-carboxaldehyde (**12**).* Yellow solid; 98% yield; mp 215–217 °C; ^{1}H NMR (DMSO) δ: 7.35 (m, 1H), 7.50 (t, 1H, ^3J = 7.7 Hz), 7.76–7.82 (m, 2H), 7.90 (dd, 1H, ^3J = 7.8 Hz, ^4J = 1.4 Hz), 8.39 (dd, 1H, ^3J = 7.7 Hz, ^4J = 1.75 Hz), 8.58 (dd, 1H, ^3J = 8.5 Hz, ^4J = 1.75 Hz), 10.2 (s, 1H), 11.8 (br s, 1H); Anal. Calcd for $C_{14}H_9NO_2$: C, 75.33; H, 4.06; N, 6.27. Found: C, 75.41; H, 3.98; N, 6.32.

*4.1.7.2. 10H-phenothiazine-1-carboxaldehyde 5,5-dioxide (**13**).* Orange solid; 97% yield; mp 198–200 °C; ^{1}H NMR (DMSO) δ: 7.25–7.40 (m, 3H), 7.63 (td, 1H, ^3J = 7.9 Hz, ^4J = 1.6 Hz), 7.99 (dd, 1H, ^3J = 7.8 Hz, ^4J = 1.4 Hz), 8.11 (dd, 1H, ^3J = 8.4 Hz, ^4J = 1.45 Hz), 8.37 (dd, 1H, ^3J = 8.4 Hz, ^4J = 1.45 Hz), 10.10 (s, 1H), 11.45 (br s, 1H); Anal. Calcd for $C_{13}H_9NO_3S$: C, 60.22; H, 3.50; N, 5.40. Found: C, 60.38; H, 3.39; N, 5.33.

*4.1.8. General procedure for synthesis of phosphonium salts **15a–e***

A solution of commercially available or described benzyl chlorides **14a–e** (85 mmol) and PPh_3 (26.2 g, 100 mmol) in toluene (110 mL) was stirred at reflux for 24 h. After cooling, the precipitate

was collected by filtration, washed with Et_2O and dried to give the corresponding phosphonium salts **15a**–**e**.

*4.1.8.1. 4-Methoxybenzyl-triphenylphosphonium chloride (**15a**).* White solid; 79% yield; mp 243–245 °C; ^{1}H NMR (DMSO) δ: 3.69 (s, 3H), 5.11 (m, 2H), 6.80 (m, 2H), 6.90 (m, 2H), 7.60 (m, 6H), 7.75 (m, 6H), 7.95 (m, 3H).

*4.1.8.2. (3,4-Dimethoxybenzyl)triphenylphosphonium chloride (**15b**).* White solid; 70% yield; mp 224–226 °C; ^{1}H NMR (DMSO) δ: 3.55 (s, 3H), 3.81 (s, 3H), 5.08 (s, 2H), 6.62–6.70 (m, 3H), 7.66–7.73 (m, 15H).

*4.1.8.3. (3,4,5-Trimethoxybenzyl)triphenylphosphonium chloride (**15c**).* White solid; 70% yield; mp 222–223 °C; ^{1}H NMR (DMSO) δ: 3.37 (s, 9H), 4.97 (s, 2H), 6.18 (m, 2H), 7.68–7.73 (m, 15H).

*4.1.8.4. (3,4-Dibenzyloxybenzyl)triphenylphosphonium chloride (**15d**).* White solid; 80% yield; mp 238–240 °C; ^{1}H NMR (DMSO) δ: 4.54 (s, 2H), 4.56 (s, 2H), 5.01 (s, 2H), 6.50 (d, 1H, ^4J = 2.1 Hz), 6.83 (d, 1H, ^3J = 8.2 Hz), 7.25 (dd, 1H, ^3J = 8.2 Hz, ^4J = 2.1 Hz), 7.32–7.43 (m, 10H), 7.75–7.80 (m, 15H).

*4.1.8.5. (3-Benzyloxy-4-methoxybenzyl)triphenylphosphonium chloride (**15e**).* White solid; 74% yield; mp 237–239 °C; ^{1}H NMR (DMSO) δ: 3.71 (s, 3H), 4.56 (s, 2H), 5.04 (s, 2H), 6.50 (d, 1H, ^4J = 2.05 Hz), 6.60 (m, 2H), 6.85 (d, 1H, ^3J = 8.2 Hz), 7.25 (dd, 1H, ^3J = 8.2 Hz, ^4J = 2.05 Hz), 7.30–7.40 (m, 3H), 7.75–7.80 (m, 12 H), 7.90 (m, 3H).

*4.1.9. General procedure for synthesis of target compounds **18a**–**e** and **19a**–**e***

A mixture of carboxaldehyde **12** or **13** (2 mmol), phosphonium chloride **15a**–**e** (2.5 mmol) and DBU (0.65 mL, 4.35 mmol) in acetonitrile was stirred at reflux for 3 h. The reaction mixture was hydrolyzed and extracted twice with ethyl acetate. The combined organic extracts were dried over $MgSO_4$, evaporated under reduced pressure and the resulting residue was purified *via* flash column chromatography with CH_2Cl_2 as eluent to afford a *Z*/*E*-isomeric mixture of the corresponding olefins **16a**–**e** and **17a**–**e**. A solution of the previously prepared olefins (0.55 mmol) in EtOH (100 mL) was stirred in the presence of 10% palladium on charcoal under an atmosphere of hydrogen at room temperature until reaction was completed as judged by TLC (12–24 h). The solution was filtered through Celite, the filtrate was evaporated under reduced pressure and the resulting residue was recrystallized from EtOH to provide the final compounds **18a**–**e** and **19a**–**e**.

*4.1.9.1. 10H-4-[2-(4-Methoxyphenyl)ethyl]acridin-9-one (**18a**).* Yellow solid; 56% yield; mp 251–253 °C; ^{1}H NMR ($CDCl_3$) δ: 3.03 (t, 2H, ^3J = 7.3 Hz), 3.17 (t, 2H, ^3J = 7.3 Hz), 3.70 (s, 3H), 6.81 (d, 2H, ^3J = 8.2 Hz), 7.08 (m, 3H), 7.23 (td, 1H, ^3J = 7.9 Hz, ^4J = 2.1 Hz), 7.50–7.63 (m, 3H), 8.42 (m, 2H); ^{13}C NMR ($CDCl_3$) δ: 33.3, 35.1, 55.2, 114.2, 116.7, 120.9, 121.3, 121.6, 125.6, 127.0, 127.2, 129.4, 133.0, 133.2, 133.5, 138.7, 140.1, 158.3, 178.6; Anal. Calcd for $C_{22}H_{19}NO_2$: C, 80.22; H, 5.81; N, 4.25. Found: C, 80.36; H, 5.74; N, 4.17.

*4.1.9.2. 10H-4-[2-(3,4-Dimethoxyphenyl)ethyl]acridin-9-one (**18b**).* Off-white solid; 60% yield; mp 206–208 °C; ^{1}H NMR ($CDCl_3$) δ: 3.05 (t, 2H, ^3J = 7.7 Hz), 3.21 (t, 2H, ^3J = 7.7 Hz), 3.72 (s, 3H), 3.77 (s, 3H), 6.70–6.90 (m, 3H), 7.10 (d, 1H, ^3J = 7.9 Hz), 7.25 (td, 1H, ^3J = 7.8 Hz, ^4J = 1.9 Hz), 7.52–7.68 (m, 3H), 8.44 (m, 2H); ^{13}C NMR ($CDCl_3$) δ: 32.8, 36.5, 55.8, 55.9, 105.3, 116.7, 120.8, 121.3, 121.4, 121.7, 125.6, 127.0, 127.3, 133.4, 136.5, 136.7, 138.9, 140.2, 153.3, 178.4; Anal. Calcd for $C_{23}H_{21}NO_3$: C, 76.86; H, 5.89; N, 3.90. Found: C, 76.98; H, 5.80; N, 4.01.

*4.1.9.3. 10H-4-[2-(3,4,5-Trimethoxyphenyl)ethyl]acridin-9-one (**18c**).* White solid; 53% yield; mp 198–201 °C; ^{1}H NMR (DMSO) δ: 2.98 (t, 2H, ^3J = 6.9 Hz), 3.31 (t, 2H, ^3J = 7.3 Hz), 3.52 (s, 3H), 3.72 (s, 6H), 6.53 (s, 2H), 7.23 (m, 2H), 7.57 (d, 1H, ^3J = 8.1 Hz), 7.69 (t, 1H, ^3J = 7.9 Hz), 7.88 (d, 1H, ^3J = 8.1 Hz), 8.15 (dd, 1H, ^3J = 8.1 Hz, ^4J = 1.3 Hz), 8.20 (dd, 1H, ^3J = 8.0 Hz, ^4J = 1.4 Hz), 10.50 (br s, 1H); ^{13}C NMR (DMSO) δ: 31.8, 35.8, 56.2, 60.3, 106.4, 118.6, 120.6, 121.2, 121.6, 124.6, 126.1, 129.3, 133.4, 133.6, 136.1, 137.2, 139.4, 141.5, 153.0, 177.5; Anal. Calcd for $C_{24}H_{23}NO_4$: C, 74.02; H, 5.95; N, 3.60. Found: C, 74.18; H, 5.85; N, 3.49.

*4.1.9.4. 10H-4-[2-(3,4-Dihydroxyphenyl)ethyl]acridin-9-one (**18d**).* Off-white solid; 60% yield; mp 206–208 °C; ^{1}H NMR (DMSO) δ: 2.92 (t, 2H, ^3J = 7.3 Hz), 3.27 (t, 2H, ^3J = 7.3 Hz), 6.62 (d, 1H, ^3J = 8.2 Hz), 6.80 (m, 2H), 7.19 (t, 1H, ^3J = 7.5 Hz), 7.25 (t, 1H, ^3J = 7.5 Hz), 7.57 (dd, 1H, ^3J = 8.1 Hz, ^4J = 2.1 Hz), 7.73 (td, 1H, ^3J = 7.5 Hz, ^4J = 1.7 Hz), 7.90 (d, 1H, ^3J = 8.1 Hz), 8.14 (dd, 1H, ^3J = 8.1 Hz, ^4J = 1.7 Hz), 8.20 (dd, 1H, ^3J = 8.1 Hz, ^4J = 1.7 Hz), 8.85 (s, 2H), 10.55 (br s, 1H); Anal. Calcd for $C_{21}H_{17}NO_3$: C, 76.12; H, 5.17; N, 4.23. Found: C, 76.05; H, 5.10; N, 4.32.

*4.1.9.5. 10H-4-[2-(3-Hydroxy-4-methoxyphenyl)ethyl]acridin-9-one (**18e**).* Off-white solid; 48% yield; mp 212–214 °C; ^{1}H NMR (DMSO) δ: 2.90 (t, 2H, ^3J = 7.1 Hz), 3.25 (t, 2H, ^3J = 7.1 Hz), 3.72 (s, 3H), 6.66 (dd, 1H, ^3J = 8.2 Hz, ^4J = 1.9 Hz), 6.75 (d, 1H, ^3J = 1.9 Hz), 6.82 (d, 1H,, ^3J = 8.2 Hz), 7.18 (t, 1H, ^3J = 7.9 Hz), 7.26 (t, 1H, ^3J = 7.9 Hz), 7.54 (dd, 1H, ^3J = 7.3 Hz, ^4J = 1.5 Hz), 7.71 (td, 1H, ^3J = 7.25 Hz, ^4J = 1.5 Hz), 7.92 (dd, 1H, ^3J = 8.3 Hz, ^4J = 1.55 Hz), 8.13 (dd, 1H, ^3J = 7.9 Hz, ^4J = 1.55 Hz), 8.21 (dd, 1H, ^3J = 8.15 Hz, ^4J = 1.55 Hz), 8.80 (s, 1H), 10.60 (br s, 1H); ^{13}C NMR (DMSO) δ: 32.0, 34.4, 56.2, 112.6, 116.5, 118.6, 119.5, 120.6, 121.2, 121.3, 121.7, 124.5, 126.1, 129.3, 133.3, 133.6, 134.3, 139.4, 141.5, 146.4, 146.7, 177.5; Anal. Calcd for $C_{22}H_{19}NO_3$: C, 76.50; H, 5.54; N, 4.06. Found: C, 76.42; H, 5.63; N, 4.17.

*4.1.9.6. 10H-1-[2-(4-Methoxyphenyl)ethyl]phenothiazine 5,5-dioxide (**19a**).* Gray solid; 51% yield; mp 188–190 °C; ^{1}H NMR ($CDCl_3$) δ: 2.91 (m, 4H), 3.70 (s, 3H), 6.72 (dd, 1H, ^3J = 8.4 Hz, ^4J = 1.6 Hz), 6.78 (d, 2H, ^3J = 8.1 Hz), 6.96 (s, 1H), 7.03 (d, 2H, ^3J = 8.1 Hz), 7.17 (m, 2H), 7.37 (m, 2H), 7.98 (m, 2H); ^{13}C NMR ($CDCl_3$) δ: 32.7, 34.9, 55.2, 114.1, 117.0, 120.7, 121.4, 121.6, 121.8, 122.8, 127.7, 129.3, 132.4, 132.5, 133.3, 135.7, 137.5, 158.2; Anal. Calcd for $C_{21}H_{19}NO_3S$: C, 69.02; H, 5.24; N, 3.83. Found: C, 69.13; H, 5.41; N, 3.91.

*4.1.9.7. 10H-1-[2-(3,4-Dimethoxyphenyl)ethyl]phenothiazine 5,5-dioxide (**19b**).* White solid; 59% yield; mp 186–188 °C; ^{1}H NMR ($CDCl_3$) δ: 2.92 (m, 4H), 3.77 (s, 3H), 3.80 (s, 3H), 6.59–6.73 (m, 4H), 6.95 (s, 1H), 7.17 (m, 2H), 7.37 (m, 2H), 7.99 (m, 2H); ^{13}C NMR ($CDCl_3$) δ: 32.4, 35.5, 55.7, 55.9, 111.1, 111.5, 116.9, 120.2, 120.4, 121.2, 121.3, 121.5, 121.7, 122.5, 127.9, 132.4, 132.9, 133.4, 135.8, 137.5, 147.5, 149.0; Anal. Calcd for $C_{22}H_{21}NO_4S$: C, 66.82; H, 5.35; N, 3.54. Found: C, 69.93; H, 5.23; N, 3.62.

*4.1.9.8. 10H-1-[2-(3,4,5-Trimethoxyphenyl)ethyl]phenothiazine 5,5-dioxide (**19c**).* White solid; 59% yield; mp 218–220 °C; ^{1}H NMR ($CDCl_3$) δ: 2.94 (m, 4H), 3.70 (s, 3H), 3.77 (6H), 6.32 (s, 2H), 6.71 (d, 1H, ^3J = 8.2 Hz), 6.99 (s, 1H), 7.18 (m, 2H), 7.39 (m, 2H), 7.99 (m, 2H); ^{13}C NMR ($CDCl_3$) δ: 32.4, 36.3, 56.1, 60.7, 77.2, 105.2, 116.7, 120.7, 121.4, 121.5, 121.9, 122.7, 127.7, 132.7, 133.3, 135.8, 136.1, 136.5, 137.4, 153.3; Anal. Calcd for $C_{23}H_{23}NO_5S$: C, 64.92; H, 5.45; N, 3.29. Found: C, 70.07; H, 5.35; N, 3.34.

*4.1.9.9. 10H-1-[2-(3,4-Dihydroxyphenyl)ethyl]phenothiazine 5,5-dioxide (**19d**).* Off-white solid; 57% yield; mp 246–248 °C; ^{1}H NMR (DMSO) δ: 2.78 (t, 2H, ^3J = 7.3 Hz), 3.06 (t, 2H, ^3J = 7.3 Hz), 6.47 (d, 1H, ^3J = 7.8 Hz), 6.65 (m, 2H), 7.06 (t, 1H, ^3J = 7.7 Hz), 7.12 (t, 1H,

$^3J = 7.7$ Hz), 7.31 (d, 1H, $^3J = 7.2$ Hz), 7.44 (t, 1H, $^3J = 7.8$ Hz), 7.54 (d, 1H, $^3J = 8.3$ Hz), 7.76 (d, 1H, $^3J = 7.8$ Hz), 7.84 (d, 1H, $^3J = 7.8$ Hz), 8.06 (br s, 2H), 9.18 (s, 1H); ^{13}C NMR (DMSO) δ: 32.1, 34.4, 115.8, 116.5, 118.7, 119.6, 120.7, 121.1, 121.6, 122.2, 122.5, 129.4, 132.2, 133.4, 133.5, 136.5, 138.9, 143.8, 145.4; Anal. Calcd for $C_{20}H_{17}NO_4S$: C, 65.38; H, 4.66; N, 3.81. Found: C, 65.47; H, 4.57; N, 3.91.

*4.1.9.10. 10H-1-[2-(3-Hydroxy-4-methoxyphenyl)ethyl]phenothiazine 5,5-dioxide (**19e**).* White solid; 51% yield; mp 199–201 °C; ^{1}H NMR ($CDCl_3$) δ: 2.91 (m, 4H), 3.74 (s, 3H), 5.64 (br s, 1H), 6.43 (dd, 1H, $^3J = 8.2$ Hz, $^4J = 2.1$ Hz), 6.62 (d, 1H, $^3J = 8.2$ Hz), 6.77 (d, 1H, $^3J = 8.3$ Hz), 6.84 (d, 1H, $^4J = 2.1$ Hz), 6.94 (s, 1H), 7.17 (m, 2H), 7.38 (m, 2H), 7.98 (m, 2H); ^{13}C NMR ($CDCl_3$) δ: 33.0, 35.5, 55.9, 110.8, 114.0, 116.8, 120.1, 121.2, 121.6, 121.7, 121.8, 122.0, 123.1, 127.4, 132.5, 133.4, 133.6, 136.0, 137.6, 145.3, 146.0; Anal. Calcd for $C_{21}H_{19}NO_4S$: C, 66.12; H, 5.02; N, 3.67. Found: C, 66.23; H, 4.94; N, 3.76.

4.2. Biological assay methods

4.2.1. Cell growth inhibitory activity

Cytotoxicity of selected drugs was tested using the MTT (thiazolyl blue tetrazolium bromide) assay based on the cellular uptake of MTT (Sigma Aldrich) and its subsequent reduction in the mitochondria of living cells to dark blue MTT formazan crystals (Mosmann, 1983). Cells were seeded on 96-well plates ((1.6–1.8) $\times$ 10^4 cells/well) in the corresponding medium supplemented with 10% FBS. Seventy-two hours later, cells were deprived of FBS. The next day, they were treated with various concentrations of each tested compound. Following the 72 h exposure to the cytotoxic drugs, cell survival was determined by incubation with MTT (4 mg/mL) for 4 h (37 °C, 5% CO_2). The cells were then lyzed in SDS/HCl and plates left in the incubator to dissolve the purple formazan crystals. The color intensity reflecting cell viability was read with a spectrophotometer Elx 800 (Biotek Instruments Inc.) at 570 nm.

4.2.2. In vitro microtubule polymerization assay

In-vitro tubulin polymerization assays were performed on a fluorescence spectrometer (Varioskan flash, Thermo Scientific, Courtaboeuf, France), equipped with a half area 96 wells microplate (Corning Costar). Excitation and emission wavelengths were 360 and 420 nm, respectively. All reagents were purchased from Cytoskeleton (Denver, CO, USA). For each inhibitor investigated, a series of ten concentrations (from 0.1 to 10 μM) was prepared in 100 μg purified bovine tubulin and buffer containing 20% glycerol, 1 mM GTP, 80 mM PIPES (pH 6.9), 2.0 mM MgCl2, 0.5 mM EGTA and 5 nM fluorescent reporter and kept at 0 °C. Then, the medium was incubated in the plate reader, at 37 °C, for 1 h. A mixture without inhibitor exhibited an increase in fluorescent intensity as a function of incubation time corresponding to the inclusion of the probe inside the tubulin polymer. On the contrary, compounds which strongly inhibited microtubule formation were identified by a fluorescent signal remaining negligible along the experiment. The effect of inhibitor concentration on tubulin polymerization rate was studied and the IC_{50} value was defined as the concentration of product which inhibits the rate of polymerization by a factor 50. It was determined from three independent assays using GraphPad Prism software (version 4).

4.2.3. Topoisomerase inhibition assay

The assays were performed as previously described [31,32]. Supercoiled puC19 DNA (0.5 μg) was incubated with 4 units of human topoisomerase I or II (TopoGen Inc.) at 37 °C for 30 min in relaxation buffer (50 mM Tris pH 7.8, 50 mM KCl, 10 mM MgCl2, 1 mM dithiothreitol, 1 mM EDTA and ATP) in the presence of varying concentrations of the drug under study. Reactions were terminated by adding SDS to 0.25% and proteinase K to 250 μg/mL. DNA samples were then added to the electrophoresis dye mixture (3 mL) and electrophoresed in a 1% agarose gel containing ethidium bromide (1 mg/mL), at room temperature for 2 h at 120 V. Gels were washed and photographed under UV light.

4.2.4. Cell cycle analysis

Cells (2.5–4 $\times$ 10^5) were seeded into a 6-well plate for 24 h incubation and then treated with compounds at different concentrations for 24 h. Supernatants and cells were collected and fixed with cold 70% ethanol for 24 h. The cells were then stained with propidium iodide (50 μg/mL, Interchim, France) and RNase solution (100 μg/mL, Sigma Aldrich) in PBS for 30 min in the dark at room temperature. Cell cycle distribution was determined on a Becton–Dickinson FACScan flow cytometer using the Cell QuestPro software and analyzed by WinMDI software.

4.3. Molecular modeling

All the calculations were realized under the Sybyl 6.9.2. molecular modeling package [33]. The tubulin structure was downloaded from the PDB data bank (http://www.rcsb.org/-PDB code: 1SA0) [34]. The studied compounds were built from the internal fragments library of Sybyl then their geometry was optimized by the Powell method available in the Maximin2 procedure to a gradient of 0.001 kcal/mol Å using the Tripos force field [35]. In order to implicitly represent a biological middle, the dielectric constant was set to 4. Atomic charges were attributed following the Gasteiger–Hückel method. They were subsequently docked into the enzyme with GOLD 5.0.1 [36]. The best of the 30 conformations generated was determined by an in house consensus scoring function base on Goldscore [36] and X-Score [37] and visual inspection of the consistency of the chosen solution.

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.ejmech.2012.10.051.

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