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Indolizine-phenothiazine hybrids as the first dual inhibitors of tubulin polymerization and farnesyltransferase with synergistic antitumor activity

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ABSTRACT

In the incessant search for innovative cancer control strategies, this study was devoted to the design, synthesis and pharmacological evaluation of dual inhibitors of farnesyltransferase and tubulin polymerization (FTI/MTIs). A series of indolizine-phenothiazine hybrids **16** (amides) and **17** (ketones) has been obtained in a 4-step procedure. The combination of the two heterocycles provided potent tubulin polymerization inhibitors with similar efficiency as the reference phenstatin and (-)-desoxypodophyllotoxin. Ketones **17** were also able to inhibit human farnesyltransferase (FTase) *in vitro*. Interestingly, three molecules **17c**, **17d** and **17f** were very effective against both considered biological targets. Next, nine indolizine-phenothiazine hybrids **16c**, **16f**, **17a-f** and **22b** were evaluated for their cell growth inhibition potential on the NCI-60 cancer cell lines panel. Ketones **17a-f** were the most active and displayed promising cellular activities. Not only they arrested the cell growth of almost all tested cancer cells, but they displayed cytotoxicity potential with GI_{50} values in the low nanomolar range. The most sensitive cell lines upon treatment with indolizine-phenothiazine hybrids were NCI-H522 (lung cancer), COLO-205 and HT29 (colon cancer), SF-539 (human glioblastoma), OVCAR-3 (ovarian cancer), A498 (renal cancer) and especially MDA-MB-435 (melanoma). Demonstrating the preclinical effectiveness of these dual inhibitors can be crucial. A single dual molecule could induce a synergy of antitumor activity, while increasing the effectiveness and reducing the toxicity of the classical combo treatments currently used in chemotherapy.

1. Introduction

Indolizine **1** (Fig. 1), formerly named pyrroline, pyrindole, 8-pyrrolopyridine and also pyrrolo[1,2-*a*]pyridine, is an aromatic heterocycle consisting of a pyridine ring condensed with a pyrrole cycle. Indolizines were discovered by Angeli in 1890 and first synthesized by Scholtz in 1912 [1]. So far, the aromatic form has not been found in the structure of natural products, while the hydrogenated form of indolizine seems to be common, many such derivatives being isolated from plants, microbes, marine organisms, insects or fungi [2]. For example, (-)-swainsonine (compound **2**, Fig. 1) is a natural compound present in some plants and fungi and exploited for the antitumor potential [3]. This compound has shown promising potential for the treatment of glioma and gastric carcinoma [4,5]. (-)-Swainsonine also enhances the *in vivo* efficacy of chemotherapeutic agents such as cisplatin and acts against tumors by stimulating macrophages, protecting the hematopoietic system of the toxicity of chemotherapeutic agents [6,7]. (+)-Castanospermine (compound **3**, Fig. 1) is extracted from *Castanospermum austral* seeds and inhibits the multiplication of human immunodeficiency virus (HIV) [8]. (-)-Ipalbidine (compound **4**, Fig. 1) is another natural compound of interest, being an analgesic preventing addiction, as well as a leukocyte respiratory burst inhibitor [9]. (R)-Antofine **5** is also a natural indolizinic alkaloid with antitumor potential [9–11]. (R)-Tylophorine **6** extracted from Tylophora exhibits potential anticancer and anti-inflammatory activity [12]. Camptothecin **7** (Fig. 1), an important representative of alkaloids inhibiting topoisomerase I, contains the partially hydrogenated indolizine nucleus in the structure [13]. (20S)-Camptothecin is extracted from the stem and bark of the decorative tree *Camptotheca acuminata*, used in Chinese traditional medicine for the treatment of cancer [14]. Moreover, many

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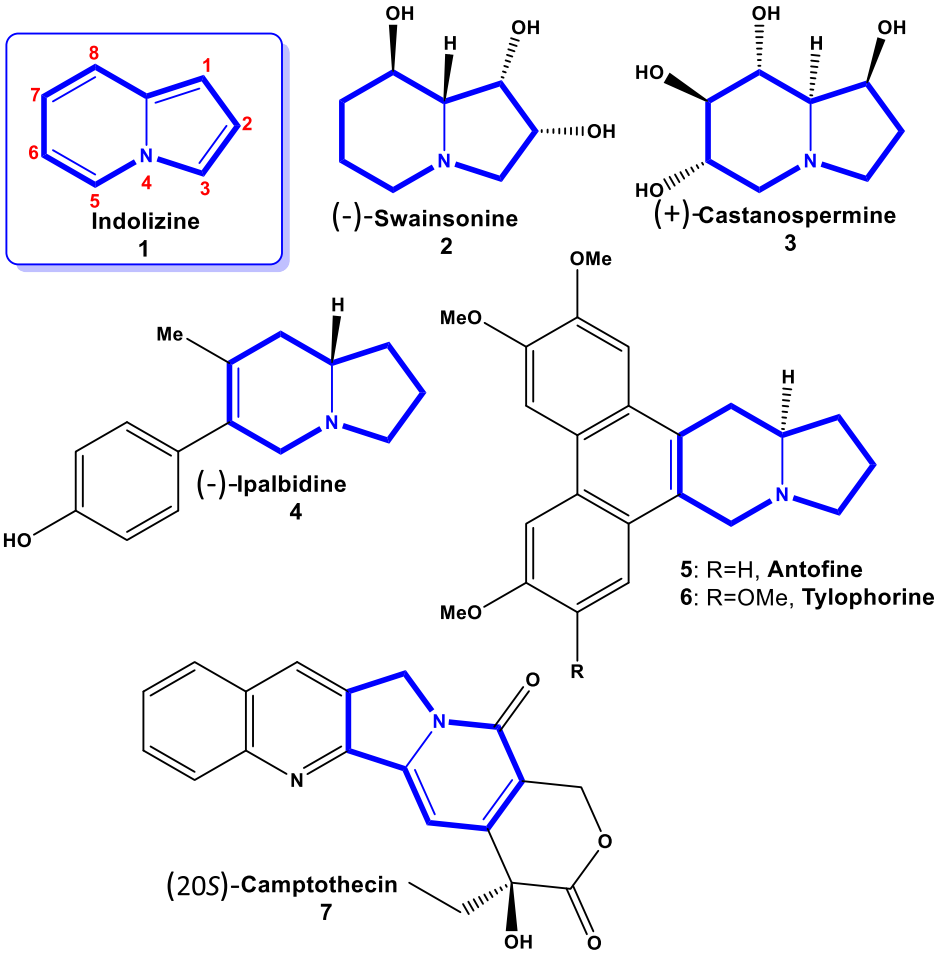


Fig. 1. Structure of indolizine **1** and of natural products containing (partially)hydrogenated forms of indolizine **2**–**7**.

analogues of this alkaloid have been synthesized in the laboratory and are used in chemotherapy [15].

Due to the pronounced similarity both structurally and chemically with the indole nucleus, the biological potential of indolizine was envisaged. Analogues of biologically active products in which indolizine took the place of the indole unit were rapidly synthesized. Thus, Carbon and Brehm [16] proposed 1-indolizinealanine as a possible antimetabolite of tryptophan. The literature highlights a variety of indolizine derivatives with biological activity such as antimicrobial, antioxidant, anti-inflammatory and anticancer [17]. In particular, many indolizines have been reported for their anticancer potential. For instance, such structures containing a cyclopropylketone in position 3 displayed *in vitro* antiproliferative activity on Hep-G2 cell lines [18], the most active in the study being compound **8** (IC_{50} (Hep-G2) = 0.2 μg/mL, Fig. 2). Other products decorated with indolizine ring are involved in the migration and proliferation of tumor cells by preventing protein–protein interaction in which the endothelial vascular growth factor (VEGF) is involved (compound **9**, Fig. 2) [19].

The first tubulin polymerization inhibitors containing an indolizine moiety were published as phenstatin analogues, known antimitotic agent (Fig. 2) [20]. Among these compounds, promising activity was reported for derivatives with an indolizine ring linked to a trimethoxyphenyl nucleus through a carbonyl group. Their cytotoxic effect was especially registered on MDA-MB-435 melanoma cells [21]. When indolizine was combined with triazine and *p*-bromobenzoyl (compound **10**, Fig. 2), an inhibition activity of the growth of SNB-75 SNC cancer cells and MDA-MB-231/ATCC breast cancer cells was observed [22]. As for indolizine-glyoxylamide **11**, it displayed cytotoxic activity against cancer cell lines which have developed resistance to chemotherapy such as Taxol-resistant HL60/TX1000 cell lines (Fig. 2) [23].

The use of indolizine ring in the conception and synthesis of some analogs of phenstatin led to promising results by providing compounds with good biological activity [21,22]. Thus, we were able to confirm that indolizine may replace successfully the cycle B of model phenstatin. In the previously reported studies, however, the active molecules were those presenting the classic 3,4,5-trimethoxyphenyl ring A (*e.g.* compound **13**, Fig. 2) [21]. In addition, our research group discovered that other indolizines inhibited the human farnesyltransferase (FTase) (*e.g.* compound **12**: IC_{50} (FTase) = 1.3 μM [24], Fig. 2).

Tubulin is a well-documented target in oncology due to its role in chromosome segregation and cell division. This requires extremely fast dynamics of microtubules polymerization and depolymerization, both mediated by the tubulin. These dynamics can be blocked by different classes of inhibitors of tubulin that, by interfering with the dynamics of the microtubules, stop the cancer cell in mitosis (arrest of cells in G2/M phase of the cell cycle), eventually leading to cell death, both through apoptosis and necrosis [25]. The FTase is another studied target in oncology. It catalyzes the addition of a lipid group to the terminal carboxyl of several proteins, including Ras proteins having a proto-oncogenic role. The substrates of this enzyme undergo a process of maturation consisting of: fixation of a prenyl chain, loss of the three terminal amino acids and methylation of the C-terminal carboxyl group. Sometimes a palmitoylation phase ends the process. The prenylation step is a key step, as its inhibition prevents proteins to attach to the target membrane [26]. Inhibitors of FTase are also capable of inhibiting bipolar spindle formation and chromosomal alignment during metaphase [27].

In the incessant search for molecules with improved antitumor

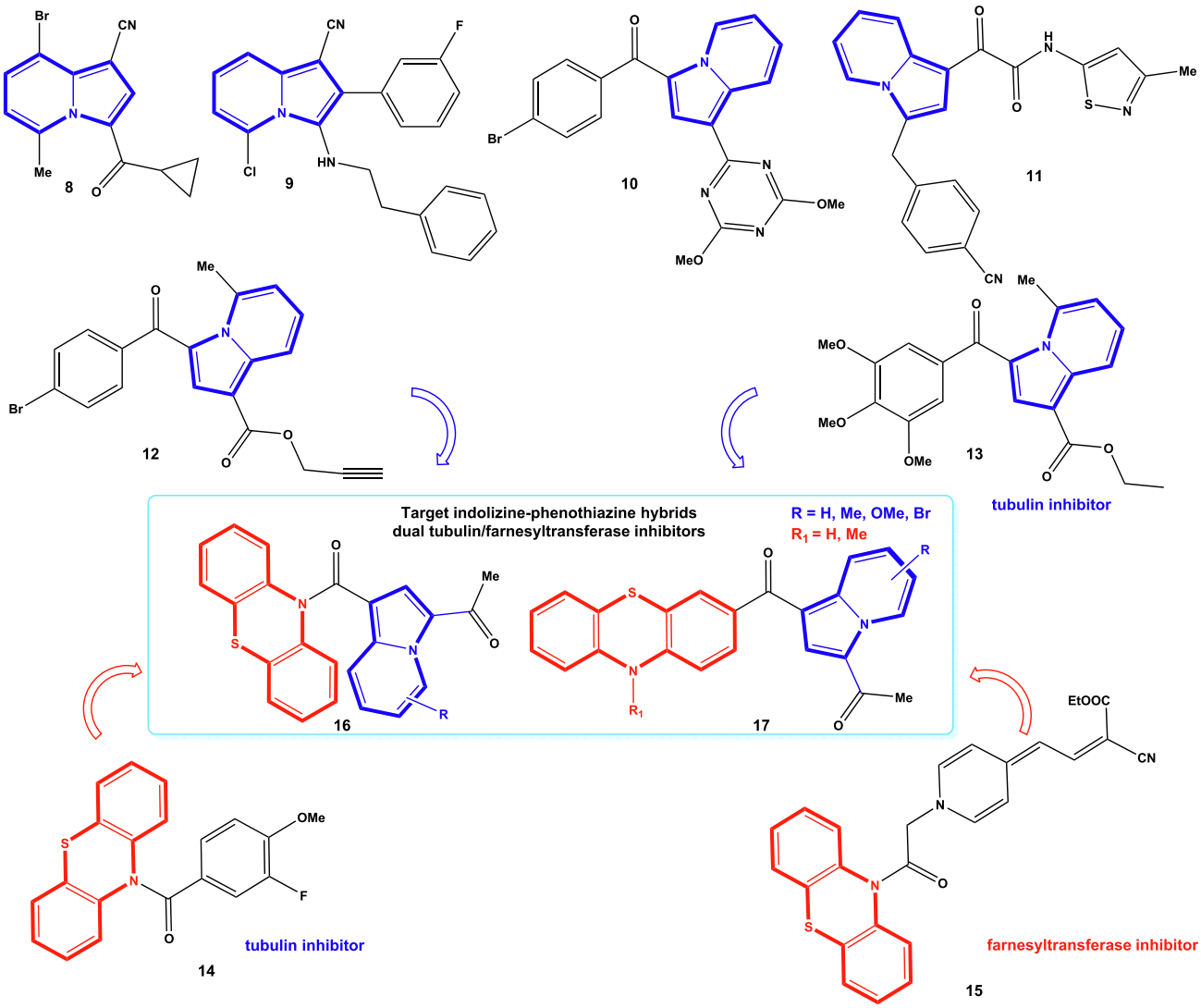


Fig. 2. Structure of previously described indolizine derivatives with antitumor activity **8–15** and structure of target indolizine-phenothiazine hybrids **16** and **17** and of reference phenstatin.

activity, a new series of compounds has been conceived in this study (target compounds, Fig. 2) where the cycle B is an indolizine unit and the cycle A is a phenothiazine. Chemistry of the latter azaheterocycle is part of the laboratory's expertise and proved to be a successful substitute for the classical trimethoxyphenyl group in the structure of antimitotic agents (*e.g.* phenothiazine derivative **14**: IC_{50} (tubulin) = 9.48 μM) [28]. Phenothiazine derivative **15** was not active on tubulin but displayed activity against human FTase (IC_{50} = 0.6 μM [29], Fig. 2).

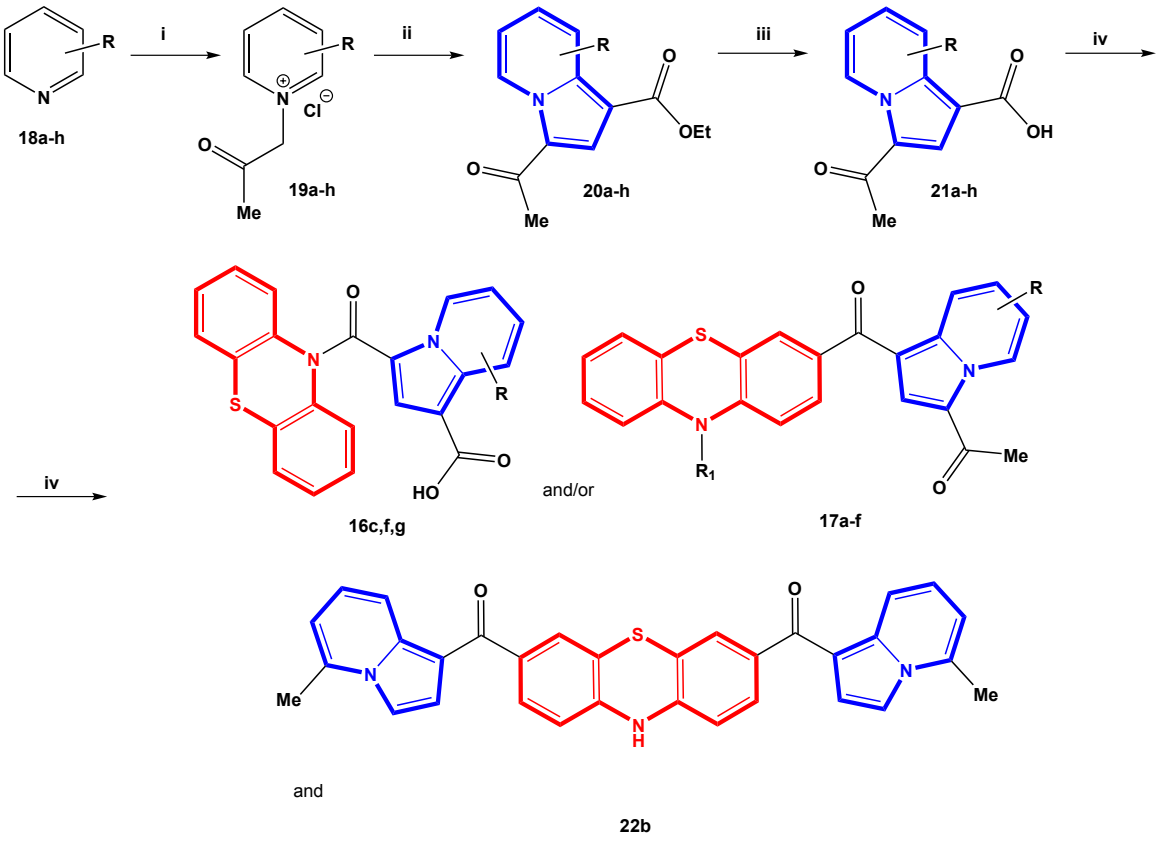
The ambition of this work was to obtain a synergy of antitumor effects using a unique compound bearing the two heterocycles that are each present in the structure of previously identified antitumor agents as phenstatin analogues as well as FTase inhibitors. These targeted indolizine-phenothiazine hybrids may constitute dual inhibitors of tubulin polymerization and farnesyltransferase. Substituents privileged on each heterocycle are those previously identified to be beneficial for the pursued biological activity: methyl, methoxy, acetyl or bromo (target compounds, Fig. 2).

The use of specific FTase inhibitors to treat different cancers has been disappointing in clinical trials due to the ability of the cancer cells to circumvent the problem of the FTase inhibition and go through FTase inhibition by using a second prenylation route mediated by structurally related geranylgeranyltransferase. Demonstrating the effectiveness of dual inhibitors of tubulin and FTase (MTI-FTI hybrids) can be crucial, leading to an innovative strategy for the design of new anticancer compounds. A single molecule may induce a synergy of antitumor action increasing the effectiveness and reducing the toxicity of the classical combo treatments.

2. Results and discussion

2.1. Chemistry

In order to obtain the indolizine-phenothiazine hybrids, a four-step synthesis described in Scheme 1 was privileged. The first step consisted in obtaining the pyridinium salts **19a-g** in very good yields (80–93%) by reaction of suitably substituted and commercially available pyridines **18a-g** with chloroacetone in THF at rt. In the second synthetic step, salts **19a-g** were subjected to a 1,3-dipolar cycloaddition in the presence of triethylamine and ethyl propiolate as dipolarophile, in order to create the indolizine ring. The best cycloaddition solvent in this study was a mixture of CH_3CN and DMF (volume ratio 8/2). The use of CH_3CN or THF resulted in lower yields. Eight ethyl esters **20a-h** were prepared in modest to good yields (22–71%). It is to be noted that the 3-bromopyridinium salt **19 g** underwent the Huisgen cycloaddition on two different accessible positions on the pyridine and allowed to obtain



Scheme 1. Synthesis of target indolizine-phenothiazine hybrids. *Reagents and conditions*: (i) chloroacetone (1.2 equiv.), THF, 24–48 h, rt; (ii) ethyl propiolate (1.5 equiv.), TEA (1.4 equiv.), CH_3CN/DMF, 24 h, 60 °C; (iii) NaOH 50% aq. solution (4 equiv.), EtOH, reflux, 3 h, then HCl 10% aq. solution; (iv) 10*H*-phenothiazine or 10-methyl-10*H*-phenothiazine (1.0 equiv.), Eaton's reagent (4.0 equiv.) (P_2O_5/CH_3SO_3H 1/10 w/w), 50 °C, 4–24 h.

two position isomers **20 g** (22% yield) and **20 h** (40% yield). Now, the saponification of the ester group furnished easily the free carboxylic acids **21a-h** in excellent yields (80–96%). The first strategy for the final step was to convert carboxylic acids into corresponding acid chlorides whose Friedel-Crafts reaction with phenothiazine or *N*-methylphenothiazine in the presence of aluminum trichloride would generate the indolizine-phenothiazine hybrids of general formula **16** and **17**. Reaction of the acid chloride of indolizine **21d** was chosen as model; however, despite a good formation of the chloride, the expected acylations failed probably due to the solubilization issues. The synthetic strategy was then modified, and the use of Eaton's reagent (10/1 $MeSO_3H/P_2O_5$) proved successful. This acid reagent is used without solvent and has the advantage to be less viscous than polyphosphoric acid, enabling efficient magnetic stirring and easier final workup of the crude. Eaton's reagent is known to promote Friedel-Crafts acylations [30–32] and functions directly with free carboxylic acids without the need to synthesize the intermediate acid chlorides. When the acylation reactions were carried out with 10-methyl-10*H*-phenothiazine as substrate, only the products acylated in the position 3 of phenothiazine were isolated in low yields (ketones **17d'** (34%) and **17e'** (24%), Table 1). The Eaton's reagent-mediated acylation of 10*H*-phenothiazine provided the acylated products in the position 3 (ketones **17a-f**, Scheme 1 and Table 1) and, in few cases, furnished also *N*-acylated indolizine-phenothiazine hybrids (compounds **16c**, **16f** and **16 g**, Table 1). A series of unexpected by-products was also isolated (indolizines **23–29**). The structures of the isolated compounds are presented in Table 1. The indolizine derivatives bearing a free carboxylic acid proved to have limited stability in the presence of Eaton's reagent and underwent decarboxylation, acetylation of the indolizine ring upon decarboxylation and/or suppression of the acetyl unit (Table 1). The 8-bromosubstituted acid **21 h** was the most sensitive and rapidly decarboxylated to furnish only the bromo-indolizine **29** in 74% yield. However, while the formation of these unexpected by-products is responsible for the modest yields of the target compounds, very interestingly they constitute new functionalized chemical platforms for further synthetic modulations.

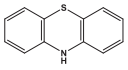
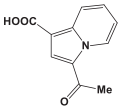
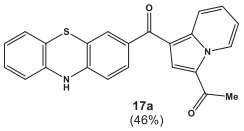
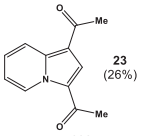
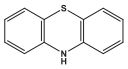
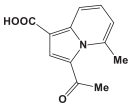
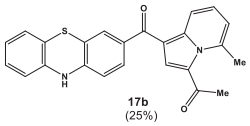
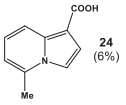
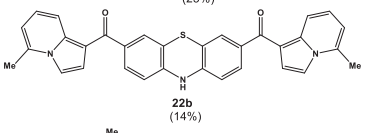
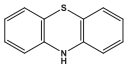
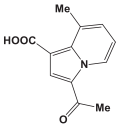
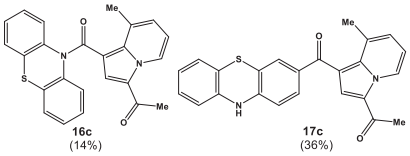
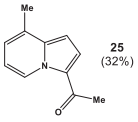
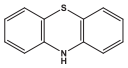
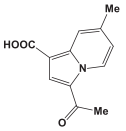
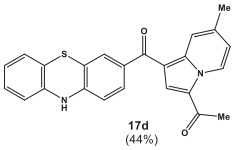
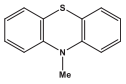
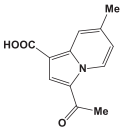
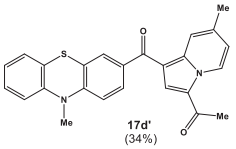
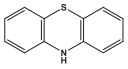
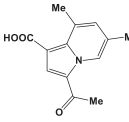
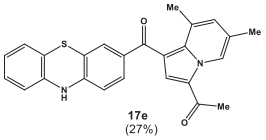
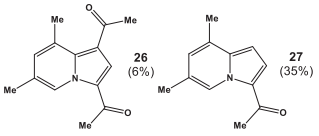
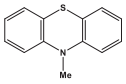
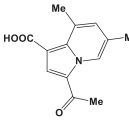
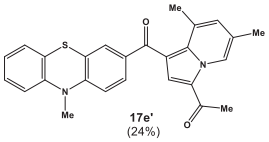
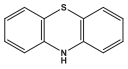
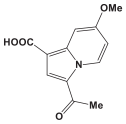
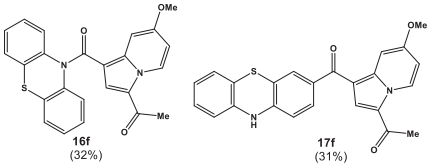
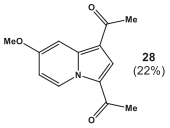
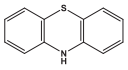
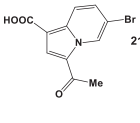
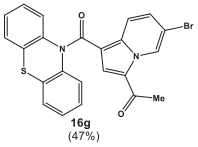
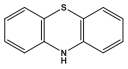
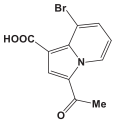
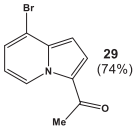
2.2. Biological evaluation of the targeted products and first SAR aspects

2.2.1. Evaluation on tubulin polymerization and on FTase

The biological evaluation of indolizine-phenothiazine hybrids **16** and **17** started by determining their potential to inhibit the tubulin polymerization using sheep brain tubulin. Compounds were diluted in DMSO to obtain a 10^{-2} M concentration then diluted in the medium to achieve the desired concentration. All compounds were first tested at a concentration of 100 μM to calculate their inhibition ratio. DMSO was used as a negative control. Phenstatin and (-)-desoxypodophyllotoxin, known strong tubulin polymerization inhibitors, were used as positive references. Only compounds that induced more than 60% inhibition of tubulin polymerization at 100 μM underwent the IC_{50} evaluation and were further evaluated on tubulin at eight different concentrations. The results are described in Table 2.

The *N*-acylation of the phenothiazine ring which provided compounds **16c**, **16f** and **16 g** was not an advantageous pharmacomodulation, none of these molecules showing notable antimitotic activity. On the contrary, the acylation of phenothiazines in position 3 was tolerated. Ketones **17c**, **17d** and **17f** displayed IC_{50} values on tubulin polymerization in the micromolar range. The best tubulin inhibitors in the current study were compounds **17d** and **17f** with similar efficiency as the reference phenstatin and (-)-desoxypodophyllotoxin. Ketones **17d** and **17f** have very similar chemical structure, the only difference being the nature of their substituent in the position 7 of the indolizine ring. Compound **17d** is decorated with a methyl substituent while the

Table 1
Isolated products and secondary products obtained by Eaton's reagent-mediated acylation of phenothiazine substrates.

Phenothiazine derivative	Carboxylic Acid	Product (isolated yield)	Secondary product (isolated yield)
	 **21a**	 **17a** (46%)	 **23** (26%)
	 **21b**	 **17b** (25%)	 **24** (6%)
		 **22b** (14%)	
	 **21c**	 **16c** (14%) **17c** (36%)	 **25** (32%)
	 **21d**	 **17d** (44%)	–
	 **21d**	 **17d'** (34%)	–
	 **21e**	 **17e** (27%)	 **26** (6%) **27** (35%)
	 **21e**	 **17e'** (24%)	–
	 **21f**	 **16f** (32%) **17f** (31%)	 **28** (22%)
	 **21g**	 **16g** (47%)	–
	 **21h**	–	 **29** (74%)

compound **17f** is substituted by a methoxy group. The slightly increased antitubulin activity of compound **17f** (IC_{50} (tubulin) = 1.11 μM, Table 2) compared to **17d** (IC_{50} (tubulin) = 3.32 μM, Table 2) is probably due to the methoxy substituent, the classic group found in the *para* position on the B cycle of reference molecules such as phenstatin or combretastatin A-4. A diminished antimitotic activity was noted for other ketones (**17a**, **17b** and **17e**) while molecules **17d'** and **17e'** bearing a *N*-methylphenothiazine unit were inactive (Table 2).

Table 2
Evaluation of synthesized molecules on tubulin polymerization and on human FTase *in vitro*.

Molecule	% TPI[a,b]	IC_{50} (tubulin) (μM)[b]	R^2[c]	% FTI[b,e]	IC_{50} (FTase) (μM)[b]	R^2
16c	46	-[d]	–	49	–	–
16f	32	–	–	37	–	–
16 g	19	–	–	65	26.48	0.9902
17a	43	–	–	95	0.35	0.9179
17b	41	–	–	73	4.80	0.8332
17c	84	27.7	0.9046	89	0.81	0.9034
17d	77	3.32	0.8844	96	0.25	0.8982
17d'	0	–	–	82	14.56	0.9662
17e	56	–	–	95	0.68	0.8829
17e'	9	–	–	81	6.46	0.8632
17f	100	1.11	0.8929	86	0.39	0.8938
22b	44	–	–	79	2.90	0.9970
Phenstatin	99	3.43	0.9378			
(-)-Desoxypodophyllotoxin	100	1.76	0.9740			
Chaetomellic acid A				100	0.183	0.9898

[a] Inhibition of tubulin polymerization at a 100 μM concentration
[b] Values represent mean of two experiments
[c] Coefficient of determination.
[d] Not determined
[e] Inhibition of human FTase at a 100 μM concentration

Consequently, the nitrogen atom of phenothiazine must imperatively remain unsubstituted in the series of ketones in order to ensure good inhibition of tubulin.

Indolizine-phenothiazine hybrids were next subjected to a biological evaluation against human FTase. DMSO and chaetomellic acid A were used as negative and positive controls, respectively. The same threshold inhibition criteria as for the tubulin polymerization assay were applied to select active molecules for IC_{50} calculation. Ten of the twelve considered compounds were able to inhibit the protein. In the series of *N*-acylated phenothiazines, only derivative **16 g** showed a weak inhibitory activity against human FTase (IC_{50} = 26.48 μM, Table 2). All tested ketones **17a-f** and **22b** displayed activity against the protein. Again, *N*-methylated phenothiazines were less active compared to nitrogen-free congeners (compare IC_{50} values on FTase of compounds **17d** *vs* **17d'** and **17e** *vs* **17e'**, Table 2). In terms of substitution of the indolizine ring, small groups are tolerated, especially in the position 7 of indolizine. Five derivatives displayed submicromolar IC_{50} values and the best FTase inhibitors in this study were **17a**, **17d** and **17f** (Table 2). The bisacylated derivative **22b** displayed unexpected micromolar activity against FTase, weaker however compared to the other 3-acylated derivatives (Table 2).

Interestingly, as imagined in the design of these indolizine-phenothiazine hybrids, dual inhibitors of tubulin and FTase were identified. Three molecules **17c**, **17d** and **17f** were active against both considered biological targets *in vitro* with IC_{50} values in the same range as for reference compounds.

2.2.2. Evaluation on cancer cell lines

Nine indolizine-phenothiazine hybrids **16c**, **16f**, **17a-f** and **22b** were selected by the National Cancer Institute (NCI) for evaluation of their cell growth inhibition potential on the NCI-60 cancer cell lines panel. All compounds submitted to the NCI were tested initially at a single high dose (10 μM) in the full NCI panel (Table 3).

The Cell One-Dose Screen confirmed that *N*-acylated phenothiazines **16c** and **16f** had very limited efficiency in inhibiting the growth of cancer cell lines. Compound **16c**, inactive in the tubulin polymerization and FTase assays, was also devoid of activity on cancer cells while compound **16f** showed modest cell growth inhibition on SR (50%), NCI-H522 (53%) and PC-3 (48%) cells. These results are in line with the results of the tests carried out on the two targets of interest.

Ketones **17a-f** were more active and displayed promising cellular activities. Not only they arrested the cell growth of almost all tested cancer cells, but they displayed cytotoxicity potential (negative values in Table 3). The cell lines on which tested compounds were the most active were NCI-H522 (lung cancer), COLO-205 and HT29 (colon cancer), SF-539 (human glioblastoma), MDA-MB-435 (melanoma), OVCAR-3 (ovarian cancer) and A498 (renal cancer) (Table 3). Compound **17b** proved non-effective in cell growth inhibition. This result corroborated well with its activity registered on the biological targets (Table 2). Only a moderate efficiency was registered on HCT-116 colon cancer cells (Table 3). The bulky derivative **22b** displayed limited efficiency probably due to solubility issues and difficulty to cross cell membranes. The log P value of compounds was next estimated with ACD software. The predicted values were: **16c** (Log P = 5.06 ± 1.34), **16f** (Log P = 4.52 ± 1.52), **17a** (Log P = 5.11 ± 1.32), **17b** (Log P = 5.57 ± 1.32), **17c** (Log P = 5.57 ± 1.32), **17d** (Log P = 5.57 ± 1.32), **17e** (Log P = 6.03 ± 1.32), **17f** (Log P = 5.02 ± 1.52), **22b** (Log P = 7.54 ± 1.45). As expected, compound **22b** had the biggest log P value in the series. This compound has low aqueous solubility, compromising bioavailability.

Only compounds which satisfied pre-determined threshold inhibition criteria in a minimum number of cell lines progressed to the full 5-dose assay (Table 4). Five phenothiazines acylated in position 3 (**17a**,**c-f**) were thus submitted to GI_{50} calculation. These experimental drugs exhibited significant growth inhibition in the nanomolar range (Table 4). All these molecules also displayed great efficiencies on both studied targets. Besides, compounds **17c**, **17d** and **17f** are the dual inhibitors identified in this report (Table 2). It is to be noted that the same ranking in terms of biological activity is observed for these dual inhibitors as previously seen in the protein assays. Compound **17f** was the most active against tubulin and had the same inhibitory efficiency on FTase as the structurally close compound **17d**, and induced the best antitumor activity against the 60-cell panel. Compound **17d** was the second most active in the dual inhibitors' series followed by compound **17c**. As expected, compound **17e**, more specific as FTase inhibitor, conserved cell growth inhibition potential, but diminished compared to dual inhibitors.

The most unexpected excellent antitumor activity was that induced by phenothiazine-ketone **17a**, the best compound of the series in the NCI evaluation. Compound **17a** displayed cell proliferation inhibition at low nanomolar concentrations (GI_{50} (NCI-H522) = 1.8 nM and (GI_{50} (MDA-MB-435) = 1.8 nM, Table 4). The fact that the most active product was not the best inhibitor of tubulin polymerization and/or FTase in the family shows that it can penetrate the cell membrane better or reach other biological target(s), one of which is FTase (IC_{50} = 0.35 μM, Table 2). The NCI Compare algorithm, a comparison

Table 3
Results of the *in vitro* human cancer cell growth inhibition for selected indolizine-phenothiazine hybrids **16c, 16f, 17a-f** and **22b** [a,b].

	Compound	16c	16f	17a	17b	17c	17d	17e	17f	22b
Cell type	**Cell line**	**GI%**[a,b]								
Leukemia	SR	11	50	91	24	87	n.t.	95	95	11
	HL-60(TB)	12	32	86	23	−5	97	−13	−10	17
	CCRF-CEM	11	12	95	17	84	89	n.t.	92	0
	K-562	7	25	n.t.[c]	27	92	95	96	93	3
Non-Small Cell Lung Cancer	NCI-H522	32	53	**−29**[d]	29	87	**−48**	**−31**	**−21**	13
	NCI-H460	0	4	97	14	94	99	99	96	0
	A549/ATCC	9	23	89	29	80	86	96	89	2
Colon Cancer	COLO 205	0	29	**−55**	0	**−44**	**−62**	**−37**	**−41**	0
	HT29	47	35	**−13**	11	88	**−57**	**−15**	**−1**	17
	HCT-116	43	39	99	42	94	−24	96	99	15
Melanoma	MDA-MB-435	4	13	**−10**	2	**−27**	**−20**	**–22**	**–33**	0
	M14	11	18	80	16	83	83	90	90	1
	SK-MEL-5	0	26	n.t.	10	92	−27	−37	−40	3
Renal cancer	A498	0	n.t.	**−12**	0	**−5**	**−16**	**−27**	**−18**	0
	RXF 393	0	5	93	0	−8	51	66	48	41
	TK-10	5	0	41	0	43	−26	65	70	0
SNC cancer	SF-539	0	10	**−38**	0	**−28**	**–32**	**−3**	**−12**	11
	SF-295	12	7	91	2	96	−2	−1	86	26
	SNB-75	12	16	87	0	76	91	−2	79	22
Ovarian Cancer	OVCAR-3	0	0	**−17**	0	**−27**	**−14**	**−26**	**−48**	0
	OVCAR-8	0	n.t.	93	12	81	96	n.t.	n.t.	0
	SK-OV-3	2	3	90	0	75	83	79	81	0
Breast cancer	MDA-MB-231/ATCC	7	34	82	21	80	97	−14	−17	1
	MCF-7	11	18	75	19	75	89	85	79	6
	MDA-MB-468	1	37	96	11	86	−3	86	−4	0
Prostate cancer	PC-3	19	48	78	14	73	82	n.t.	76	12
	DU-145	0	0	98	14	86	99	98	−18	0

[a] Data obtained from NCI's *in vitro* 60-cell one dose screen (experiments conducted with 10 μM concentration of tested molecule).
[b] GI% is the percentage of growth inhibition of tumor cells.
[c] Not tested.
[d] A value of -x means x% cancer cells lethality of preexisting cells (cytotoxic effect).

tool that analyzes the profile of cellular growth inhibition from the NCI-60 cell line panel of tested compounds to assist in the identification of molecules with similar activity profiles, was used to try to obtain other clues about the potential targets achieved. The results indicated that molecule **17a** has a very similar profile as known cytotoxic agents used in clinics: doxorubicin (DNA intercalant), vinblastine and taxol (tubulin inhibitors).

The most sensitive cancer cell line to all the tested experimental drugs was MDA-MB-435 melanoma cells but tested compounds have also shown great potential on other cancer cell lines such as HL-60 (TB) and K-562 (leukemia), NCI-H522 (lung cancer), and OVCAR-3 (ovarian cancer), respectively (Table 4).

2.3. Docking

To investigate how the indolizine-phenothiazine hybrids interact with the biological targets, molecular modeling studies were performed on selected compounds: a specific strong inhibitor of FTase (**17a**), a dual tubulin/FTase inhibitor (**17d**) and a modest FTase inhibitor (**17d'**). First, docking studies were conducted in the colchicine binding site of tubulin, compared to Combretastatin A-4 (CA-4) as reference compound (Fig. 3).

Compound **17a** displayed a single conformation with the tricycle on the left side of the binding site while the indolizine ring occupied the entry into the smaller side pocket which is generally occupied by the cycle B (3′-hydroxy-4′-methoxyphenyl) of CA-4 (Fig. 3(a)). Since compound **17a** has rather rigid structure, this was the only possibility to fit in the active site, although the contact between the carbonyl group and the protein surface is not optimal (Fig. 3(b)). This is in accordance with the experimental results obtained in the tubulin polymerization assay. Compound **17d** had a behavior like **17a**. The additional methyl on the indolizine fitted into the side pocket, thus adding a positive hydrophobic contact and partially mimicking the B-ring of CA-4 (Fig. 3(c)). These interactions may explain the antitubulin potential of this molecule.

Only one conformation well overlapped with the previous molecule was found for indolizine-phenothiazine **17d'**, *N*-methylated analogue of **17d** (Fig. 3(d)). The additional methylation of phenothiazine could provide better hydrophobic contact with the binding site. However, the bulkier structure of **17d'** compared to **17d** seems to prevent the correct positioning in the active site.

The behavior of the same molecules **17a**, **17d** and **17d'** was next studied in the active site of FTase (Fig. 3(e)-(h)). Derivative **17a** displayed 25 conformations that conveniently overlapped, with the phenothiazine tricycle slightly offset in front of the zinc cation of FTase (Fig. 3(e)). Its analog **17d** with an additional methyl grafted on the indolizine heterocycle was positioned in the same way, having 27 overlapping conformations out of 30 and better site compatibility (Fig. 3(f)). As a reminder, molecule **17d** was identified as a dual inhibitor of both tubulin and FTase. The results of molecular modeling agree with the experimental results. The addition of one methyl to the molecule on the phenothiazine nitrogen atom in compound **17d'** (Fig. 3(g) and (h)), affected the positioning of the compound in the FTase site. For this molecule, there were two possibilities of binding: with the phenothiazine close to the zinc cation such as the preceding molecules (Fig. 3(g)) or vice versa, with a weak or even absent interaction with the metal cation (Fig. 3(h)).

3. Conclusions

A new series of indolizine-phenothiazine hybrids has been designed, synthesized and evaluated on tubulin polymerization and human FTase. An NCI screen was performed on nine synthesized derivatives. This screen utilized 60 different human tumor cell lines, representing leukemia, melanoma and cancers of the lung, colon, brain, ovary, breast, prostate, and kidney cancers.

Table 4
Results of the NCI 60 Cell Five-Dose Screen for selected compounds **17a** and **17c-f**[a,b].

	Compound	17a	17c	17d	17e	17f
Cell type	**Cell line**	**GI_{50} (nM)**[a,b]				
Leukemia	SR	< **3.3**[c]	118	38.6	302	56.7
	HL-60(TB)	< **2.4**	296	47.1	263	26
	CCRF-CEM	< **3.3**	314	257	394	39
	K-562	< **3.3**	53.7	41	206	46.2
Non-Small Cell Lung Cancer	NCI-H522	**1.8**	34.1	41.2	197	48.7
	NCI-H460	< **3.3**	133	46.5	301	35.4
	A549/ATCC	< **3.3**	206	64.8	310	42.4
Colon Cancer	COLO 205	**3.3**	147	59.8	202	24.5
	HT29	< **3.3**	252	62.7	325	33
	HCT-116	< **3.3**	183	41.4	332	28.5
Melanoma	MDA-MB-435	**1.8**	**31.9**	**29.7**	**46.8**	**19**
	M14	< **3.3**	146	90.6	267	30.9
	SK-MEL-5	< **3.3**	77.7	122	406	46.2
Ovarian Cancer	OVCAR-3	**3.7**	62.5	32.3	222	26.2
	OVCAR-8	< **3.3**	361	451	379	50.3
	SK-OV-3	**4.4**	194	189	279	37.2
Renal Cancer	A498	**2.3**	99.8	43.1	66.4	30.2
	RXF 393	**3.3**	227	41.1	162	28.2
	CAKI-1	**3.7**	n.t.[d]	53	529	52.8
Prostate Cancer	PC-3	< **3.3**	259	50.6	321	47.4
	DU-145	< **3.3**	386	188	407	42.6
CNS Cancer	SF-539	**2.8**	203	58.1	185	21.2
	SF-295	**2.8**	93.7	36	187	25.5
	SNB-75	**2.0**	158	41.6	134	25.9
Breast Cancer	MDA-MB-231/ATCC	**6.5**	351	348	472	57.8
	MCF7	**3.4**	49.7	34.5	209	36.7
	MDA-MB-468	**2.6**	102	66.3	487	33.1

[a] Data obtained from NCI's *in vitro* 60 cell 5-dose screen.
[b] GI_{50} is the molar concentration of synthetic compound causing 50% growth inhibition of tumor cells.
[c] Compound **17a** has been tested at concentrations 5.10^{-5}M, 5.10^{-6}M, 5.10^{-7}M, 5.10^{-8}M and 5.10^{-9}M. On some cell lines, the GI_{50} could not be calculated since the compound exhibited an inhibitory activity still greater than 50% inhibition (see SI for full dose–response curves).
[d] Not tested.

Compounds were obtained in a 4-step procedure, and Eaton's reagent was essential to obtain target hybrids in the final step. It generated reaction products that were in some cases different depending on the nature of indolizine substituents (phenothiazine-ketones **17a-f** and **22b** or phenothiazine-amides **16c**, **16f-g**). It also led to the formation of numerous by-products **23–29** that explained the low yields of the compounds of interest. The structures of by-products obtained were elucidated. These indolizine derivatives constitute new functionalized chemical platforms that may interest the medicinal chemistry community for further modulations.

The final compounds are characterized by a new cycle A (phenothiazine substituted in position 3) and a new cycle B (acetylindolizine) linked by the characteristic carbonyl connector as the reference phenstatin. The combination of the two heterocycles provided phenothiazine-ketones as inhibitors of tubulin polymerization with the same activity as the parent drug. The condition to maintain the antitubulin potential was to keep the phenothiazine unsubstituted on the nitrogen atom and the indolizine substituted with a methyl or methoxy group in position 7. The grafting of a methyl group on the nitrogen atom of phenothiazine suppressed the activity. The obtained phenothiazine-amides **16c**, **16f** and **16 g** did not exhibit any antitubulin activity.

The hybrids of this series have also inhibited the FTase activity and emphasized similar structure–activity relationships. However, this enzyme was less sensitive to the substituents present on the indolizine. It can be concluded that the optimal structural changes for the activity on FTase were: i) the placing of the indolizine heterocycle in the position 3 of the phenothiazine through a carbonyl bridge; ii) maintaining the phenothiazine nitrogen atom unsubstituted and iii) the substitution on the indolizine ring by a methyl or a methoxy group on positions 6, 7 and 8.

Thus, small molecular weight tubulin-FTase inhibitors were obtained (molecules **17c**, **17d** and **17f**). To the best of our knowledge this is the first time when dual inhibitors (MTI-FTI hybrids) acting on tubulin polymerization and on FTase is realized. This duality of actions offers new perspectives and highlights the importance of such inhibitors in the design of new anticancer drugs. As expected, they were active in the screening on the 60 tumor cell lines performed at NCI especially on MDA-MB-435 melanoma cell lines.

The most promising antitumor activity in the NCI evaluation was that induced by phenothiazine-ketone **17a**, displaying cell proliferation inhibition at low nanomolar concentrations on multiple cancer cell lines (*e.g.* GI_{50} (NCI-H522) = 1.8 nM; GI_{50} (MDA-MB-435) = 1.8 nM). This compound was less effective against tubulin compared to dual inhibitors. The excellent cellular activity may be due to binding to another biological target(s) and/or better cell penetration. All these aspects require further investigation.

4. Experimental section

4.1. Materials and methods

Starting materials are commercially available and were used without further purification (suppliers: Carlo Erba Reagents S.A.S., Tokyo Chemical Industry Co. Ltd. and Acros Organics). Melting points were measured on a MPA 100 OptiMelt® apparatus and are uncorrected. Nuclear Resonance Magnetic (NMR) were acquired at 400 MHz for ^{1}H NMR and at 100 MHz for ^{13}C NMR, on a Varian 400-MR spectrometer or at 500 MHz for ^{1}H NMR and at 125 MHz for ^{13}C NMR, on a Bruker Avance III 500 MHz spectrometer with tetramethylsilane (TMS) as internal standard, at 25 °C. Chemical shifts (δ) are expressed in ppm relative to TMS. Splitting patterns are designed: s, singlet; d, doublet; dd, doublet of doublet; t, triplet; m, multiplet; quint, quintuplet; br s, broaden singlet; br t, broaden triplet. Coupling constants (*J*) are reported in Hertz (Hz). Thin layer chromatography (TLC) was realized on Macherey Nagel silica gel plates with fluorescent indicator and were visualized under a UV-lamp at 254 nm and 365 nm. Flash chromatography was performed with a CombiFlash R*f* Companion (Teledyne-Isco System) using RediSep packed columns. IR spectra were recorded on a Varian 640-IR FT-IR or on a FTIR Bruker Tensor 27 spectrometer. Elemental analyses (C, H, N) of new compounds were determined on a Thermo Electron apparatus by "Pole Chimie Moleculaire-Welience", Faculte des Sciences Mirande, Dijon, France.

4.2. General procedure for the synthesis of pyridinium salts ***19a-g***

Chloroacetone (1.2 equiv.) was added to a solution of pyridine **18a-g** (1 equiv.) in THF. The resulting mixture was stirred at rt for 24–48 h. The precipitate formed was filtered, washed with cold acetone and dried to provide pure pyridinium salt **19a-g** in very good yields.

The physico-chemical characterization of salts **19a-h** corresponded to that described in the literature [33].

4.3. General 1,3-dipolar cycloaddition procedure for the synthesis of indolizines ***20a-h***

The suitably substituted pyridinium salt **19a-g** (1 equiv.) was added to a mixture of CH_3CN/DMF (volume ratio 8/2). Triethylamine (1.4 equiv.) was then added to the resulting suspension. After few minutes of magnetic stirring, ethyl propiolate (1.5 equiv.) has been added and the mixture was stirred under heating at 60 °C for 24 h. The volatiles were then evaporated. The crude was then dissolved in a mixture of ethyl

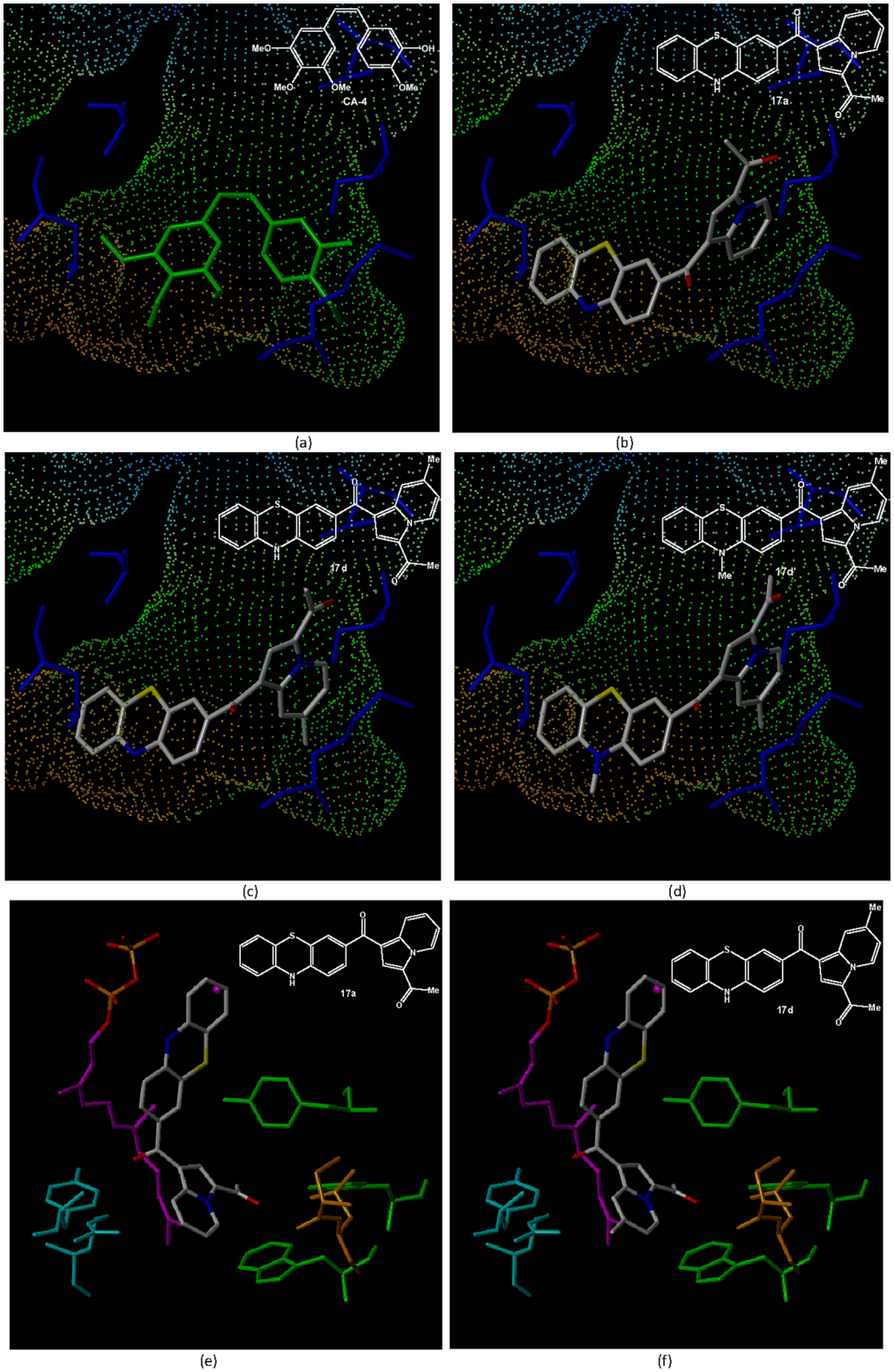
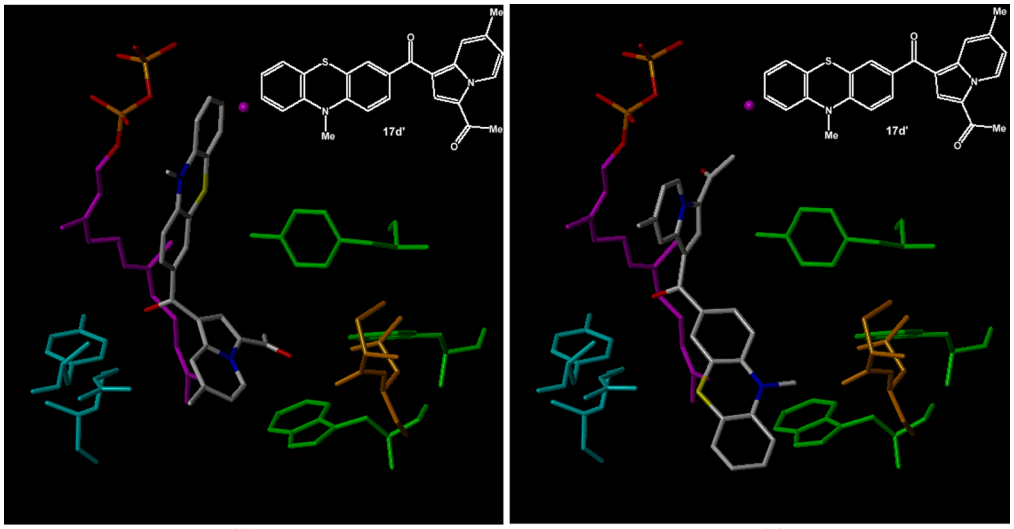


Fig. 3. Structure and docking of indolizine-phenothiazine hybrids in the active site of: tubulin (colchicine binding site) (a) combretastatin A-4, (b) compound **17a**, (c) compound **17d**, (d) compound **17d'** and FTase (e) compound **17a**, (f) compound **17d**, (g) compound **17d'** and (h) compound **17d'**.



(g) (h)

Fig. 3. (*continued*)

acetate and distilled water. The organic layer was collected, and the aqueous layer was extracted twice with ethyl acetate. The combined organic layers were dried over anhydrous Na_2SO_4. After the filtration of hydrated Na_2SO_4, the filtrate was concentrated and then purified by flash chromatography on silica prepacked column (chromatographic eluent: gradient *n*-heptane/EtOAc) to obtain pure indolizines **20a-h.**

Indolizines **20a-f** presented the same physico-chemical properties as previously reported [33].

*4.3.1. 3-Acetyl-1-carboxyethyl-6-bromoindolizine (**20 g**)*

The general procedure was used with 4.9 g (19.56 mmol) of *N*-(methylcarbonylmethyl)-3-bromopyridinium chloride **19 g** in a mixture of 24 mL CH_3CN and 6 mL DMF. Yellow solid; 22% yield (1.27 g); mp (EtOAc:*n*-heptane) 159–161 °C; ^{1}H NMR ($CDCl_3$, 500 MHz) δ (ppm): 1.43 (t, *J* = 7.0 Hz, 3H, C*H*$_3$), 2.59 (s, 3H, C*H*$_3$), 4.40 (q, *J* = 7.0 Hz, 2H, C*H*$_2$), 7.45 (d, *J* = 9.0, 2.0 Hz, 1H, Ar*H*), 7.95 (s, 1H, Ar*H*), 8.41 (d, *J* = 9.5 Hz, 1H, Ar*H*), 10.08 (s, 1H, Ar*H*); ^{13}C NMR ($CDCl_3$, 125 MHz) δ (ppm): 14.7 (CH_3), 24.5 (CH_3), 60.5 (CH_2), 106.7 (C), 110.4 (C), 120.0 (CH), 122.9 (C), 126.0 (CH), 129.2 (CH), 130.4 (CH), 137.4 (C), 163.8 (C), 188.1 (C); IR ν (cm^{-1}): 3115, 2985, 1708, 1687, 1643, 1514, 1476, 1426, 1362, 1255, 1213, 1185, 1047, 1018, 989, 978, 932, 869, 821, 773, 728, 642, 629, 576, 443, 424.

*4.3.2. 3-Acetyl-1-carboxyethyl-8-bromoindolizine (**20 h**)*

Product obtained in the same reaction with **20 g**; yellow solid; 40% yield (2.31 g); mp (EtOAc:*n*-heptane) 105–107 °C; ^{1}H NMR ($CDCl_3$, 500 MHz) δ (ppm): 1.43 (t, *J* = 7.0 Hz, 3H, C*H*$_3$), 2.60 (s, 3H, C*H*$_3$), 4.41 (q, *J* = 7.0 Hz, 2H, C*H*$_2$), 6.85(t, *J* = 7.0 Hz, 1H, Ar*H*), 7.60 (d, *J* = 7.5 Hz, 1H, Ar*H*), 7.94 (s, 1H, Ar*H*), 9.98 (d, *J* = 7.0 Hz, 1H, Ar*H*); ^{13}C NMR ($CDCl_3$, 125 MHz) δ (ppm): 14.5 (CH_3), 27.7 (CH_3), 61.1 (CH_2), 109.6 (C), 112.2 (C), 114.8 (CH), 122.4 (C), 127.3 (CH), 128.0 (CH), 131.5 (CH), 135.0 (C), 163.9 (C), 187.8 (C); IR ν (cm^{-1}): 3120, 2981, 1708, 1643, 1520, 1471, 1422, 1382, 1367, 1344, 1221, 1204, 1166, 1120, 1048, 987, 938, 865, 780, 764, 651, 620, 576, 560.

*4.4. General procedure for the saponification of ethyl esters and generation of carboxylic acids **21a-h***

Indolizine ethyl ester **20a-h** (1 equiv.) was suspended in absolute ethanol. NaOH 50% aqueous solution (4 equiv.) was added and the mixture was refluxed for 3 h. The medium became homogenous (this corresponded to the complete formation of the sodium carboxylate; reaction monitored by ^{1}H NMR). After cooling to rt, HCl 10% aqueous solution was added to the crude to obtain a pH slightly acid. The precipitate formed was filtered, washed with cold absolute ethanol and dried to provide pure carboxylic acid **21a-h**.

*4.4.1. 3-Acetylindolizin-1-yl-carboxylic acid (**21a**)*

The general procedure was used with 3.0 g (12.97 mmol) of indolizine ethyl ester **20a** in 50 mL of absolute EtOH. White solid; 80% yield (2.1 g); mp (EtOH) 252–253 °C; ^{1}H NMR (DMSO-d_6, 500 MHz) δ (ppm): 2.55 (s, 3H, C*H*$_3$), 7.21 (t, *J* = 7.0 Hz, 1H, Ar*H*), 7.53 (t, *J* = 8.0 Hz, 1H, Ar*H*), 8.11 (s, 1H, Ar*H*), 8.28 (d, *J* = 7.5 Hz, 1H, Ar*H*), 9.78 (d, *J* = 7.0 Hz, 1H, Ar*H*), 12.49 (br s, 1H, COO*H*); ^{13}C NMR (DMSO-d_6, 125 MHz) δ (ppm): 27.3 (CH_3), 105.6 (C), 115.6 (CH), 118.9 (CH), 122.2 (C), 126.4 (CH), 127.6 (CH), 128.3 (CH), 138.5 (C), 164.8 (C), 187.7 (C); IR ν (cm^{-1}): 3120, 3110, 1674, 1637, 1519, 1489, 1466, 1442, 1370, 1336, 1284, 1245, 1202, 1161, 1046, 1001, 932, 911, 784, 748, 693, 636, 562, 457, 422.

*4.4.2. 3-Acetyl-5-methylindolizin-1-yl-carboxylic acid (**21b**)*

The general procedure was used with 2.3 g (9.38 mmol) of indolizine ethyl ester **20b** in 30 mL of absolute EtOH. White solid; 91% yield (1.85 g); mp (EtOH) 219–224 °C; ^{1}H NMR (DMSO-d_6, 500 MHz) δ (ppm): 2.51 (s, 3H, C*H*$_3$), 2.62 (s, 3H, C*H*$_3$), 7.06 (t, *J* = 7.0 Hz, 1H, Ar*H*), 7.50 (dd, *J* = 7.0, 2.0 Hz, 1H, Ar*H*), 8.14 (s, 1H, Ar*H*), 8.24 (d, *J* = 7.0 Hz, 1H, Ar*H*), 12.41 (br s, 1H, COO*H*); ^{13}C NMR (DMSO-d_6, 125 MHz) δ (ppm): 27.3 (CH_3), 105.6 (C), 115.6 (CH), 118.9 (CH), 122.2 (C), 126.4 (CH), 127.6 (CH), 128.3 (CH), 138.5 (C), 164.8 (C), 187.7 (C); IR ν (cm^{-1}): 2742, 2607, 1672, 1651, 1629, 1519, 1485, 1450, 1432, 1388, 1346, 1318, 1261, 1188, 1093, 1077, 1020, 945, 883, 867, 790, 774, 733, 703, 635, 588, 546, 521, 446.

*4.4.3. 3-Acetyl-8-methylindolizin-1-yl-carboxylic acid (**21c**)*

The general procedure was used with 2.7 g (11.01 mmol) of indolizine ethyl ester **20c** in 50 mL of absolute EtOH. White solid; 96% yield (2.3 g); mp (EtOH) 182–185 °C; ^{1}H NMR (DMSO-d_6, 500 MHz) δ (ppm): 2.55 (s, 3H, C*H*$_3$), 2.71 (s, 3H, C*H*$_3$), 7.11 (t, *J* = 7.1 Hz, 1H, Ar*H*), 7.29 (d, *J* = 7.1 Hz, 1H, Ar*H*), 8.11 (s, 1H, Ar*H*), 9.79 (d, *J* = 7.2 Hz, 1H, Ar*H*), 12.20 (br s, 1H, COO*H*); ^{13}C NMR (DMSO-d_6, 125 MHz) δ (ppm): 21.7 (CH_3), 27.8 (CH_3), 108.4 (C), 115.7 (CH), 121.7 (C), 126.4 (CH), 128.6 (2CH), 129.6 (C), 137.6 (C), 165.2 (C), 187.7 (C); IR ν (cm^{-1}): 2936, 2716, 1662, 1632, 1512, 1480, 1438, 1350, 1267, 1234, 1223, 1148, 1027, 995, 946, 867, 767, 738, 706, 681, 646, 568, 487, 434.

*4.4.4. 3-Acetyl-7-methylindolizin-1-yl-carboxylic acid (**21d**)*

The general procedure was used with 1.5 g (6.11 mmol) of

indolizine ethyl ester **20d** in 20 mL of absolute EtOH. White solid; 91% yield (1.2 g); mp (EtOH) 248–250 °C; ^{1}H NMR (DMSO-d_6, 500 MHz) δ (ppm): 2.51 (s, 3H, C*H*$_3$), 2.58 (s, 3H, C*H*$_3$), 7.14 (dd, *J* = 7.2, 1.6 Hz, 1H, Ar*H*), 8.12 (s, 1H, Ar*H*), 8.14 (s, 1H, Ar*H*), 9.73 (d, *J* = 7.2 Hz, 1H, Ar*H*), 12.47 (br s, 1H, COO*H*); ^{13}C NMR (DMSO-d_6, 125 MHz) δ (ppm): 21.0 (CH_3), 27.0 (CH_3), 104.4 (C), 117.5 (CH), 117.8 (CH), 121.8 (C), 126.7 (CH), 127.7 (CH), 138.6 (C), 139.0 (C), 164.8 (C), 187.3 (C); IR ν (cm^{-1}): 1659, 1627, 1514, 1485, 1464, 1364, 1338, 1294, 1277, 1236, 1195, 1182, 1148, 1117, 1044, 1013, 939, 875, 816, 778, 749, 669, 622, 552, 507, 419.

*4.4.5. 3-Acetyl-6,8-dimethylindolizin-1-yl-carboxylic acid (**21e**)*

The general procedure was used with 1.5 g (5.78 mmol) of indolizine ethyl ester **20e** in 20 mL of absolute EtOH. White solid; 83% yield (1.1 g); mp (EtOH) 209–211 °C; ^{1}H NMR (DMSO-d_6, 500 MHz) δ (ppm): 2.32 (s, 3H, C*H*$_3$), 2.54 (s, 3H, C*H*$_3$), 2.71 (s, 3H, C*H*$_3$), 7.19 (s, 1H, Ar*H*), 8.07 (s, 1H, Ar*H*), 9.63 (s, 1H, Ar*H*), 12.30 (br s, 1H, COO*H*); ^{13}C NMR (DMSO-d_6, 125 MHz) δ (ppm): 17.3 (CH_3), 21.2 (CH_3), 27.4 (CH_3), 107.7 (C), 121.0 (C), 123.8 (CH), 124.7 (C), 128.0 (CH), 128.5 (C), 131.1 (CH), 136.1 (C), 164.8 (C), 187.1 (C); IR ν (cm^{-1}): 1667, 1632, 1514, 1482, 1442, 1418, 1367, 1344, 1236, 1209, 1177, 1086, 1024, 982, 949, 926, 851, 781, 760, 688, 661, 584, 556, 505, 423.

*4.4.6. 3-Acetyl-7-methoxyindolizin-1-yl-carboxylic acid (**21f**)*

The general procedure was used with 1.93 g (7.39 mmol) of indolizine ethyl ester **20f** in 30 mL of absolute EtOH. White solid; 91% yield (1.56 g); mp (EtOH) 245–246 °C; ^{1}H NMR (DMSO-d_6, 500 MHz) δ (ppm): 2.52 (s, 3H, C*H*$_3$), 3.92 (s, 3H, C*H*$_3$), 6.93 (dd, *J* = 7.6, 2.8 Hz, 1H, Ar*H*), 7.62 (d, *J* = 2.8 Hz, 1H, Ar*H*), 8.03 (s, 1H, Ar*H*), 9.65 (d, *J* = 7.6 Hz, 1H, Ar*H*), 12.36 (br s, 1H, COO*H*); ^{13}C NMR (DMSO-d_6, 125 MHz) δ (ppm): 26.3 (CH_3), 55.3 (CH_3), 96.7 (CH), 103.4 (C), 108.5 (CH), 121.0 (C), 126.7 (CH), 129.4 (CH), 140.8 (C), 158.3 (C), 164.5 (C), 186.4 (C); IR ν (cm^{-1}): 1669, 1635, 1524, 1497, 1472, 1434, 1352, 1309, 1282, 1232, 1190, 1105, 1045, 1019, 942, 889, 839, 814, 775, 759, 669, 644, 628, 532, 484, 435.

*4.4.7. 3-Acetyl-6-bromoindolizin-1-yl-carboxylic acid (**21 g**)*

The general procedure was used with 0.63 g (2.03 mmol) of indolizine ethyl ester **20 g** in 15 mL of absolute EtOH. White solid; 80% yield (0.58 g); mp (EtOH) 204–205 °C; ^{1}H NMR (DMSO-d_6, 500 MHz) δ (ppm): 2.57 (s, 3H, C*H*$_3$), 7.67 (dd, *J* = 9.6, 1.6 Hz, 1H, Ar*H*), 8.13 (s, 1H, Ar*H*), 8.24 (d, *J* = 9.2 Hz, 1H, Ar*H*), 9.94 (s, 1H, Ar*H*), 12.66 (br s, 1H, COO*H*); ^{13}C NMR (DMSO-d_6, 125 MHz) δ (ppm): 27.7 (CH_3), 107.2 (C), 110.0 (C), 120.4 (CH), 122.8 (C), 126.7 (CH), 128.2 (CH), 130.3 (CH), 137.0 (C), 164.9 (C), 188.7 (C); IR ν (cm^{-1}): 2937, 2755, 2600, 1640, 1511, 1481, 1361, 1280, 1237, 1185, 1036, 993, 933, 810, 657, 630, 479, 422.

*4.4.8. 3-Acetyl-8-bromoindolizin-1-yl-carboxylic acid (**21 h**)*

The general procedure was used with 2.13 g (6.87 mmol) of indolizine ethyl ester **20 h** in 25 mL of absolute EtOH. White solid; 93% yield (1.8 g); mp (EtOH) 196–197 °C; ^{1}H NMR (DMSO-d_6, 500 MHz) δ (ppm): 2.58 (s, 3H, C*H*$_3$), 7.07 (t, *J* = 8.7 Hz, 1H, Ar*H*), 7.77 (d, *J* = 8.4 Hz, 1H, Ar*H*), 8.13 (s, 1H, Ar*H*), 9.86 (d, *J* = 6.8 Hz, 1H, Ar*H*), 12.66 (br s, 1H, COO*H*); ^{13}C NMR (DMSO-d_6, 125 MHz) δ (ppm): 28.1 (CH_3), 110.5 (C), 111.7 (C), 115.7 (CH), 122.3 (C), 127.6 (CH), 127.7 (CH), 131.7 (CH), 134.0 (C), 165.0 (C), 188.2 (C); IR ν (cm^{-1}): 2924, 2569, 1672, 1641, 1515, 1477, 1429, 1345, 1246, 1209, 1183, 1044, 996, 938, 867, 766, 727, 691, 653, 635, 477.

*4.5. General procedure for the synthesis of acylphenothiazines **16c,f,g** and **17a-f***

Eaton's reagent was freshly prepared (4 equiv.) by mixing phosphorus pentoxide with methanesulfonic acid (1:10, w:w) under nitrogen atmosphere and magnetic stirring for 10 min at 60 °C. The carboxylic acid **21a-h** (1 equiv.) and 10*H*-phenothiazine or 10-methyl-10*H*-phenothiazine (1 equiv.) were then added to the Eaton's reagent. The medium was stirred at 50 °C for 4–24 h. After cooling to rt, the Eaton's reagent from the crude has been carefully neutralized by adding slowly an aqueous solution of $NaHCO_3$ (exothermic reaction). Ethyl acetate was then added. The organic layer was over anhydrous Na_2SO_4. After the filtration of hydrated Na_2SO_4, the filtrate was concentrated and then purified by flash chromatography on silica prepacked column (chromatographic eluent: gradient *n*-heptane/EtOAc 100/0 to 0/100) to obtain pure indolizine-phenothiazine hybrids **16c,f,g** and **17a-f.**

*4.5.1. 1-[(10H-Phenothiazin-3-yl)carbonyl]-3-acetylindolizine (**17a**)*

The general procedure was used with 0.91 g (4.47 mmol) of carboxylic acid **21a** and 0.89 g (4.47 mmol) of 10*H*-phenothiazine. Orange solid; 46% yield (0.92 g); mp (EtOAc/*n*-heptane) 228–229 °C; ^{1}H NMR ($CDCl_3$, 400 MHz) δ (ppm): 2.59 (s, 3H, C*H*$_3$), 6.04 (s, 1H, N*H*), 6.57 (d, *J* = 8.0 Hz, 1H, Ar*H*), 6.62 (d, *J* = 8.0 Hz, 1H, Ar*H*), 6.87 (t, *J* = 7.7 Hz, 1H, Ar*H*), 6.99 (t, *J* = 8.8 Hz, 2H, Ar*H*), 7.11 (t, *J* = 7.7 Hz, 1H, Ar*H*), 7.48–7.54 (m, 3H, Ar*H*), 7.77 (s, 1H, Ar*H*), 8.53 (d, *J* = 8.8 Hz, 1H, Ar*H*), 9.96 (d, *J* = 6.8 Hz, 1H, Ar*H*); ^{13}C NMR ($CDCl_3$, 100 MHz) δ (ppm): 27.37 (CH_3), 112.9 (CH), 116.4 (CH), 116.7 (CH), 117.1 (CH), 119.6 (C), 120.2 (CH + C), 120.5 (C), 122.4 (CH), 122.8 (C), 127.0 (C), 128.6 (CH), 129.2 (CH), 131.3 (CH), 132.8 (CH), 133.0 (CH), 133.5 (CH), 135.8 (C), 138.8 (C), 140.4 (C), 187.4 (C), 187.9 (C); IR ν (cm^{-1}): 3302, 2360, 1560, 1497, 1463, 1357, 1309, 1267, 1226, 1194, 1156, 1106, 1035, 860, 744, 637, 535, 424. Elem. Analysis calcd. for $C_{23}H_{16}N_2O_2S$: C, 71.86; H, 4.20; N, 7.29. Found: C, 72.18; H, 4.13; N, 7.15%.

*4.5.2. 1,3-Bisacetylindolizine (**23**)*

By-product from the synthesis of ketone **17a**. Yellow solid; 26% yield (0.17 g); mp (EtOAc/*n*-heptane) 110–112 °C; ^{1}H NMR ($CDCl_3$, 400 MHz) δ (ppm): 2.49 (s, 3H, C*H*$_3$), 2.61 (s, 3H, C*H*$_3$), 7.05 (td, *J* = 6.8, 1.2 Hz, 1H, Ar*H*), 7.44 (td, *J* = 6.8, 1.2 Hz, 1H, Ar*H*), 7.99 (s, 1H, Ar*H*), 8.45 (d, *J* = 8.8 Hz, 1H, Ar*H*), 9.88 (d, *J* = 8.8 Hz, 1H, Ar*H*); ^{13}C NMR ($CDCl_3$, 100 MHz) δ (ppm): 8.5 (CH_3), 24.8 (CH_3), 110.6 (C), 113.3 (CH), 117.1 (CH), 120.3 (C), 121.9 (CH), 125.8 (CH), 126.5 (CH), 135.0 (C), 182.6 (C), 185.3 (C); IR ν (cm^{-1}): 1622, 1509, 1481, 1440, 1411, 1359, 1333, 1306, 1260, 1205, 1167, 1106, 1017, 935, 876, 843, 759, 672, 634, 561, 421. Elem. Analysis calcd. for $C_{12}H_{11}NO_2$: C, 71.63; H, 5.51; N, 6.96. Found: C, 71.91; H, 5.42; N, 7.08%.

*4.5.3. 1-[(10H-Phenothiazin-3-yl)carbonyl]-3-acetyl-5-methylindolizine (**17b**)*

The general procedure was used with 2.31 g (10.64 mmol) of carboxylic acid **21b** and 2.12 g (10.64 mmol) of 10*H*-phenothiazine. Orange solid; 25% yield (1.0 g); mp (EtOAc/*n*-heptane) 212–214 °C; ^{1}H NMR (DMSO-d_6, 400 MHz) δ (ppm): 2.50 (s, 3H, C*H*$_3$), 2.63 (s, 3H, C*H*$_3$), 6.72 (d, *J* = 8.2 Hz, 1H, Ar*H*), 6.77 (d, *J* = 8.2 Hz, 1H, Ar*H*), 6.88 (d, *J* = 7.2 Hz, 1H, Ar*H*), 7.16 (d, *J* = 3.2 Hz, 1H, Ar*H*), 7.25 (dd, *J* = 9.1, 7.1 Hz, 1H, Ar*H*), 7.28 (d, *J* = 1.5 Hz, 1H, Ar*H*), 7.44 (d, *J* = 2.0 Hz, 1H, Ar*H*), 7.47 (d, *J* = 1.6 Hz, 1H, Ar*H*), 7.50 (d, *J* = 3.1 Hz, 1H, Ar*H*), 7.60 (dd, *J* = 7.8, 2.0 Hz, 1H, Ar*H*), 8.21 (d, *J* = 8.9 Hz, 1H, Ar*H*), 9.45 (s, 1H, N*H*); ^{13}C NMR (DMSO-d_6, 100 MHz) δ (ppm): 18.8 (CH_3), 26.6 (CH_3), 111.9 (CH), 112.4 (CH), 113.5 (C), 114.5 (CH), 114.6 (CH), 116.3 (C), 116.5 (C), 117.6 (CH), 117.9 (CH), 125.0 (CH), 126.9 (CH), 127.2 (CH), 129.4 (CH), 129.7 (CH), 131.7 (C), 135.5 (C), 135.6 (C), 136.9 (C), 143.0 (C), 145.3 (C), 186.9 (C), 195.6 (C); IR ν (cm^{-1}): 3272, 1665, 1589, 1557, 1482, 1434, 1347, 1322, 1261, 1226, 1205, 1148, 958, 917, 807, 781, 742, 716, 580, 442. Elem. Analysis calcd. for $C_{24}H_{18}N_2O_2S$: C, 72.34; H, 4.55; N, 7.03. Found: C, 72.70; H, 4.84; N, 6.98%.

*4.5.4. Bis-(5-methylindolizin-1-yl)carbonyl(10H-phenothiazin-3,7-diyl) (**22b**)*

By-product from the synthesis of ketone **17b**. Orange solid; 14%

yield (0.7 g); mp (EtOAc/*n*-heptane) 215–218 °C; ^{1}H NMR (DMSO-d_6, 400 MHz) δ (ppm): 2.55 (s, 6H, 2CH_3), 6.80 (d, J = 8.4 Hz, 2H, ArH), 6.89 (dt, J = 6.9, 1.1 Hz, 2H, ArH), 7.18 (d, J = 3.3 Hz, 2H, ArH), 7.25 (dd, J = 9.1, 6.7 Hz, 2H, ArH), 7.31 (d, J = 2.4 Hz, 2H, ArH), 7.48 (dd, J = 8.2, 1.8 Hz, 2H, ArH), 7.51 (d, J = 3.3 Hz, 2H, ArH), 8.22 (d, J = 9.1 Hz, 2H, ArH), 9.38 (s, 1H, NH); ^{13}C NMR (DMSO-d_6, 100 MHz) δ (ppm): 18.8 (2CH_3), 112.0 (2CH), 112.4 (2CH), 113.5 (2C), 114.4 (2CH), 116.3 (2CH), 117.6 (2CH), 117.9 (2CH), 124.9 (2CH), 127.3 (2C), 129.7 (2C), 135.1 (2CH), 135.6 (2C), 136.9 (2C), 143.7 (2C), 186.9 (2C); IR ν (cm^{-1}): 3301, 1633, 1585, 1555, 1479, 1427, 1352, 1287, 1193, 1151, 1124, 1069, 916, 829, 781, 734, 704, 680, 630, 575, 437. Elem. Analysis calcd. for $C_{32}H_{23}N_3O_2S$: C, 74.83; H, 4.51; N, 8.18. Found: C, 75.20; H, 4.75; N, 8.36%.

4.5.5. 5-Methylindolizin-1-carboxylic acid (**24**)

By-product from the synthesis of ketone **17b**. Orange solid; 6% yield (0.11 g); mp (EtOAc/*n*-heptane) 181–184 °C; ^{1}H NMR ($CDCl_3$, 400 MHz) δ (ppm): 2.59 (s, 3H, CH_3), 6.63 (d, J = 6.4 Hz, 1H, ArH), 7.10 (t, J = 6.8 Hz, 1H, ArH), 7.19 (d, J = 2.8 Hz, 1H, ArH), 7.40 (d, J = 2.8 Hz, 1H, ArH), 8.21 (d, J = 8.8 Hz, 1H, ArH); ^{13}C NMR ($CDCl_3$, 100 MHz) δ (ppm): 17.7 (CH_3), 103.2 (C), 109.5 (CH), 110.8 (CH), 115.5 (CH), 116.5 (CH), 121.4 (CH), 133.0 (C), 135.4 (C), 166.4 (C); IR ν (cm^{-1}): 1645, 1523, 1475, 1452, 1365, 1290, 1259, 1192, 1151, 1110, 1048, 989, 910, 790, 763, 737, 706, 570, 513, 439. Elem. Analysis calcd. for $C_{10}H_9NO_2$: C, 68.56; H, 5.18; N, 8.00. Found: C, 68.72; H, 5.34; N, 8.19%.

4.5.6. 1-[(10H-Phenothiazin-3-yl)carbonyl]-3-acetyl-5-methylindolizine (**17c**)

The general procedure was used with 1.99 g (9.18 mmol) of carboxylic acid **21c** and 1.83 g (9.18 mmol) of 10*H*-phenothiazine. Yellow solid; 36% yield (1.2 g); mp (EtOAc/*n*-heptane) 200–202 °C; ^{1}H NMR (DMSO-d_6, 400 MHz) δ (ppm): 2.27 (s, 3H, CH_3), 2.53 (s, 3H, CH_3), 6.72 (m, J = 8.4, 8.0, 1.6 Hz, 2H, ArH), 6.80 (t, J = 8.0 Hz, 1H, ArH), 6.92 (d, J = 7.6 Hz, 1H, ArH), 7.01 (t, J = 8.0 Hz, 1H, ArH), 7.18 (t, J = 6.8 Hz, 1H, ArH), 7.31 (d, J = 6.8 Hz, 1H, ArH), 7.40 (br s, 1H, ArH), 7.52 (dd, J = 8.0, 2.0 Hz, 1H, ArH), 7.89 (s, 1H, ArH), 9.16 (br s, 1H, NH), 9.80 (d, J = 7.6 Hz, 1H, ArH); ^{13}C NMR (DMSO-d_6, 100 MHz) δ (ppm): 20.7 (CH_3), 27.8 (CH_3), 113.9 (CH), 114.0 (C), 115.4 (CH), 115.7 (CH), 116.0 (C), 116.4 (CH), 116.7 (CH), 121.6 (C), 123.2 (CH), 126.3 (C), 126.7 (CH), 127.5 (CH), 128.2 (CH), 128.2 (C), 129.4 (CH), 131.5 (CH), 132.7 (C), 137.5 (C), 140.5 (C), 146.3 (C), 187.8 (C), 188.0 (C); IR ν (cm^{-1}): 3315, 1610, 1591, 1561, 1504, 1470, 1425, 1397, 1304, 1214, 1145, 1072, 949, 927, 836, 753, 703, 635, 582. Elem. Analysis calcd. for $C_{24}H_{18}N_2O_2S$: C, 72.34; H, 4.55; N, 7.03. Found: C, 72.11; H, 4.25; N, 6.77%.

4.5.7. 1-[(10H-Phenothiazin-10-yl)carbonyl]-3-acetyl-8-methylindolizine (**16c**)

By-product from the synthesis of ketone **17c**. Yellow solid; 14% yield (0.49 g); mp (EtOAc/*n*-heptane) 203–206 °C; ^{1}H NMR (DMSO-d_6, 400 MHz) δ (ppm): 2.48 (s, 3H, CH_3), 2.59 (s, 3H, CH_3), 6.26 (d, J = 8.1 Hz, 2H, ArH), 6.78–6.92 (m, 6H, ArH), 7.00 (dd, J = 7.3, 1.9 Hz, 2H, ArH), 7.59 (s, 1H, ArH), 9.89 (d, J = 6.5 Hz, 1H, ArH); ^{13}C NMR (DMSO-d_6, 100 MHz) δ (ppm): 18.0 (CH_3), 27.4 (CH_3), 114.4 (CH + C), 115.3 (C), 115.9 (2CH), 119.8 (2C), 121.3 (C), 122.5 (2CH), 124.8 (CH), 125.2 (CH), 126.5 (CH), 126.6 (2CH), 127.0 (2CH), 128.2 (2C), 135.6 (C), 144.5 (C), 186.6 (C); IR ν (cm^{-1}): 1629, 1587, 1460, 1433, 1337, 1306, 1278, 1238, 1066, 1035, 951, 773, 745, 651, 562, 450. Elem. Analysis calcd. for $C_{24}H_{18}N_2O_2S$: C, 72.34; H, 4.55; N, 7.03. Found: C, 72.49; H, 4.58; N, 6.93%.

4.5.8. 3-Acetyl-8-methylindolizine (**25**)

By-product from the synthesis of ketone **17c**. Orange oil; 32% yield (0.5 g); ^{1}H NMR ($CDCl_3$, 400 MHz) δ (ppm): 2.48 (s, 3H, CH_3), 2.55 (s, 3H, CH_3), 6.48 (d, J = 4.4 Hz, 1H, ArH), 6.79 (t, J = 7.2 Hz, 1H, ArH), 6.92 (d, J = 6.8 Hz, 1H, ArH), 7.49 (d, J = 4.4 Hz, 1H, ArH), 9.73 (d, J = 7.2 Hz, 1H, ArH); ^{13}C NMR ($CDCl_3$, 100 MHz) δ (ppm): 18.1 (CH_3), 27.1 (CH_3), 100.3 (CH), 113.6 (CH), 122.9 (CH), 123.2 (C), 123.3 (CH), 126.4 (CH), 127.6 (C), 139.4 (C), 186.5 (C). Elem. Analysis calcd. for $C_{11}H_{11}NO$: C, 76.28; H, 6.40; N, 8.09. Found: C, 76.54; H, 6.64; N, 8.18%.

4.5.9. 1-[(10H-Phenothiazin-3-yl)carbonyl]-3-acetyl-7-methylindolizine (**17d**)

The general procedure was used with 0.63 g (2.91 mmol) of carboxylic acid **21d** and 0.58 g (2.91 mmol) of 10*H*-phenothiazine. Green solid; 44% yield (0.44 g); mp (EtOAc/*n*-heptane) 122–125 °C; ^{1}H NMR (DMSO-d_6, 400 MHz) δ (ppm): 2.52 (s, 3H, CH_3), 2.54 (s, 3H, CH_3), 6.70–6.82 (m, 3H, ArH), 6.93 (d, J = 8.3 Hz, 1H, ArH), 7.01 (t, J = 8.9 Hz, 1H, ArH), 7.17 (d, J = 8.9 Hz, 1H, ArH), 7.34 (s, 1H, ArH), 7.52 (d, J = 8.9 Hz, 1H, ArH), 7.98 (s, 1H, ArH), 8.26 (s, 1H, ArH), 9.06 (s, 1H, NH), 9.74 (d, J = 7.7 Hz, 1H, ArH); ^{13}C NMR (DMSO-d_6, 100 MHz) δ (ppm): 21.0 (CH_3), 27.1 (CH_3), 111.3 (C), 113.5 (CH), 114.7 (CH), 115.9 (CH), 116.3 (CH), 118.1 (CH), 118.6 (C), 121.7 (C), 122.5 (CH), 126.2 (C), 126.8 (C), 127.4 (C), 127.7 (CH), 129.5 (C), 132.8 (CH), 139.6 (C), 139.8 (CH), 140.4 (2CH), 145.0 (C), 186.5 (C), 187.5 (C); IR ν (cm^{-1}): 3275, 2919, 1634, 1591, 1563, 1503, 1462, 1420, 1362, 1336, 1289, 1228, 1197, 1146, 1083, 1013, 932, 883, 827, 799, 774, 740, 701, 620, 550, 422. Elem. Analysis calcd. for $C_{24}H_{18}N_2O_2S$: C, 72.34; H, 4.55; N, 7.03. Found: C, 72.62; H, 4.55; N, 7.36%.

4.5.10. 1-[(10-Methyl-10H-phenothiazin-3-yl)carbonyl]-3-acetyl-7-methylindolizine (**17d'**)

The general procedure was used with 1.8 g (8.30 mmol) of carboxylic acid **21d** and 1.77 g (8.30 mmol) of 10-methyl-10*H*-phenothiazine. Orange solid; 34% yield (1.13 g); mp (EtOAc/*n*-heptane) 144–145 °C; ^{1}H NMR ($CDCl_3$, 500 MHz) δ (ppm): 2.50 (s, 3H, CH_3), 2.54 (s, 3H, CH_3), 3.46 (s, 3H, CH_3), 6.87 (d, J = 7.5 Hz, 1H, ArH), 6.90 (d, J = 8.5 Hz, 1H, ArH), 6.95 (dd, J = 7.0, 2.0 Hz, 1H, ArH), 6.99 (td, J = 7.5, 1.0 Hz, 1H, ArH), 7.17 (dd, J = 7.5, 1.5 Hz, 1H, ArH), 7.21 (td, J = 8.0, 1.5 Hz, 1H, ArH), 7.50 (d, J = 1.5 Hz, 1H, ArH), 7.69 (dd, J = 8.0, 2.0 Hz, 1H, ArH), 7.71 (s, 1H, ArH), 8.39 (t, J = 1.5 Hz, 1H, ArH), 9.83 (d, J = 7.0 Hz, 1H, ArH); ^{13}C NMR ($CDCl_3$, 125 MHz) δ (ppm): 21.7 (CH_3), 27.3 (CH_3), 35.8 (CH_3), 112.5 (C), 113.5 (CH), 114.6 (CH), 118.7 (CH), 119.3 (CH), 122.5 (C), 123.1 (C), 123.3 (CH), 123.6 (C), 127.5 (CH), 127.8 (CH), 127.9 (CH), 128.1 (CH), 128.7(CH), 129.3 (CH), 134.5 (C), 140.4 (C), 140.9 (C), 144.9 (C), 148.9 (C), 187.6 (C), 188.7 (C); IR ν (cm^{-1}): 1704, 1614, 1573, 1505, 1462, 1334, 1286, 1251, 1231, 1197, 1141, 1105, 1013, 931, 885, 832, 805, 763, 745, 619, 55, 425. Elem. Analysis calcd. for $C_{25}H_{20}N_2O_2S$: C, 72.79; H, 4.89; N, 6.79. Found: C, 72.93; H, 5.10; N, 6.98%.

4.5.11. 1-[(10H-Phenothiazin-3-yl)carbonyl]-3-acetyl-6,8-dimethylindolizine (**17e**)

The general procedure was used with 1.8 g (7.78 mmol) of carboxylic acid **21e** and 1.55 g (7.78 mmol) of 10*H*-phenothiazine. Orange solid; 27% yield (0.91 g); mp (EtOAc/*n*-heptane) 199–202 °C; ^{1}H NMR (DMSO-d_6, 500 MHz) δ (ppm): 2.35 (s, 6H, 2CH_3), 2.50 (s, 3H, CH_3), 6.71 (d, J = 8.0 Hz, 1H, ArH), 6.72 (d, J = 8.5 Hz, 1H, ArH), 6.80 (t, J = 7.5 Hz, 1H, ArH), 6.91 (d, J = 7.5 Hz, 1H, ArH), 7.00 (t, J = 7.5 Hz, 1H, ArH), 7.20 (s, 1H, ArH), 7.38 (d, J = 2.0 Hz, 1H, ArH), 7.50 (dd, J = 8.0, 2.0 Hz, 1H, ArH), 7.82 (s, 1H, ArH), 9.15 (s, 1H, NH), 9.65 (s, 1H, ArH); ^{13}C NMR (DMSO-d_6, 125 MHz) δ (ppm): 17.9 (CH_3), 20.2 (CH_3), 27.4 (CH_3), 113.5 (CH), 114.9 (CH), 115.1 (C), 115.9 (CH), 116.0 (C), 116.2 (C), 120.9 (C), 122.8 (CH), 123.7 (CH), 125.0 (C), 126.3 (CH), 127.0 (CH), 127.7 (CH), 128.4 (C), 130.6 (CH), 131.0 (CH), 132.3 (C), 135.9 (C), 140.1 (C), 145.8 (C), 187.2 (C), 187.4 (C); IR ν (cm^{-1}): 3275, 2919, 1634, 1591, 1563, 1503, 1462, 1420, 1362, 1336, 1289, 1228, 1197, 1146, 1083, 1013, 932, 883, 827, 799, 774, 740, 701, 620, 550, 422. Elem. Analysis calcd. for $C_{25}H_{20}N_2O_2S$: C, 72.79; H,

4.89; N, 6.79. Found: C, 73.08; H, 5.01; N, 6.99%.

4.5.12. 1,3-Bisacetyl-6,8-dimethylindolizine (26)

By-product from the synthesis of ketone **17e**. Orange solid; 6% yield (0.1 g); mp (EtOAc/*n*-heptane) 141–144 °C; ^{1}H NMR ($CDCl_3$, 500 MHz) δ (ppm): 2.35 (s, 3H, C*H*$_3$), 2.46 (s, 3H, C*H*$_3$), 2.59 (s, 3H, C*H*$_3$), 2.63 (s, 3H, C*H*$_3$), 7.04 (s, 1H, Ar*H*), 7.98 (s, 1H, Ar*H*), 9.69 (s, 1H, Ar*H*); ^{13}C NMR ($CDCl_3$, 100 MHz) δ (ppm): 12.4 (CH_3), 18.4 (CH_3), 21.4 (CH_3), 27.6 (CH_3), 115.3 (C), 121.9 (C), 125.0 (CH), 125.6 (C), 126.8 (CH), 129.3 (C), 132.0 (CH), 135.3 (C), 186.5 (C), 187.4 (C). IR ν (cm^{-1}): 2918, 1667, 1639, 1513, 1478, 1432, 1404, 1357, 1277, 1225, 1176, 1142, 1101, 1040, 982, 950, 886, 855, 798, 754, 661, 575, 524, 481. Elem. Analysis calcd. for $C_{14}H_{15}NO_2$: C, 73.34; H, 6.59; N, 6.11. Found: C, 73.50; H, 6.88; N, 6.39%.

4.5.13. 3-Acetyl-6,8-dimethylindolizine (27) [34]

By-product from the synthesis of ketone **17e**. Yellow solid; 35% yield (0.51 g); mp (EtOAc/*n*-heptane) 57–59 °C; ^{1}H NMR ($CDCl_3$, 400 MHz) δ (ppm): 2.31 (s, 3H, C*H*$_3$), 2.44 (s, 3H, C*H*$_3$), 2.53 (s, 3H, C*H*$_3$), 6.42 (d, *J* = 4.4 Hz, 1H, Ar*H*), 6.79 (s, 1H, Ar*H*), 7.41 (d, *J* = 4.8 Hz, 1H, Ar*H*), 9.56 (s, 1H, Ar*H*); ^{13}C NMR ($CDCl_3$, 100 MHz) δ (ppm): 17.7 (CH_3), 18.3 (CH_3), 26.9 (CH_3), 99.8 (CH), 122.0 (C), 122.7 (CH), 123.1 (C), 124.1 (CH), 126.0 (CH), 126.5 (C), 138.0 (C), 186.1 (C). IR ν (cm^{-1}): 2920, 1613, 1511, 1464, 1434, 1381, 1343, 1286, 1224, 1197, 1169, 1135, 1105, 1035, 970, 948, 912, 841, 780, 744, 672, 582, 479, 443. Elem. Analysis calcd. for $C_{12}H_{13}NO$: C, 76.98; H, 7.00; N, 7.48. Found: C, 77.21; H, 7.13; N, 7.26%.

4.5.14. 1-[(10H-Phenothiazin-3-yl)carbonyl]-3-acetyl-6,8-dimethylindolizine (17e')

The general procedure was used with 0.58 g (2.51 mmol) of carboxylic acid **21e** and 0.54 g (2.53 mmol) of 10-methyl-10*H*-phenothiazine. Green solid; 24% yield (0.24 g); mp (EtOAc/*n*-heptane) 150–151 °C; ^{1}H NMR ($CDCl_3$, 400 MHz) δ (ppm): 2.37 (s, 3H, C*H*$_3$), 2.47 (s, 3H, C*H*$_3$), 2.50 (s, 3H, C*H*$_3$), 3.44 (s, 3H, C*H*$_3$), 6.85 (d, *J* = 8.5 Hz, 2H, Ar*H*), 6.97 (td, *J* = 7.8, 1.3 Hz, 1H, Ar*H*), 7.04 (s, 1H, Ar*H*), 7.13 (dd, *J* = 7.8, 1.6 Hz, 1H, Ar*H*), 7.18 (td, *J* = 7.8, 1.6 Hz, 1H, Ar*H*), 7.54 (s, 1H, ArH), 7.71 (d, *J* = 2.0 Hz, 1H, Ar*H*), 7.75 (dd, *J* = 8.3, 2.0 Hz, 1H, Ar*H*), 9.73 (s, 1H, ArH); ^{13}C NMR ($CDCl_3$, 100 MHz) δ (ppm): 18.4 (CH_3), 20.9 (CH_3), 27.4 (CH_3), 35.7 (CH_3), 113.2 (CH), 114.5 (CH), 115.5 (C), 121.4 (C), 122.8 (C), 123.2 (C), 123.3 (CH), 124.9 (CH), 125.4 (C), 127.3 (CH), 127.3 (CH), 127.6 (CH), 127.7 (CH), 129.0 (CH), 129.2 (C), 131.3 (CH), 133.7 (C), 137.2 (C), 144.6 (C), 149.5 (C), 187.2 (C), 188.8 (C); IR ν (cm^{-1}): 2920, 1620, 1572, 1505, 1462, 1414, 1335, 1253, 1234, 1207, 1174, 1139, 1039, 980, 948, 925, 849 822, 770, 740, 664, 648, 584, 546, 470, 436. Elem. Analysis calcd. for $C_{26}H_{22}N_2O_2S$: C, 73.21; H, 5.20; N, 6.57. Found: C, 73.44; H, 5.50; N, 6.75%.

4.5.15. 1-[(10H-Phenothiazin-3-yl)carbonyl]-3-acetyl-7-methoxyindolizine (17f)

The general procedure was used with 0.9 g (3.86 mmol) of carboxylic acid **21f** and 0.77 g (3.86 mmol) of 10*H*-phenothiazine. Green solid; 31% yield (0.48 g); mp (EtOAc/*n*-heptane) 202–205 °C; ^{1}H NMR ($CDCl_3$, 400 MHz) δ (ppm): 2.51 (s, 3H, C*H*$_3$), 3.94 (s, 3H, C*H*$_3$), 6.02 (br s, 1H, N*H*), 6.55 (dd, *J* = 7.9, 1.0 Hz, 1H, Ar*H*), 6.60 (d, *J* = 8.3 Hz, 1H, Ar*H*), 6.75 (dd, *J* = 7.5, 2.8 Hz, 1H, Ar*H*), 6.86 (td, *J* = 7.4, 1.2 Hz, 1H, Ar*H*), 6.95–7.00 (m, 2H, Ar*H*), 7.46 (d, *J* = 1.7 Hz, 1H, Ar*H*), 7.48 (dd, *J* = 7.9, 1.7 Hz, 1H, Ar*H*), 7.67 (s, 1H, Ar*H*), 7.96 (d, *J* = 2.7 Hz, 1H, Ar*H*), 9.76 (d, *J* = 7.9 Hz, 1H, Ar*H*); ^{13}C NMR ($CDCl_3$, 100 MHz) δ (ppm): 27.2 (CH_3), 56.0 (CH_3), 98.5 (CH), 109.7 (CH), 111.4 (CH), 113.8 (CH), 115.1 (CH), 116.6 (C), 117.0 (C), 121.8 (CH), 122.6 (CH), 126.4 (C), 127.1 (C), 127.8 (CH), 127.9 (CH), 129.4 (CH), 130.2 (CH), 133.5 (C), 141.0 (C), 142.6 (C), 145.4 (C), 160.1 (C), 187.0 (C), 187.1 (C); IR ν (cm^{-1}): 3264, 1643, 1616, 1569, 1493, 1470, 1427, 1362, 1334, 1301, 1274, 1217, 1189, 1113, 1073, 1028, 944, 822, 741, 688, 624, 535, 437. Elem. Analysis calcd. for $C_{24}H_{18}N_2O_3S$: C, 69.55; H, 4.38; N, 6.76. Found: C, 69.94; H, 4.60; N, 7.13%.

4.5.16. 1-[(10H-Phenothiazin-10-yl)carbonyl]-3-acetyl-7-methoxyindolizine (16f)

By-product from the synthesis of ketone **17f**. Pink solid; 32% yield (0.5 g); mp (EtOAc/*n*-heptane) 222–225 °C; ^{1}H NMR (DMSO-d_6, 400 MHz) δ (ppm): 2.14 (s, 3H, C*H*$_3$), 3.91 (s, 3H, C*H*$_3$), 6.65 (s, 1H, Ar*H*), 6.87 (dd, *J* = 7.9, 2.8 Hz, 1H, Ar*H*), 7.30–7.33 (m, 4H, Ar*H*), 7.53 (d, *J* = 2.8 Hz, 1H, Ar*H*), 7.61–7.64 (m, 4H, Ar*H*), 9.52 (d, *J* = 7.9 Hz, 1H, Ar*H*); ^{13}C NMR (DMSO-d_6, 100 MHz) δ (ppm): 26.7 (CH_3), 56.2 (CH_3), 97.6 (CH), 105.8 (CH), 109.5 (C), 120.9 (CH), 126.6 (CH), 127.2 (2CH), 127.6 (2CH), 127.7 (2CH), 128.0 (2CH), 129.9 (C), 132.3 (2C), 139.7 (2C), 141.9 (C), 158.8 (C), 162.9(C), 186.5 (C); IR ν (cm^{-1}): 1622, 1519, 1455, 1425, 1362, 1303, 1238, 1190, 1128, 1082, 1023, 931, 837, 808, 757, 725, 690, 661, 629, 526, 477. Elem. Analysis calcd. for $C_{24}H_{18}N_2O_3S$: C, 69.55; H, 4.38; N, 6.76. Found: C, 69.90; H, 4.53; N, 7.01%.

4.5.17. 1,3-Bisacetyl-7-methoxyindolizine (28)

By-product from the synthesis of ketone **17f**. White-off solid; 22% yield (0.2 g); mp (EtOAc/*n*-heptane) 117–118 °C; ^{1}H NMR ($CDCl_3$, 400 MHz) δ (ppm): 2.48 (s, 3H, C*H*$_3$), 2.56 (s, 3H, C*H*$_3$), 3.93 (s, 3H, C*H*$_3$), 6.71 (d, *J* = 8.0 Hz, 1H, Ar*H*), 7.79 (s, 1H, Ar*H*), 7.90 (s, 1H, Ar*H*), 9.71 (d, *J* = 8.0 Hz, 1H, Ar*H*); ^{13}C NMR ($CDCl_3$, 100 MHz) δ (ppm): 11.0 (CH_3), 26.9 (CH_3), 55.8 (CH_3), 97.6 (CH), 109.6 (CH), 111.6 (C), 122.3 (C), 125.1 (CH), 130.4 (CH), 140.3 (C), 160.4 (C), 185.2 (C), 187.2 (C). IR ν (cm^{-1}): 1648, 1615, 1511, 1459, 1427, 1361, 1303, 1266, 1239, 1195, 1154, 1122, 1087, 1023, 944, 908, 840, 815, 741, 683, 632, 599, 510, 428. Elem. Analysis calcd. for $C_{13}H_{13}NO_3$: C, 67.52; H, 5.67; N, 6.06. Found: C, 67.67; H, 6.03; N, 6.41%.

4.5.18. 1-[(10H-Phenothiazin-10-yl)carbonyl]-3-acetyl-6-bromoindolizine (16 g)

The general procedure was used with 0.37 g (1.31 mmol) of carboxylic acid **21 g** and 0.26 g (1.31 mmol) of 10*H*-phenothiazine. White-pink solid; 47% yield (0.27 g); mp (EtOAc/*n*-heptane) 107–108 °C; ^{1}H NMR ($CDCl_3$, 400 MHz) δ (ppm): 2.31 (s, 3H, C*H*$_3$), 6.81 (t, *J* = 7.6 Hz, 1H, Ar*H*), 7.02 (s, 1H, Ar*H*), 7.13–7.15 (m, 4H, Ar*H*), 7.42–7.44 (m, 2H, Ar*H*), 7.46–7.51 (m, 2H, Ar*H*), 7.55 (d, *J* = 8.6 Hz, 1H, Ar*H*), 9.81 (d, *J* = 7.8 Hz, 1H, Ar*H*); ^{13}C NMR ($CDCl_3$, 100 MHz) δ (ppm): 27.3 (CH_3), 111.8 (C), 112.8 (C), 114.3 (CH), 122.1 (C), 123.5 (CH), 126.6 (2CH), 127.0 (2CH), 127.2 (2CH), 127.5 (2CH), 127.6 (CH), 129.3 (CH), 132.3 (2C), 134.4 (C), 139.1 (2C), 164.1 (C), 187.4 (C); IR ν (cm^{-1}): 1668, 1650, 1631, 1530, 1460, 1428, 1395, 1363, 1339, 1311, 1282, 1253, 1198, 1180, 1126, 1084, 1005, 943, 825, 754, 729, 688, 657, 630, 453. Elem. Analysis calcd. for $C_{23}H_{15}BrN_2O_2S$: C, 59.62; H, 3.26; N, 6.05. Found: C, 59.78; H, 3.43; N, 6.09%.

4.5.19. 3-Acetyl-8-bromoindolizine (29) [34]

The general procedure was used with 0.91 g (3.22 mmol) of carboxylic acid **21 h** and 0.64 g (3.22 mmol) of 10*H*-phenothiazine. White solid; 74% yield (0.57 g); mp (EtOAc/*n*-heptane) 74–75 °C; ^{1}H NMR ($CDCl_3$, 400 MHz) δ (ppm): 2.55 (s, 3H, C*H*$_3$), 6.64 (d, *J* = 4.6 Hz, 1H, Ar*H*), 6.68 (t, *J* = 7.4 Hz, 1H, Ar*H*), 7.29 (d, *J* = 7.4 Hz, 1H, Ar*H*), 7.49 (d, *J* = 4.6 Hz, 1H, Ar*H*), 9.77 (d, *J* = 7.0 Hz, 1H, Ar*H*); ^{13}C NMR ($CDCl_3$, 100 MHz) δ (ppm): 27.3 (CH_3), 103.3 (CH), 112.6 (C), 113.3 (CH), 123.6 (CH), 124.1 (C), 125.8 (CH), 127.3 (CH), 137.4 (C), 187.2 (C). IR ν (cm^{-1}): 1633, 1506, 1463, 1423, 1378, 1358, 1327, 1281, 1234, 1187, 1166, 1048, 1014, 932, 885, 780, 756, 708, 663, 568. Elem. Analysis calcd. for $C_{10}H_8BrNO$: C, 50.45; H, 3.39; N, 5.88. Found: C, 50.89; H, 3.72; N, 6.13%.

4.6. Tubulin polymerization assay

Sheep brain tubulin was purified according to the method of

Shelanski [35] by two cycles assembly–disassembly and then dissolved in the assembly buffer containing 0.1 M MES, 0.5 mM $MgCl_2$, 1 mM EGTA, and 1 mM of GTP (pH 6.6) to give a tubulin concentration of about 2–3 mg/mL. Tubulin assembly was monitored by fluorescence according to reported procedure [36] using DAPI as fluorescent molecule. Assays were realized on 96-well plates prepared with Biomek NKMC and Biomek 3000 from Beckman coulter and read at 37 °C on Wallac Victor fluorimeter from Perkin–Elmer. Each measurement was reproduced twice (two independent experiments on different 96-well plates) in duplicate. The IC_{50} value of each compound was determined as tubulin polymerization inhibition by 50% compared to the rate in the absence of compound. The IC_{50} values for all compounds were compared to the IC_{50} of phenstatin and (-)-desoxypodophyllotoxin and measured the same day under the same conditions.

4.7. Human FTase assay [37]

Assays were realized in 96-well plates, prepared with a Biomek NKMC and a Biomek 3000 from Beckman Coulter and read on a Wallac Victor fluorimeter from PerkineElmer. Per well, 20 μL of farnesyl pyrophosphate (10 μM) was added to 180 μL of a solution containing 2 μL of varied concentrations of potential inhibitors (dissolved in DMSO) and 178 μL of a solution composed by 10 μL of partially purified recombinant human FTase (5 mg/mL) and 1.0 mL of Dansyl-GCVLS peptide (in the following buffer: 5.6 mM DTT, 5.6 mM $MgCl_2$, 12 μM $ZnCl_2$ and 0.2% (w/v) octyl-ß-D-glucopyranoside, 52 mM Tris/HCl, pH 7.5). Fluorescence was recorded for 15 min (0.7 s per well, 20 repeats) at 30 °C with an excitation filter at 340 nm and an emission filter of 486 nm. Each measurement was reproduced twice (two independent experiments on different 96-well plates) in duplicate. The kinetic experiments were realized under the same conditions, either with FPP as varied substrate with a constant concentration of Dns-GCVLS of 2.5 μM, or with Dns-GCVLS as varied substrate with a constant concentration of FPP of 10 μM. Nonlinear regressions were performed with Excel software.

4.8. Cell proliferation assay

The compounds were tested against a panel of 60 human cancer cell lines at the National Cancer Institute, Germantown, MD, USA [38]. The cytotoxicity studies were conducted using a 48 h exposure protocol using the sulforhodamine B assay [39].

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Author contributions

A.G. wrote the manuscript, performed experiments on tubulin polymerization and on FTase *in vitro* and analyzed the data; I.-M. M. performed the organic synthesis and characterization of molecules; J.D. performed experiments on tubulin polymerization; A.F. performed molecular modeling on tubulin and FTase; E.B. supervised the organic synthesis and contributed to the writing of the manuscript.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bioorg.2020.104184.

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