Published in final edited form as:
J Med Chem. 2008 October 9; 51(19): 5953–5957. doi:10.1021/jm800203e.

Novel Microtubule-Interacting Phenoxy Pyridine and Phenyl Sulfanyl Pyridine Analogues for Cancer Therapy

Ravi Kumar Anchoori[†], **Madeleine Susanne Quirine Kortenhorst**[†,‡], **Manuel Hidalgo**[§], **Taradas Sarkar**[‖], **Gurulingappa Hallur**[†], **Ruoli Bai**[‖], **Paul J. Van Diest**[‡], **Ernest Hamel**[‖], and **Saeed R. Khan**[*,†]

†*Division of Chemical Therapeutics, The Sidney Kimmel Comprehensive Cancer Center at Johns Hopkins, 1650 Orleans Street, CRB-I, 162 C, Baltimore, Maryland 21231*

‡*Pathology Department, University Medical Center Utrecht, Heidelberglaan 100, 3584 CX, Utrecht, Netherlands*

§*Gastrointestinal Cancer Program, The Sidney Kimmel Comprehensive Cancer Center at Johns Hopkins, 1650 Orleans Street, CRB-I, 162 A, Baltimore, Maryland 21231*

‖*Toxicology and Pharmacology Branch, Developmental Therapeutics Program, Division of Cancer Treatment and Diagnosis, National Cancer Institute at Frederick, National Institutes of Health, Frederick, Maryland 21702*

Abstract

Current microtubule inhibitory agents used in the clinic to treat cancer have severe side effects, and development of resistance is frequent. We have evaluated the antitumor effect of a novel 30-compound library of phenoxy pyridine and phenyl sulfanyl pyridine derivatives. MTT assays revealed that, of all 30 compounds tested, compounds **2** and **3** showed the largest decrease in proliferation (low μM range) against Panc1 and HS766T human pancreatic cancer cells. Flow cytometry experiments with MCF7 breast cancer cells showed a G2/M arrest comparable to that of colcemid. Immunofluorescence staining demonstrated complete disappearance of intracellular microtubules. Tubulin assembly assays, however, showed a dose-dependent decrease in tubulin assembly with compound **3** that seemed limited to about 50% of the control reaction. With compound **2** treatment, there was only a delay in the onset of assembly, with no effect on the extent of the reaction. Taken together, our results show that these novel microtubule inhibitors have promising anticancer activity and can be potentially used to overcome paclitaxel resistance in the clinical setting.

Introduction

Cancer is a major worldwide health problem. Improvements in treatment and prevention have led to a decrease in cancer deaths, but the number of new diagnoses continues to rise. Treatment of cancer cells with agents that interfere with microtubule assembly causes mitotic arrest and eventually cell death. Current microtubule inhibitory agents used in the clinic have severe side effects, and development of resistance is frequent. We have designed and synthesized a novel 30-compound library of phenoxy pyridine (PP[a]) and phenyl sulfanyl pyridine (PSP) derivatives and studied their effects in pancreatic cancer, breast cancer, and Burkitt lymphoma cells.

*To whom correspondence should be addressed. Phone: (410)614-0200. Fax: (410)614-8397. E-mail: khansa@jhmi.edu.

Supporting Information Available: Spectral data for all the compounds and experimental procedures for biological evaluation. This material is available free of charge via the Internet at http:// pubs.acs.org.

Our strategy, as outlined in this paper, was to discover new microtubule inhibitors using a small molecule library of compounds that contained the PP or PSP core structures. Previously, we published work from our laboratory related to design, synthesis, and evaluation of novel benzoylphenylurea (BPU) compounds as microtubule inhibitors.[1,2] BPU and its derivatives were originally developed as insecticides,[3,4] but they were later found to possess cytotoxic activity.[4] BPU derivatives are known to inhibit tubulin polymerization, cause microtubule depolymerization in vitro, and demonstrate activity against solid tumors.[5,6]

N,N-dimethylamino-benzoylphenylurea **1**[6] is a novel, small-molecule, orally available, tubulin-interactive agent that is currently undergoing phase I clinical evaluation in refractory solid tumors in humans. When administered on a continuous weekly schedule, the dose-limiting toxicity (DLT) of NSC 639829 was severe myelosuppression. This DLT correlated with continual accumulation of the parent compound and cytotoxic metabolites. An alternative approach of an interrupted schedule (6 weeks on/2 weeks off) appears to prevent severe myelosuppression while maintaining antitumor efficacy.

---

[a]Abbreviations

MTT 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

PP phenoxy pyridine

PSP phenyl sulfanyl pyridine

BPU benzoyl phenyl urea

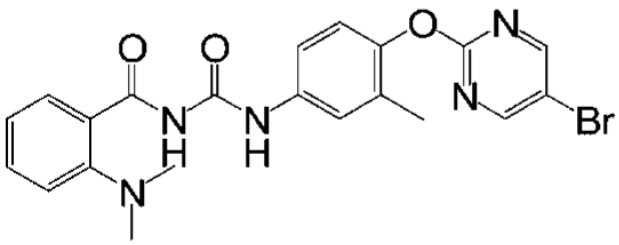
DLT dose limiting toxicity

DMF dimethyl formamide

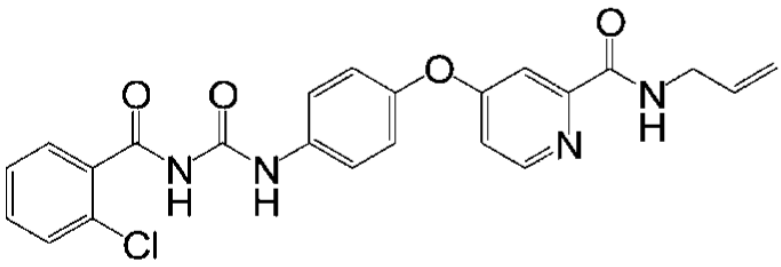
$SOCl_2$ thionyl chloride

MPM2 mitotic protein monoclonal 2

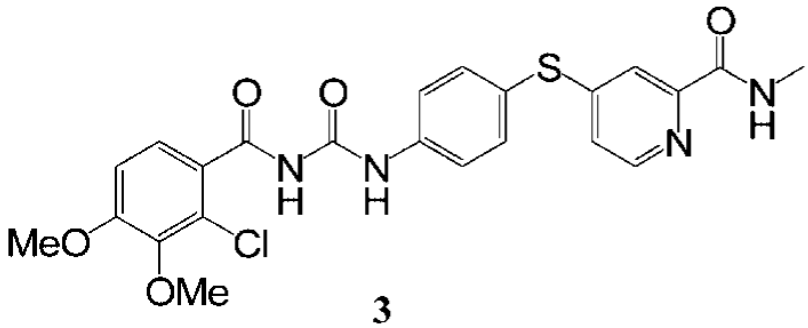
DAPI 4,6-diamidino-2-phenylindole



1 (NSC 639829)



2



3

In the meantime, BPU analogues are being synthesized to optimize potency and improve physicochemical properties. This effort has led to the development of a highly potent, novel series of modified sulfur BPU compounds. These analogues were synthesized in excellent yield by replacing the urea moiety with thiourea and the ether moiety with sulfide, sulfoxide, or sulfone groups by coupling corresponding benzoylisothiocyanate and aniline derivatives. Preliminary investigation of the most active of the sulfur analogues demonstrated excellent in vitro and in vivo efficacy against pancreas, prostate, and breast cancer models.[1] Sulfur analogues of BPU appear to be promising compounds as a successor to **1**. However, development of the sulfur analogues is limited by their poor solubility in solvents generally used in formulations. The sulfur analogues are also difficult to formulate for oral administration.

On the basis of these findings, we concluded that it would be desirable to change the scaffold of the BPU molecule while keeping the carbonyl urea moiety intact. A literature search led us to the molecule sorafenib from the Bayer Company. Sorafenib is structurally related to the BPUs, but it is a raf-kinase inhibitor and is currently marketed for the treatment of cancer. We therefore modified our sulfur BPU analogue by introducing substituted phenoxy or thiophenyl

moieties and synthesized a library of 30 PP and PSP compounds (Chart 1). We evaluated these compounds for in vitro activity against pancreatic cancer cell lines.

The compounds differ in substitution pattern and in the presence of hetero atoms, but in all cases the carbonyl urea moiety is present. As expected, these compounds show activity in the low μM range on pancreatic cell lines. The most active compounds were **2** and **3**, and they were selected for further studies.

Results and Discussion

Chemistry

Synthesis of the library compounds was based in Scheme 1. 2-Picolinic acid **4** was treated with $SOCl_2$ in DMF, yielding acid chloride **5** as the HCl salt, which on treatment with the indicated amines in methanol yielded compounds of general structure **6**. Further treatment of compound **6** with 4-amino phenol and 4-amino thiophenol of general formula **7** in the presence of potassium *tert*-butoxide yielded anilines **8** as the major and **9** as the minor products. Substituted isocyanates **12** were synthesized by treating corresponding amides **11** with oxalyl chloride in the presence of dichloroethane. The free amine functionality of general structure **8** treated with corresponding isocyanates **12** and isothiocyanate (purchased from SIGMA) **13** in the presence of dioxane yielded target compounds **10**. The structures of the library compounds are shown in Chart 1. All compounds were characterized by spectral data that confirmed the assigned structures.

Biology. PP and PSP Analogues in the Low μM Range Decrease Proliferation of Pancreatic Cancer Cells

Table 1 compares the cytotoxicity of the PP and PSP derivatives in an initial screen at 1 and 10 μM against human pancreatic cancer cell lines Panc1 and HS766T, as determined with the MTT assay. Because only **2** and **3** had submicromolar IC_{50}s in both cell lines, further evaluation was limited to these two agents. These studies showed that **2** had IC_{50} values of 0.8 and 0.15 μM against Panc1 and HS766T cells, respectively, and that **3** had IC_{50} values of 0.7 and 0.5 μM against Panc1 and HS766T cells, respectively.

PP and PSP Analogues Cause G2/M Arrest

Because the compounds were designed to bind to tubulin, flow cytometry experiments[7] were carried out to determine whether compound treatment would lead to cell cycle arrest at G2/M. MCF7 breast cancer cells were treated for 15 h with 5 μM **2** or 5 μM **3**. As a positive control, 0.1 μg/mL (about 0.25 μM) colcemid was used. After incubation, cells were harvested and fixed prior to labeling of DNA with propidium iodide and labeling of mitotic cells with the monoclonal antibody MPM2. MPM2 specifically recognizes the phosphorylated epitopes of proteins that are phosphorylated at the onset of mitosis. Figure 1 shows that treatment of MCF7 cells for 15 h with **2** or **3** caused 10–13 fold increase in mitotic cells, comparable to the effect observed with colcemid.

We next wished to confirm that tubulin was the direct target of **2** and **3**, and we examined the effects of these agents on the polymerization of purified tubulin. We used a reaction condition[8] that has been used successfully with many different antitubulin agents binding at various sites on the tubulin molecule, both in its unpolymerized $\alpha\beta$-heterodimer form and in microtubules. Generally, a concentration that inhibits extent of assembly at 20 min by 50%, as measured by turbidity development, is determined[8] (see Figure 2C for a typical inhibition pattern, obtained with 1.5–4.0 μM colchicine; a series of such experiments yielded an IC_{50} of 2.2 μM for colchicine). However, this parameter could not be measured for **2**. As shown in Figure 2A, with selected concentrations of **2**, assembly was progressively delayed in its onset

with up to about 4 μM **2**. In addition, initially there were also decreasing rates of polymer propagation with increasing concentrations of **2**. At no concentration was a significant effect observed on extent of assembly. At concentrations over about 4 μM, there was a seemingly paradoxical reversal of the effects on assembly onset and propagation rates, although at all concentrations examined, there was some effect on the overall assembly reaction. This may indicate formation of a polymer of aberrant morphology, as occurs with a variety of antitubulin compounds. These unusual effects may also be caused by limited solubility of **2** in the 0.8 M glutamate required to induce assembly.

A somewhat different pattern was observed with **3** (Figure 2B) in that the turbidity readings with 4–5 μM compound were below 50% of the control value, but all higher concentrations of **3** reached an apparent limit of about 40% of the control value. There was a slight suggestion in some experiments, as shown in Figure 2B, of a similar paradoxical rise in the turbidity readings at the highest concentrations of **3**. The greater, although still limited, effect of **3** on tubulin assembly may indicate greater solubility of this compound as compared with **2** in 0.8 M glutamate.

The difficulty of quantitating inhibition of the assembly reaction based on its extent, especially with **2**, led us to determine instead compound concentrations that inhibited the maximum propagation rate by 50%. The turbidity data were evaluated with Microcal Origin, which was used to calculate the first derivative for each reading (4 readings per sample per minute). The maximum value was determined for the controls and for each compound concentration. We obtained the following results, expressed in terms of IC_{50} ± standard deviation, versus control reaction: for colchicine, 0.99 ± 0.01 μM; for **2**, 4.0 ± 0.1 μM; for **3**, 1.7 ± 0.3 μM.

As shown in the figure, we did not find any cells in metaphase, anaphase or telophase. Figure 3B therefore shows only a control cell in metaphase. Not all cells were in mitosis after the 15 h treatment. Some interphase cells displayed several micronuclei, indicating escape from the mitotic spindle checkpoint. Colcemid at 0.25 μM was again used as a positive control.

Immunofluorescence studies were done because of the seeming contradiction between robust G2/M arrest and the equivocal tubulin assembly data of Figure 2.

PP and PSP Analogues Cause Complete Disassembly of Cellular Microtubule

To obtain further information about the mitotic arrest caused in MCF7 cells by **2** and **3**, we performed immunofluorescence experiments[7] with an antibody against R-tubulin, with cellular DNA stained with 4,6-diamidino-2-phenylindole (DAPI). Concordant with our flow cytometry experiments, a large number of cells were arrested in mitosis after 15 h treatment with either 5 μM **2** or 5 μM **3**. All mitotic cells were arrested in prometaphase of the cell cycle (Figure 3A).

Effect of 3 Compared to Paclitaxel on Growth of SKOV3 and SKOV3—Paclitaxel-Resistant Cells

We further studied dose response of SKOV3 and SKOV3—paclitaxel-resistant cells to **3** in comparison with paclitaxel for 7 days (Figure 4). Both **3** and paclitaxel were evaluated by using standard cytotoxicity assays in SKOV3 and SKOV3—paclitaxel-resistant ovarian cancer cell lines. Even though **3** is less effective in SKOV3 cell lines, it showed excellent cytotoxicity compared to that of the paclitaxel at equivalent doses in paclitaxel-resistant cell lines. Further mechanistic studies are ongoing.

Conclusion

On the basis of our antiproliferative study of the library compounds, it appears that the substituent pattern on the "lefthand" (see structural diagram) phenyl ring is critical for activity, with the chlorine atom at position 2 being particularly important, but not sufficient, for activity. This —Cl group was present in both of the most active compounds, and it was the sole substituent on the phenyl ring in **2**, but this same —Cl substituent was also present in many of the less active agents. It should also be noted that the amide substituent differed in **2** and **3**, and the former was an ether ("X" group in the diagram) and the latter a thioether. **18**, the cognate thio ether of **2**, was less active than **2**, but the cognate ether of **3** has not yet been prepared.

In conclusion, we have synthesized a series of PP and PSP analogues. Some of these had excellent growth inhibition activity against both pancreatic and breast cancer cell lines, and **3** and **2** were the most potent. Cell cycle analysis on breast cancer MCF7 cells treated with **2** or **3** revealed that both agents caused mitotic arrest. These compounds are currently being evaluated for their in vivo efficacy in animal models. These findings have encouraged us to continue the development and testing of novel analogues and to conduct further studies to investigate SAR and their mechanisms of action.

Further, **3** showed significant cytotoxicty against a paclitaxel-resistant cell lines as compared with paclitaxel at equivalent doses. Our results show that these analogues inhibited cell survival in both the paclitaxel-sensitive and paclitaxel-resistant ovarian cancer cells (SKOV3) with equivalent activity. Further mechanistic evaluation of the most potent analogues is underway. In summary, we have identified a small molecule inhibitor with promising anticancer activity that retains the ability to induce cell death in a paclitaxel-resistant cell line.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgment

We gratefully acknowledge the financial support of grants from FAMRI and NIH. We thank the Medicinal Chemistry Center of the SKCCC at Johns Hopkins for conducting the NMR and LC-MS studies. In addition, we thank Susan Dalrymple and Dr. Antonio Jimeno, Sidney Kimmel Comprehensive Cancer Center at Johns Hopkins, for help in cell culture analysis.

References

(1). Hallur G, Jimeno A, Dalrymple S, Zhu T, Jung MK, Hidalgo M, Isaacs JT, Sukumar S, Hamel E, Khan SR. Benzoylphenylurea sulfur analogs with potent antitumor activity. J. Med. Chem 2006;49:2357–2360. [PubMed: 16570932]

(2). Jimeno A, Hallur G, Chan A, Zhang X, Cusatis G, Chan F, Shah P, Chen R, Hamel E, Garrett-Myer E, Khan SR, Hidalgo M. Development of two novel benzoylphenylurea sulphur analogues and evidence that the microtubule-associated protein tau is predictive of their activity in pancreatic cancer. Mol. Cancer Ther 2007;6:1509–1516. [PubMed: 17483439]

(3). Tasheva M, Hristeva V. Comparative study on the effects of five benzoylphenylurea insecticides on haematological parameters in rats. J. Appl. Toxicol 1993;13:67–68. [PubMed: 8440876]

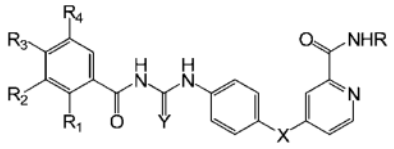
(4). Okada H, Koyanagi T, Yamada N, Haga T. Synthesis and antitumor activities of novel benzoyl phenylurea derivatives. Chem. Pharm. Bull. (Tokyo) 1991;39:2308–2315. [PubMed: 1804543]

(5). Okada H, Kato M, Koyanagi T, Mizuno K. Synthesis and antitumor activities of water-soluble benzoylphenylureas. Chem. Pharm. Bull. (Tokyo) 1999;47:430–433. [PubMed: 10212393]

(6). Hollingshead MG, Sackett DL, Alley MC, Waud WR, Dykes DJ, Hamel E, Sausville EA. The anticancer activity of six benzoylphenylurea compounds and their interaction with tubulin. Proc. Amer. Assoc. Cancer Res 1998;39:164.

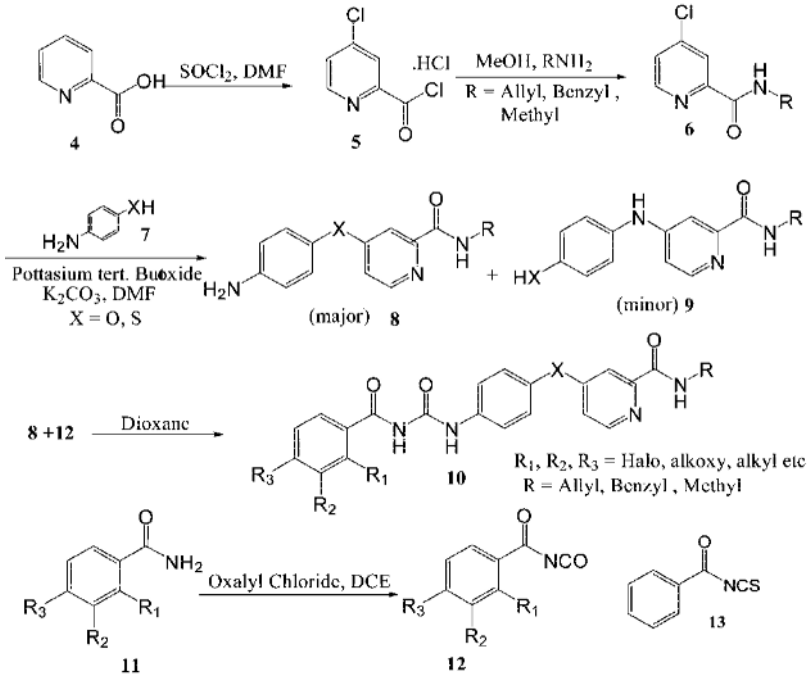
(7). Frances M, Davis T, Tsao Y, Susan KF, Potu NR. Monoclonal antibodies to mitotic cells. Proc. Natl. Acad. Sci. U.S.A 1983;80(10):2926–2930. [PubMed: 6574461]

(8). Hamel E. Evaluation of antimitotic agents by quantitative comparisons of their effects on the polymerization of purified tubulin. Cell. Biochem. Biophys 2003;38:1–22. [PubMed: 12663938]



14 R = $C_{10}H_{21}$, $R_1 = R_3 = H$, $R_2 = R_4 = OMe$, X = S, Y = O
15 R = Allyl, $R_1 = NO_2$, $R_2 = R_3 = R_4 = H$, X = S, Y = O
16 R = Allyl, $R_1 = R_3 = H$, $R_2 = R_4 = OMe$, X = Y = O
17 R = Allyl, $R_1 = NO_2$, $R_2 = R_3 = R_4 = H$, X = Y = O
18 R = Allyl, $R_1 = Cl$, $R_2 = R_3 = R_4 = H$, X = S, Y = O
19 R = Allyl, $R_1 = R_2 = R_3 = R_4 = H$, X = Y = O
2 R = Allyl, $R_1 = Cl$, $R_2 = R_3 = R_4 = H$, X = Y = O
20 R = $C_{10}H_{21}$, $R_1 = R_3 = H$, $R_2 = R_4 = OMe$, X = SO_2, Y = O
21 R = Benzyl, $R_1 = NO_2$, $R_2 = R_3 = R_4 = H$, X = S, Y = O
22 R = Benzyl, $R_1 = Cl$, $R_2 = R_3 = R_4 = H$, X = S, Y = O
23 R = Benzyl, $R_1 = R_2 = R_3 = R_4 = H$, X = S, Y = O
24 R = Allyl, $R_1 = Cl$, $R_2 = R_3 = OMe$, $R_4 = H$, X = S, Y = O
25 R = Allyl, $R_1 = Cl$, $R_2 = R_3 = OMe$, $R_4 = H$, X = O, Y = O
26 R = Benzyl, $R_1 = Cl$, $R_2 = R_3 = OMe$, $R_4 = H$, X = S, Y = O
27 R = $C_{10}H_{21}$, $R_1 = Cl$, $R_2 = R_3 = OMe$, $R_4 = H$, X = S, Y = O
28 R = Allyl, $R_1 = R_3 = H$, $R_2 = R_4 = OMe$, X = S, Y = O
29 R = Allyl, $R_1 = R_2 = R_3 = R_4 = H$, X = O, Y = S
3 R = Methyl, $R_1 = Cl$, $R_2 = R_3 = OMe$, $R_4 = H$, X = S, Y = O
30 R = Methyl, $R_1 = R_3 = H$, $R_2 = R_4 = OMe$, X = S, Y = O
31 R = Methyl $R_1 = NO_2$, $R_2 = R_3 = R_4 = H$, X = S, Y = O
32 R = Methyl, $R_1 = R_2 = R_3 = R_4 = H$, X = S, Y = O
33 R = Methyl, $R_1 = R_2 = R_3 = R_4 = H$, X = Y = S
34 R = Methyl $R_1 = Cl$, $R_2 = R_3 = R_4 = H$, X = S, Y = O
35 R = Allyl, $R_1 = R_2 = R_3 = R_4 = H$, X = S, Y = O
36 R = $C_{10}H_{21}$, $R_1 = NO_2$, $R_2 = R_3 = R_4 = H$, X = S, Y = O

Chart 1.
Structure of PP and PSP Analogues



Scheme 1.

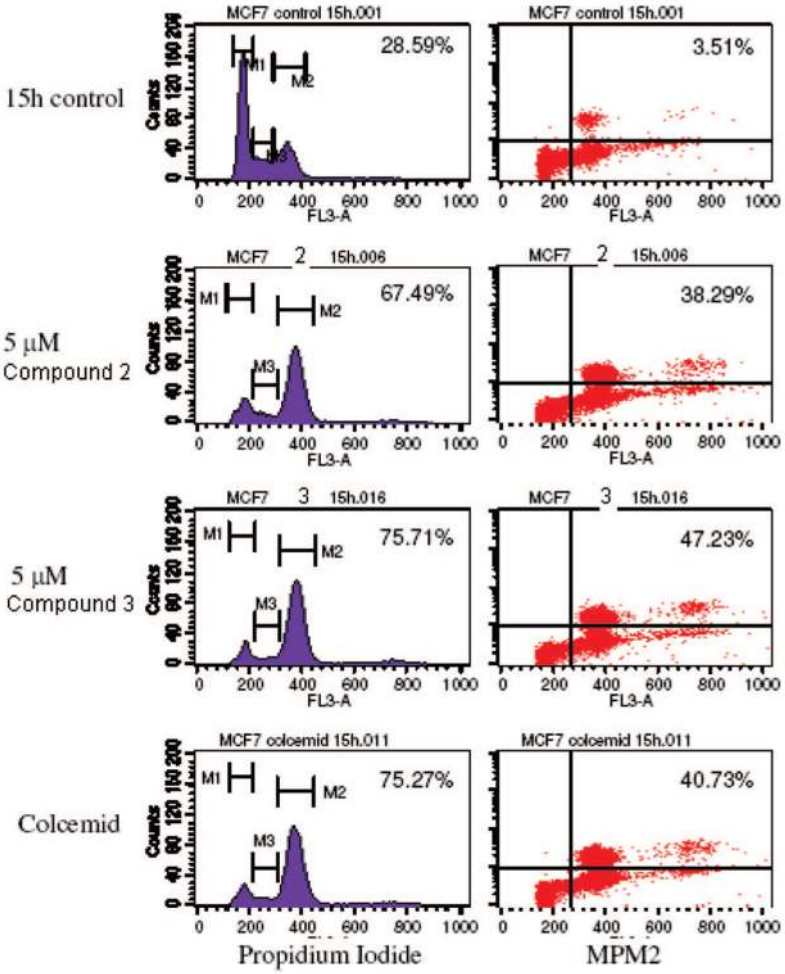


Figure 1.

Treatment of MCF7 cells for 15 h with either 5 μM compound **2** or 5 μM compound **3** results in a 10–15-fold increase in mitotic cells. Colcemid (0.25 μM) was used as a positive control. In the propidium iodide scans, M1 indicates G1 cells, M3 cells in S phase, and M2 cells at G2/M.

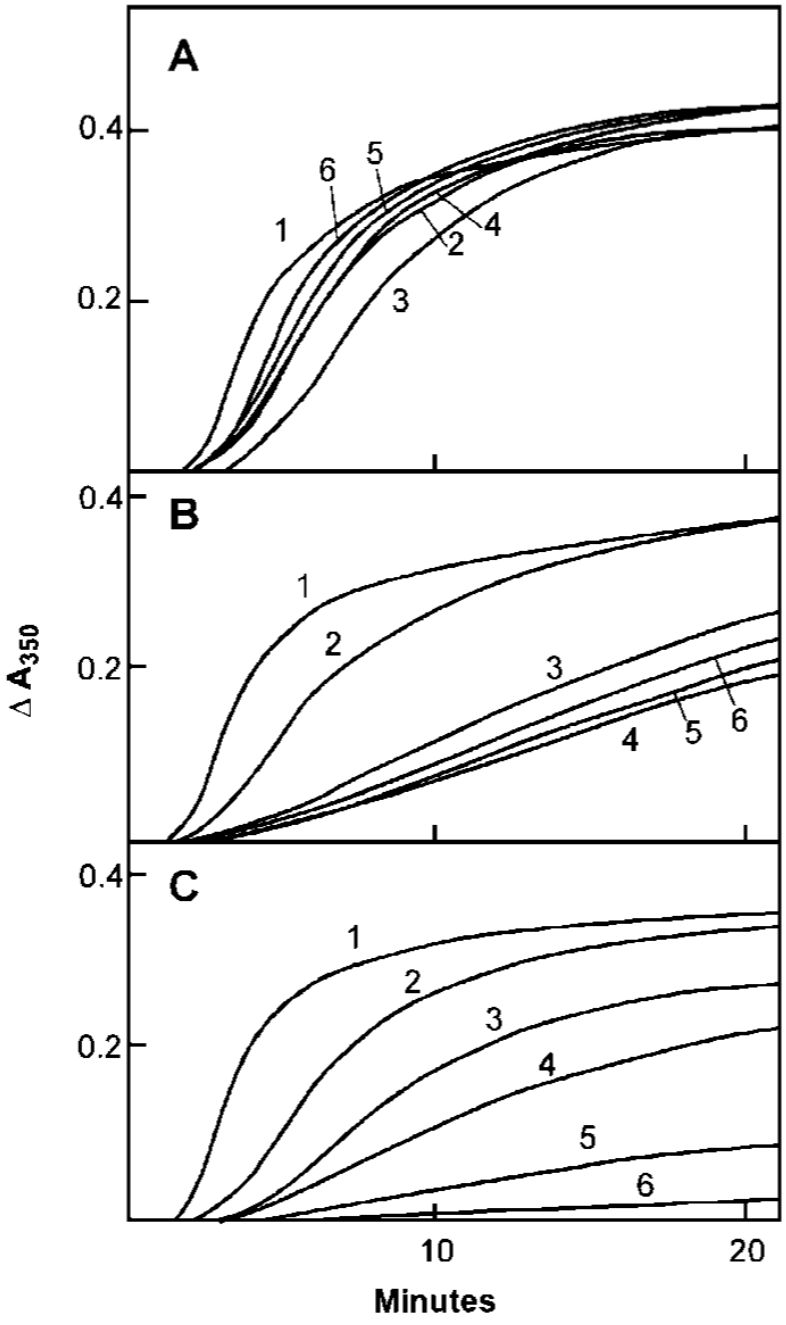


Figure 2.

Effects of varying concentrations of **2** (A), **3** (B), and colchicine (C) on tubulin assembly. At zero time, the temperature was rapidly increased from 0 to 30 °C. Compound concentrations were as follows: (A) curve 1, control (no compound); curves 2–6, **2** at 1.5, 4.0, 10, 20, and 40 μM, respectively; (B) curve 1, control (no compound); curves 2–6, **3** at 1.5, 4.0, 10, 20, and 40 μM, respectively; (C) curve 1, control (no compound); curves 2–6, colchicine at 1.0, 1.5, 2.0, 3.0, and 4.0 μM, respectively.

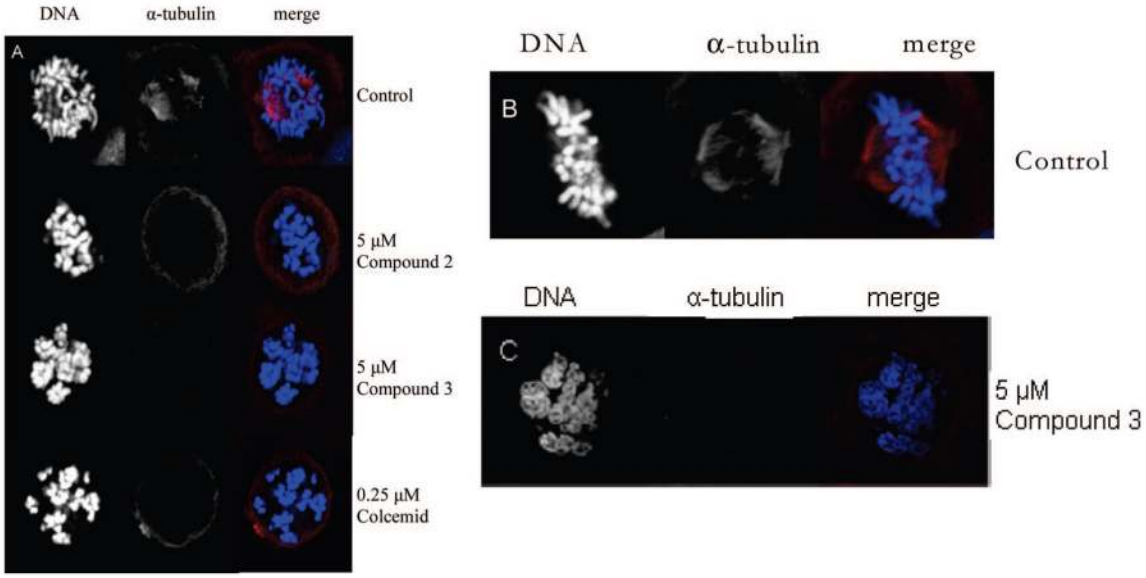


Figure 3.

Immunofluorescence staining of MCF7 cells treated for 15 h with either 5 μM **2**, 5 μM **3**, or 0.25 μM colcemid. Tubulin was labeled with primary antibody against α-tubulin from Molecular Probes and secondary antibody Alexa-568. DNA was stained with DAPI. (A) Loss of cellular microtubules and prometaphase arrest in MCF7 cells treated for 15 h. (B) Metaphase plate formation in untreated control cells. (C) Micronuclei formation in treated interphase cells that bypassed the mitotic checkpoint.

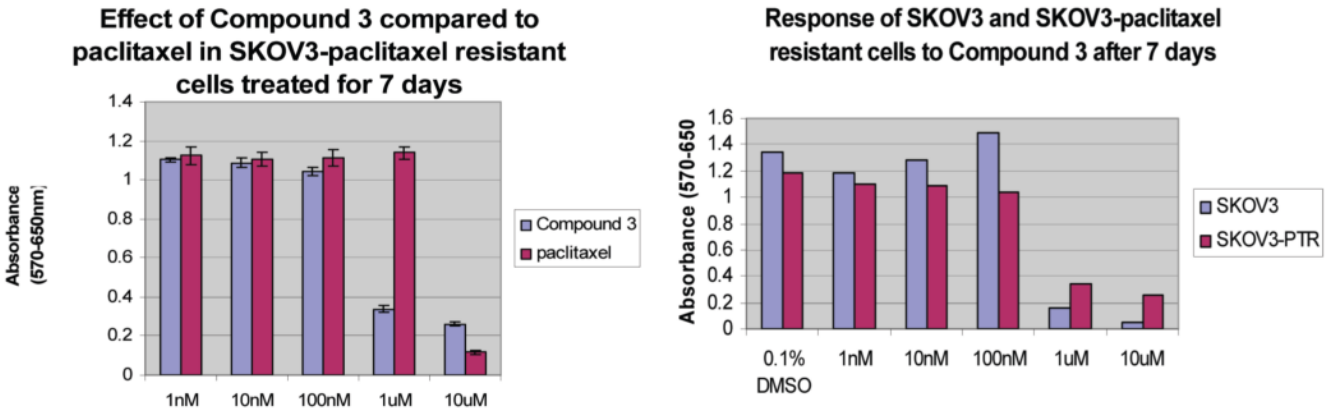


Figure 4.
Cytotoxicity assay of **3** and paclitaxel on SKOV3 and SKOV3—paclitaxel-resistant ovarian cell lines.

Table 1

Growth Inhibition of Pancreatic Cancer Cell Lines by PP and PSP Analogues Compared with Untreated Control

analogues	Panc1				HS766T			
	1 μM	SD	10 μM	SD	1 μM	SD	10 μM	SD
14	74	11	58	7	77	18	66	18
15	70	12	44	5	50	10	27	5
16	58	15	68	16	107	40	64	7
17	76	24	55	17	55	7	30	4
18	69	2	46	6	109	34	36	6
19	66	7	51	6	76	18	70	14
2	47	7	38	4	36	5	23	2
20	48	6	43	7	71	16	56	16
22	86	7	66	5	95	10	74	15
23	67	11	53	6	79	9	73	14
24	88	8	44	3	101	19	23	2
25	61	10	53	5	67	15	50	12
26	89	7	51	8	97	21	75	15
27	63	14	51	8	79	15	62	10
28	62	12	41	8	105	18	87	8
29	97	6	68	8	88	9	73	15
3	35	4	33	4	30	6	21	2
30	77	11	52	7	54	12	31	3
31	60	7	38	4	63	5	23	2
32	65	9	41	2	77	19	26	3
33	98	9	63	3	106	19	31	2
35	63	5	60	6	81	12	79	13
36	64	9	43	5	86	8	66	7
37	54	12	42	4	74	21	93	7
38	72	14	66	11	78	9	83	12
39	53	5	58	4	80	16	68	6
40	80	12	81	12	97	13	101	14
41	96	12	88	9	96	14	97	14
42	36	3	79	11	87	16	26	1