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ScienceDirect
Bioorganic & Medicinal Chemistry 14 (2006) 7761–7773
Bioorganic & Medicinal Chemistry

Discovery of substituted 4-anilino-2-(2-pyridyl)pyrimidines as a new series of apoptosis inducers using a cell- and caspase-based high throughput screening assay. Part 1: Structure–activity relationships of the 4-anilino group

Nilantha Sirisoma, Shailaja Kasibhatla, Bao Nguyen, Azra Pervin, Yang Wang, Gisela Claassen, Ben Tseng, John Drewe and Sui Xiong Cai*

EpiCept Corporation, 6650 Nancy Ridge Drive, San Diego, CA 92121, USA

Received 24 June 2006; revised 1 August 2006; accepted 3 August 2006
Available online 21 August 2006

Abstract—A series of 4-anilino-2-(2-pyridyl)pyrimidines has been discovered as a new class of potent inducers of apoptosis using a cell-based HTS assay. Compound **5a** was found to arrest T47D cells in G_2/M and induced apoptosis. SAR studies showed that a small and electron-donating group at the *meta*-position of the anilino ring is important for activity. A 20-fold increase in potency, from hit compound 4-(3-methoxyanilino)-2-(2-pyridinyl)-6-(trifluoromethyl)pyrimidine (**5a**) to lead compound 4-(2,5-dimethoxyanilino)-2-(2-pyridinyl)-6-(trifluoromethyl)pyrimidine (**5l**), was obtained through the SAR studies. Compound **5l** is highly active with an EC_{50} value of 18 nM in the caspase activation assay in T47D breast cells. Interestingly, **5a** and other *meta*-mono-substituted compounds were active against T47D cells but were not active against H1299 and HT29 cells, while **5l** and other 2,5-disubstituted compounds were active against all the three cells. In a tubulin polymerization assay, compound **5l** inhibited tubulin polymerization with an IC_{50} value of <0.5 μM, while **5a** was not active up to 50 μM.

1. Introduction

Programmed cell death, or apoptosis, is a highly regulated process of cellular suicide.[1] It is known that inappropriate apoptosis induction results in excessive cell death, and could be the cause of degenerative diseases.[2] Inadequate apoptosis, on the other hand, results in over-proliferation of cells, and could be the cause of cancer.[3] The basic mechanism of apoptosis involves the activation of caspases,[4] a family of cysteine proteases that is essential for the initiation as well as execution of apoptosis. Among them, caspase-3 has been identified as one of the key executioner caspases that cleave multiple protein substrates in cells and lead to irreversible cell death.[5]

Since inadequate apoptosis is one of the hallmarks of many cancer cells,[3] and many chemotherapeutics are known to induce apoptosis, promoting apoptosis is a promising strategy for cancer drug discovery.[6] Novel approaches to promoting apoptosis include the development of soluble and truncated tumor-necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL), and agonistic antibodies against TRAIL death receptors such as HGS-ETR1 and HGS-ETR2 that induce apoptosis in tumor cells,[7] as well as the discovery of BCL-2 inhibitors,[8,9] XIAP inhibitors,[10] and MDM2 antagonists.[11]

We have been interested in the discovery and development of apoptosis inducers as potential anticancer agents. Toward this goal, we have developed a cell-based high throughput screening (HTS) system for inducers of apoptosis[12] using novel fluorescent caspase-3 substrates.[13,14] The advantage of cell-based HTS assay is that it can identify molecules that perturb the apoptosis signal pathway and induce apoptosis, through known mechanism or molecular targets, as well as by novel mechanism or molecular targets. We have reported recently the discovery and SAR studies of

Keywords: Inducers of apoptosis; HTS; 4-Anilino-2-(2-pyridyl)pyrimidines; SAR; Tubulin inhibitors; Anticancer agents.
* Corresponding author. Tel.: +1 858 202 4006; fax: +1 858 202 4000; e-mail: scai@epicept.com

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doi:10.1016/j.bmc.2006.08.002

N-phenyl nicotinamide (**1a**),[15] indole-2-carboxylic acid benzylidene-hydrazide (**1b**),[16] 2-amino-3-cyano-7-dimethylamino-4-aryl-4*H*-chromene (**2**),[17,18] gambogic acid (**3**),[19] and 3-aryl-5-aryl-1,2,4-oxadiazoles[20] as novel apoptosis inducers (Chart 1). The 4-aryl-4*H*-chromenes were found to bind at or close to the binding site for colchicine and several of these chromenes have been found to have vascular targeting activity with good efficacy in several anticancer animal models.[21,22] Gambogic acid was identified as a fast inducer of apoptosis and novel derivatives with good in vivo activity have been identified. Based on the SAR of gambogic acid, novel reagents such as gambogyl-biotin have been designed and synthesized, leading to the identification of transferrin receptor as its molecular target.[23] The 3-aryl-5-aryl-1,2,4-oxadiazoles were found to induce apoptosis selectively in certain tumor types, and are not active against primary normal cells. A novel analog, 5-(3-chlorothiophen-2-yl)-3-(5-chloro-pyridin-2-yl)-1,2,4-oxadiazole (**4a**), was identified as a lead compound with in vivo anticancer activity. A photoaffinity labeling agent (**4b**) was designed and synthesized, resulting in the identification of TIP47, an insulin growth factor II (IGF II) receptor binding protein, as the molecular target for this class of novel apoptosis inducers.[24] Thus, our chemical genetics approach for the discovery of apoptosis inducers, starting with a cell-based HTS assay, through SAR studies and target identification, results in the identification of potential anticancer agents as well as their targets, including known and novel targets. Herein, we report the discovery of 4-(3-methoxyanilino)-2-(2-pyridinyl)-6-(trifluoromethyl)pyrimidine (**5a**) as a novel inducer of apoptosis, and the structure–activity relationship of various substituted 4-anilino-2-(2-pyridinyl)-pyrimidines, using our cell- and caspase-based apoptosis HTS assay.

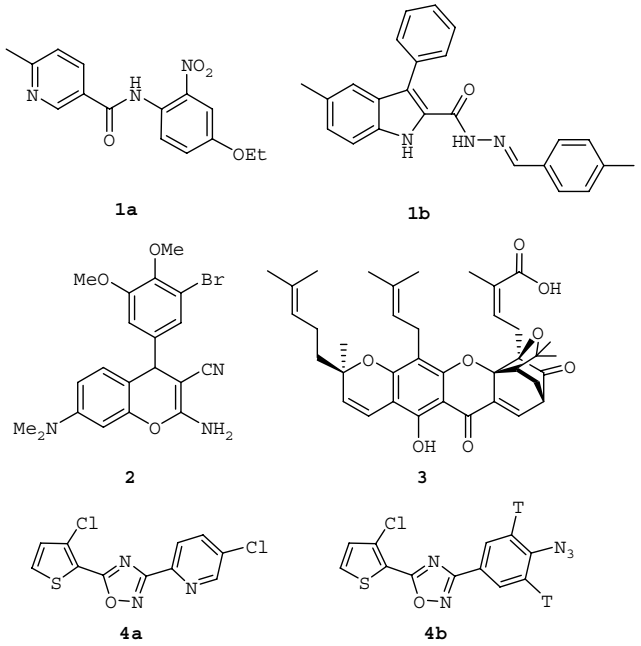


Chart 1.

2. Results and discussion

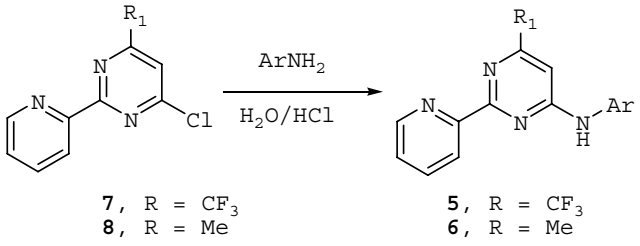
2.1. Chemistry

The hit compound 4-(3-methoxyanilino)-2-(2-pyridinyl)-6-(trifluoromethyl)pyrimidine (**5a**) was obtained from a commercial library (Buttpark, UK). Other 4-anilino-2-(2-pyridinyl)-pyrimidines were synthesized as shown in Scheme 1, by reaction of 4-chloro-2-(2-pyridinyl)-6-(trifluoromethyl)pyrimidine (**7**) or 4-chloro-6-methyl-2-(2-pyridinyl)pyrimidine (**8**)[25] with a substituted aniline. Compounds **7** and **8** were obtained from Key Organics (UK).

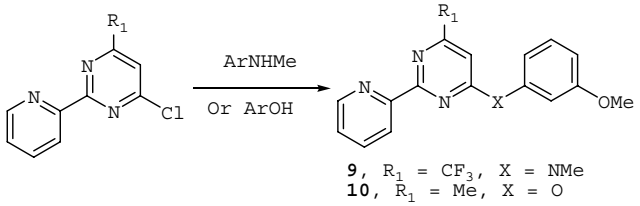
The *N*-Me analog **9** was prepared similarly via reaction of 4-chloro-2-(2-pyridinyl)-6-(trifluoromethyl)pyrimidine with *N*-methyl-3-methoxyaniline. The phenoxy analog **10** was prepared via reaction of 4-chloro-6-methyl-2-(2-pyridinyl)pyrimidine with 3-methoxyphenol (Scheme 2).

2.2. HTS assays

Compound **5a** was identified as an inducer of apoptosis from our compound library using our cell-based apoptosis induction HTS assay in human breast T47D cancer cells as described previously.[15] Briefly, human T47D cells, in a 384-well microtiter plate containing 10 μM of test compound, were incubated for 24 h at 37 °C. Caspase-3 fluorogenic substrate *N*-(Ac-DEVD)-*N*′-ethoxycarbonyl-R110[26] was then added to cells and the samples were mixed by agitation and incubated at room temperature for 3 h. Using a fluorescent plate reader, employing excitation at 485 nm and emission at 525 nm, the fluorescence was measured and the amount of caspase activation was determined. Compounds that induce apoptosis and activate the caspases yield a fluorescent signal higher than the background (signal/background ratio). Compounds found to give a ratio of >3 are considered active and retested in triplicate for confir-



Scheme 1.



Scheme 2.

mation in T47D cells. Compounds confirmed to be active are then tested at several concentrations to provide a dose response and the caspase activation activity (EC_{50}) calculated. Compound **5a** was found to induce apoptosis and activate caspase in T47D cells with a ratio of around 10 over untreated cells, which are higher than that of substituted *N*-phenyl nicotinamides[15] and 4-aryl-4*H*-chromenes.[17] Those compounds have a ratio of around 6. Compound **5a** was found to have an EC_{50} value of 0.36 μM.

2.3. Characterization of compound 5a

The apoptosis-inducing activity of compound **5a** was characterized by cell cycle analysis. T47D cells were treated with 0.7 μM of compound **5a** for 24 h or 48 h at 37 °C. Cells were then stained with propidium iodide and analyzed by flow cytometry. Figure 1A shows that control cells (treated with solvent DMSO) were mostly in the G_1 phase of the cell cycle at 24 h, and control cells at 48 h had almost identical distribution as at 24 h (data not shown). A large increase in G_2/M content (from 16.9% to 66.5%) was observed after 24 h of treatment with **5a**, together with an increased sub-G_1 population (from 3.4% to 13.6%), indicating apoptotic cells (Fig. 1A vs B). After 48 h of treatment, sub-G_1 population increased to 73.6%, indicating that most of the cells are apoptotic (Fig. 1C). When cells were treated with a broad-spectrum caspase inhibitor **13** (MX1013, benzyloxycarbonyl-Val-Asp-fluoromethylketone)[27] before treatment with **5a**, sub-G_1 apoptotic cell numbers decreased both at 24 h (from 13.6% to 8.7%, Fig. 1B vs D) and 48 h (from 73.6% to 29.9%, Fig. 1C vs E), and instead cells were accumulated in the G_2/M (72.8% and 53.9%, respectively). Cells treated with caspase inhibitor **13** alone without **5a** behave similar to control cells, showing most of the cells in the G_1 phase at both 24 h and 48 h (data not shown). These results indicate that treatment of cells with **5a** results in G_2/M arrest, accompanied by induction of apoptosis, and that apoptosis induced by **5a** is caspase dependent and can be inhibited by a broad-spectrum caspase inhibitor.

2.4. Structure–activity relationship (SAR) studies

The cell-based caspase HTS assay was used to test analogs of **5a** for SAR studies. A panel of three different cell lines, human breast cancer cell line T47D, human non-small cell lung cancer cell line H1299, and human colon cancer cells HT29, were used for these experiments. The caspase activation assays were run in 384-well microtiter plates as described previously.[15] The caspase activation activity (EC_{50}) of compound **5a** and its analogs in the three cancer cell lines is summarized in Table 1.

Table 1 shows that compound **5a** has an EC_{50} for caspase activation of 0.36 μM in T47D, and is inactive up to 10 μM against H1299 and HT29 cells, showing that **5a** is selective against certain cancer cell lines. Compound **6a**, with the trifluoromethyl group in the 6-position of pyrimidine replaced by a methyl group, was found to have similar activity as that of **5a** against T47D, and also was not active against H1299 and HT29. Since the starting material, 4-chloro-6-methyl-2-(2-pyridinyl)pyrimidine (**8**), for **6a** is commercially available and can be obtained easily, we decided to explore the SAR of **6a** first using various substituted anilines. Compound **6b**, with the 3-methoxy group removed, was not active up to 10 μM, indicating that the methoxy in the *meta*-position is important for activity. Moving the methoxy group from the 3-position to 2-position (**6c**) and 4-position (**6d**), resulted in large reduction of potency and the compounds were not active up to 10 μM, indicating that the position of methoxy group also is important for activity. Replacement of the 3-methoxy group by other groups was then explored. Compound **6e**, with a 3-ethoxy group, was not active up to 10 μM in T47D cells, suggesting that there might be a size-limited pocket in that position. Compounds **6f** (3-benzyloxy) and **6g** (3-trifluoromethoxy) were both not active up to 10 μM, supporting that the pocket in the 3-position might be size limited. Compounds **6h**–**6l**, with an electron-withdrawing group in the *meta*-position (fluoro, cyano, trifluoromethyl, methylketone, and benzoyl), were all not active up to 10 μM, suggesting that electron-donating group is preferred in the 3-position.

We then explored compounds with two methoxy groups substituted in the aniline ring. Compound **6m**, with methoxy group in both 3- and 5-position, is about 7-fold more potent than **6a**, confirming the importance of methoxy group in the *meta*-position (3- and 5-position). The 2,4-dimethoxy analog **6n** was not active up to 10 μM, again confirming the importance of the *meta*-methoxy group. Compound **6o**, with methoxy group in both 2- and 5-position, is about 6-fold more potent than **6a**, suggesting that the *ortho*-methoxy group plays an important role for activity. Interestingly, the 2,3-dimethoxy analog **6p** was not active up to 10 μM. The difference in activity between **6o** and **6p** most probably is due to the steric interaction between the *ortho*-methoxy group and the pyrimidine ring. As shown in Chart 2, to avoid the unfavorable steric interaction between the *ortho*-methoxy group and the H in the pyrimidyl ring, the preferred position for the *ortho*-methoxy group in **6o** should be in the 10 o'clock position, which will put the *meta*-methoxy group in the 4 o'clock position. Similarly, the preferred position for the *ortho*-methoxy group in **6p** should be in the 10 o'clock position, which, however, will put the *meta*-methoxy group in the 12 o'clock position. Since **6o** is >200-fold more active than **6p**, these results suggested that the orientation of the *meta*-methoxy group in the 4 o'clock position is critical for the activity of these compounds. Interestingly, compound **11** (Gleevec, STI 571) also has a similar 2,5-disubstituted aniline ring, and it is known that the *ortho*-methyl group is important for the selectivity of **11**.[28,29] Compound **12**, a close analog of compound **11**, was found by X-ray crystal structure determination to bind to the Abl-kinase with the *meta*-amido group oriented in the 4 o'clock position.[30] Although the pyridyl, pyrimidyl, and aniline rings in **6o** is attached to each other in a different way from that of compound **11**, a striking structural similarity between **6o** and the left portion of compound **11** can be observed.

Entry	M1 (sub)	M2 (G_1)	M3 (S)	M4 (G_2/M)
1A	3.4	71.0	8.0	16.9
1B	13.6	10.7	9.2	66.5
1C	73.6	5.8	5.3	13.4
1D	8.7	11.0	7.7	72.8
1E	29.9	4.4	12.0	53.9
1F	26.3	19.4	10.9	40.4
1G	58.5	17.5	4.5	17.8
1H	19.2	18.3	16.3	44.5
1I	28.0	37.9	10.6	20.5

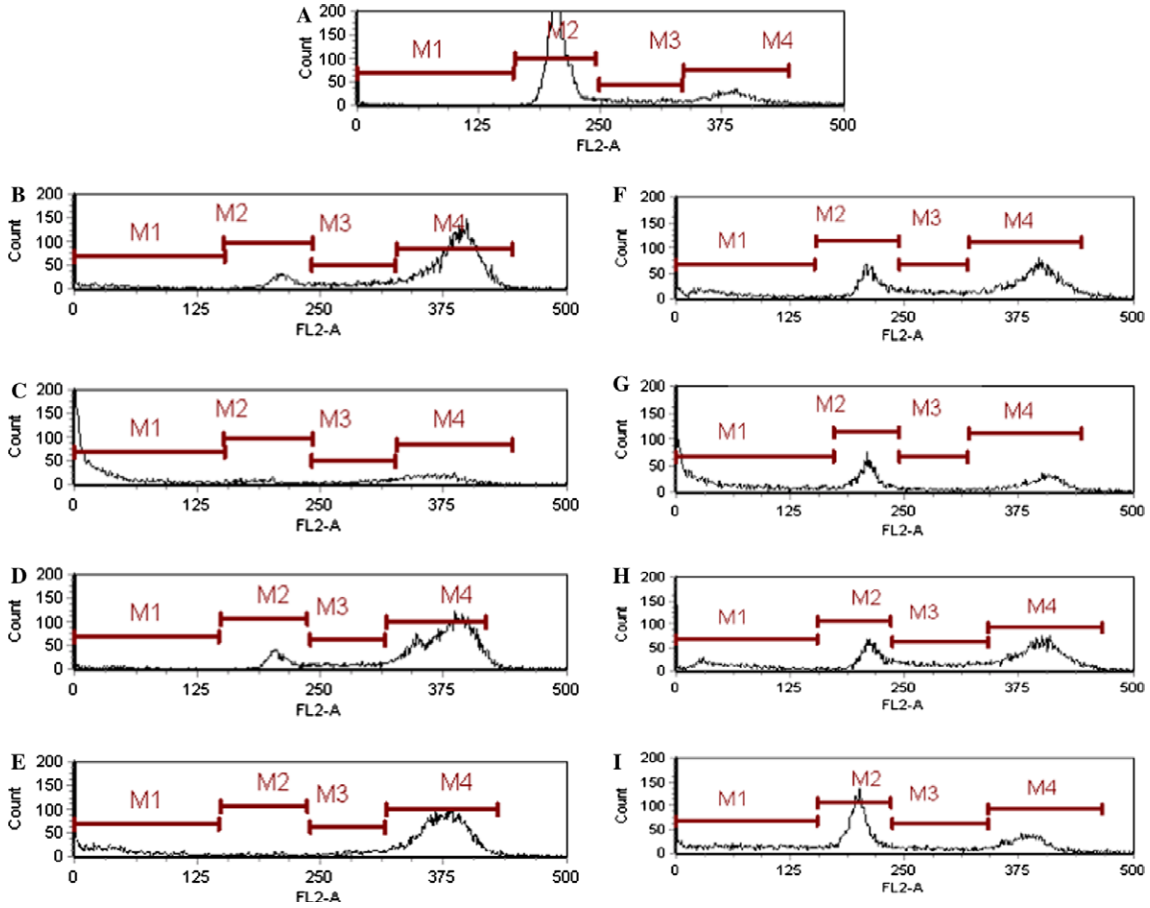
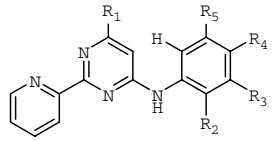
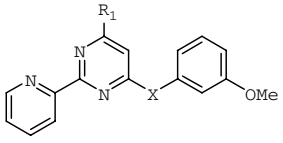


Figure 1. (A–I) Graphs showing drug-induced cell cycle arrest and apoptosis in T47D cells by compounds **5a** and **5l**. (A) Control cells treated with solvent DMSO, showing most of the cells in G_1 (M2). (B) cells treated with 250 nM of 4-(3-methoxyanilino)-2-(2-pyridinyl)-6-(trifluoromethyl)pyrimidine (**5a**) for 24 h, showing most of the cells in G_2/M (M4). (C) Cells treated with 250 nM of compound **5a** for 48 h, showing most of the cells are apoptotic with sub-diploid DNA content (M1). (D) Cells treated with 250 nM of compound **5a** for 24 h in the presence of 10 μM of caspase inhibitor **13**, showing most of the cells in G_2/M (M4), with some apoptotic cells (M1). (E) Cells treated with 250 nM of compound **5a** for 48 h in the presence of 10 μM of caspase inhibitor **13**, showing most of the cells in G_2/M (M4), or are apoptotic (M1). (F) Cells treated with 50 nM of 4-(2,5-dimethoxyanilino)-2-(2-pyridinyl)-6-(trifluoromethyl)pyrimidine (**5l**) for 24 h, showing most of the cells in G_2/M (M4), or are apoptotic (M1). (G) Cells treated with 50 nM of compound **5l** for 48 h, showing most of the cells are apoptotic with sub-diploid DNA content (M1). (H) Cells treated with 50 nM of compound **5l** for 24 h in the presence of 10 μM of caspase inhibitor **13**, showing most of the cells in G_2/M (M4), or are apoptotic (M1). (I) Cells treated with 50 nM of compound **5l** for 48 h in the presence of 10 μM of caspase inhibitor **13**, showing most of the cells in G_1 (M2), or G_2/M (M4), or are apoptotic (M1).

Table 1. SAR of 4-anilino-2-(2-pyridyl)pyrimidines in the caspase activation assay



Compound	R_1	R_2	R_3	R_4	R_5	EC_{50}[a] (μM)		
						T47D	H1299	HT29
5a	CF_3	H	OMe	H	H	0.36 ± 0.17	>10	>10
6a	Me	H	OMe	H	H	0.31 ± 0.01	>10	>10
6b	Me	H	H	H	H	>10	>10	>10
6c	Me	OMe	H	H	H	>10	>10	>10
6d	Me	H	H	OMe	H	>10	>10	>10
6e	Me	H	OEt	H	H	>10	>10	>10
6f	Me	H	OBz	H	H	>10	>10	>10
6g	Me	H	OCF_3	H	H	>10	>10	>10
6h	Me	H	F	H	H	>10	>10	>10
6i	Me	H	CN	H	H	>10	>10	>10
6j	Me	H	CF_3	H	H	>10	>10	>10
6k	Me	H	COMe	H	H	>10	>10	>10
6l	Me	H	COPh	H	H	>10	>10	>10
6m	Me	H	OMe	H	OMe	0.046 ± 0.003	>10	>10
6n	Me	OMe	H	OMe	H	>10	>10	>10
6o	Me	OMe	H	H	OMe	0.050 ± 0.005	0.27 ± 0.01	0.46 ± 0.06
6p	Me	OMe	OMe	H	H	> 10	>10	>10
5b	CF_3	H	H	H	H	>10	>10	>10
5c	CF_3	H	OEt	H	H	0.65 ± 0.07	>10	>10
5d	CF_3	H	OH	H	H	0.32 ± 0.02	>10	>10
5e	CF_3	H	SMe	H	H	>10	>10	>10
5f	CF_3	H	OCF_3	H	H	>10	>10	>10
5g	CF_3	H	F	H	H	>10	>10	>10
5h	CF_3	H	CF_3	H	H	>10	>10	>10
5i	CF_3	H	Cl	H	H	>10	>10	>10
5j	CF_3	H	H	OMe	H	>10	>10	>10
5k	CF_3	H	OMe	H	OMe	0.050 ± 0.006	0.23 ± 0.04	0.68 ± 0.02
5l	CF_3	OMe	H	H	OMe	0.018 ± 0.001	0.15 ± 0.02	0.19 ± 0.04
5m	CF_3	Me	H	H	OMe	0.055 ± 0.006	0.37 ± 0.06	0.54 ± 0.02
5n	CF_3	Cl	H	H	OMe	0.18 ± 0.02	1.66 ± 0.18	2.78 ± 0.10
5o	CF_3	Cl	H	H	Cl	>10	>10	>10
5p	CF_3	Me	H	OMe	OMe	0.061 ± 0.009	1.94 ± 0.59	1.36 ± 0.01
5q	CF_3	OMe	H	Cl	OMe	0.12 ± 0.01	0.38 ± 0.03	0.52 ± 0.04



Compound	R_1	X	EC_{50}[a]		
			T47D	H1299	HT29
9	CF_3	NMe	>10	>10	>10
10	Me	O	>10	>10	>10

[a] Data are means of three or more experiments and are reported as means ± standard error of the mean (SEM).

We then came back to explore the SAR of compound **5a**. Similar to the SAR of **6a**, the 3-methoxy group was found to be important for the activity of **5a**. Compound **5b**, without the 3-methoxy group, was not active up to 10 μM. The 3-ethoxy analog **5c** has good activity and is about 2-fold less active than **5a**. This is different from **6e**, the inactive ethoxy analog of **6a**, suggesting that the SAR of the CF_3 series (**5a**) may not be exactly same as the CH_3 series (**6a**). The 3-hydroxy analog **5d** is about as active as **5a**, suggesting that a small and electron-donating group is preferred in the 3-position. Replacement of the 3-methoxy group by other groups (3-methylthiol, trifluoromethoxy, fluoro, trifluoromethyl, and chloro, compounds **5e**–**5i**) all resulted in compounds which were inactive up to 10 μM. Similarly, the 4-methoxy analog **5j** also was inactive.

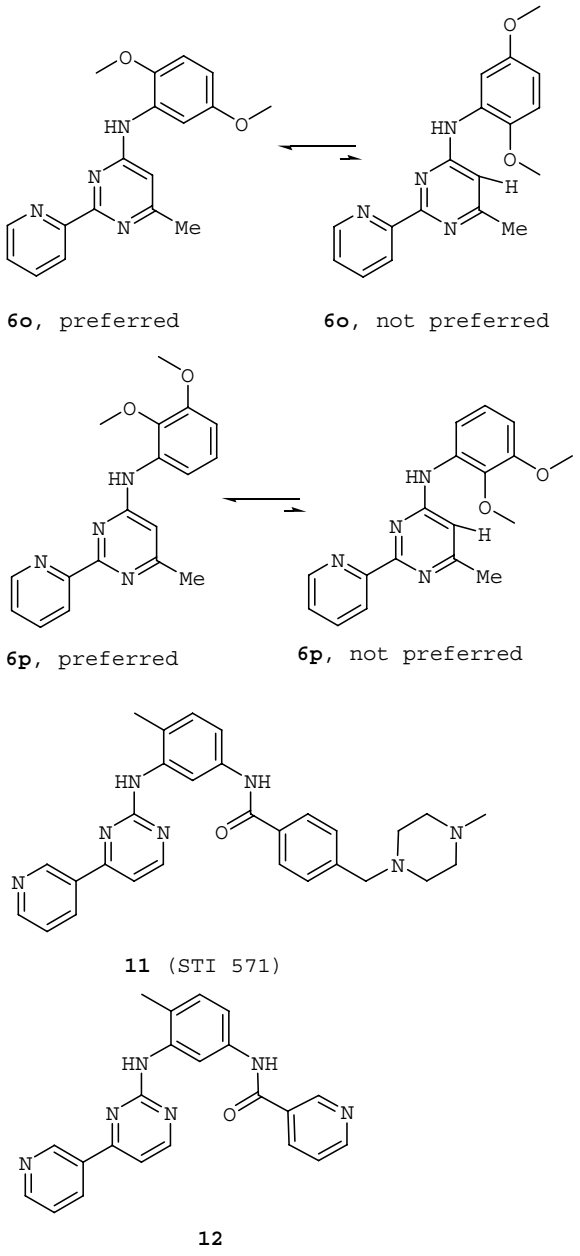


Chart 2.

The disubstituted analogs of **5a** were found to have similar SAR as that of **6a**. The 3,5-dimethoxy compound **5k** is about 7-fold more potent than **5a**, confirming the importance of *meta*-methoxy group for activity. The 2,5-dimethoxy compound **5l**, with an EC_{50} value of 0.018 μM at T47D cells, is the most active compound in this series, and is 20-fold more potent than **5a**. Compound **5m**, with a methyl group in the 2-position, also was highly active with an EC_{50} value of 0.055 μM, and is about 3-fold less active than **5l**. Interestingly, compound **5n**, with a chloro group in the 2-position, is 10-fold less active than **5l**, suggesting that an electron-donating group is preferred in the *ortho*-position. The 2,5-dichloro analog **5o** was inactive up to 10 μM, indicating that an electron-donating methoxy group in the *meta*-position is important for activity of disubstituted compounds.

Compound **5p**, with an additional methoxy group in the 4-position of compound **5m**, is about as active as that of **5m**, indicating that the 4-methoxy group contributes little to the activity. Compound **5q**, with an additional chloro group in the 4-position of compound **5l**, is >6-fold less active than **5l**, probably because chloro is an electron withdrawing group which is not preferred. Overall, these results suggested that there is no advantage of trisubstituted compounds versus disubstituted compounds.

We then explored the modification of the linker NH group. Compound **9**, with an NMe group replacing the NH group in compound **5a**, was not active up to 10 μM. Therefore, compound **9** is >25-fold less active than **5a**, indicating that the NH group is critical for the apoptosis-inducing activity of **5a**. Compound **10**, with an O group replacing the NH group in compound **6a**, also was not active up to 10 μM, confirming the importance of the NH linker group. These data suggested that the NH group most probably forms a hydrogen bond with its target protein and replacing the NH group with an NMe or O group results in the breaking up of this important interaction. Interestingly, the corresponding NH group in compound **12**, a close analog of compound **11**, was shown to form an important hydrogen bond when binding to Abl tyrosine kinase.[30]

The activities of these compounds toward the human non-small cell lung cancer cells H1299 and human colon cancer cells HT29 were somewhat different from that of T47D cells. Compounds that are inactive against T47D cells also are all inactive against H1299 and HT29 cells. Active compounds **5a**, **5c**, **5d** and **6a**, with a mono-substituent in the *meta*-position, are active against T47D, but inactive against H1299 and HT29 cells up to 10 μM, indicating that these compounds are selective against certain cancer cells. However, 2,5-disubstituted compounds, including **6o**, **5l**–**5n**, and 2,4,5-trisubstituted compounds such as **5p** and **5q**, are active against T47D cells as well as H1299 and HT29 cells, indicating that these compounds are not selective against certain cancer cells. Since the non-selective compounds and selective compounds are structurally related and have similar chemical properties, it is unlikely that the observed different activities against cancer cells are due to different metabolism or uptake/excretion, and suggests that the 2,5-disubstituted compounds such as **6o** and **5l** might have a different mechanism of action in inducing apoptosis from that of mono-*meta*-substituted compounds such as **5a** and **6a**. Interestingly, 3,5-dimethoxy analog **5k** is active against T47D cells as well as H1299 and HT29 cells, while 3,5-dimethoxy analog **6m** is only active against T47D cells, again suggesting that the SAR of the CF_3 series (**5a**) is not exactly same as the CH_3 series (**6a**).

The apoptosis-inducing activity of potent analog **5l** was also characterized by cell cycle analysis similar to compound **5a**. T47D cells were treated with 50 nM of compound **5l** for 24 h or 48 h at 37 °C, then stained with propidium iodide and analyzed by flow cytometry. Similar to compound **5a**, an increase in G_2/M population

Table 2. Comparison of caspase activation activity and inhibition of cell proliferation activity of 4-anilino-2-(2-pyridyl)pyrimidines

Compound	T47D			H1299		
	EC_{50}[a] (μM)	GI_{50}[b] (μM)	GI_{50}/ EC_{50}	EC_{50}[a] (μM)	GI_{50}[b] (μM)	GI_{50}/EC_{50}
5a	0.36 ± 0.17	0.57 ± 0.17	1.6	>10	>10	NA
6a	0.31 ± 0.01	1.61 ± 0.31	5.2	>10	>10	NA
6o	0.050 ± 0.005	0.21 ± 0.06	4.2	0.27 ± 0.01	0.74 ± 0.19	2.7
5c	0.65 ± 0.07	0.47 ± 0.10	0.72	>10	>10	NA
5l	0.018 ± 0.001	0.20 ± 0.04	11	0.15 ± 0.02	0.12 ± 0.02	0.8
5m	0.055 ± 0.006	0.29 ± 0.12	5.3	0.37 ± 0.06	0.47 ± 0.02	1.3

[a] Data are means of three or more experiments and are reported as means ± standard error of the mean (SEM).
[b] Data are means of three experiments and are reported as means ± standard error of the mean (SEM).

(from 16.9% to 40.4%) was observed after 24-h treatment with **5l**, together with a large increased apoptotic sub-G_1 population (from 3.4% to 26.3%, Fig. 1A vs F). Sub-G_1 population increased to 58.5% after 48 h treatment with **5l**, indicating that most of the cells are apoptotic (Fig. 1G). These data confirm that compound **5l** is more potent than **5a** as an inducer of apoptosis. Treatment with the broad-spectrum caspase inhibitor **13** also resulted in reduction of sub-G_1 apoptotic cells at 24 h (from 26.3% to 19.2%, Fig. 1F vs H) and 48 h (from 58.5% to 28.0%, Fig. 1G vs I), and cells were observed both in the G_1 (18.3% and 37.9%, Fig. 1H and I) and G_2/M. In comparison, cells treated with **5a** show few cells in the G_1 (11.0% and 4.4%, Fig. 1D and E). These results indicated that treatment of cells with **5a** and **5l** both led to G_2/M arrest, followed by induction of apoptosis, and most of the cell death can be inhibited by a pan-caspase inhibitor. However, the mechanism of action may not be exactly the same for these two compounds.

Selected compounds were also tested by the traditional inhibition of cell proliferation (GI_{50}) assay to confirm that the active compounds can inhibit tumor cell growth, as well as to determine whether there is a correlation between the activity from the caspase activation assay and the cell proliferation assay. The growth inhibition assays in T47D and H1299 cells were run in a 96-well microtiter plate as described previously.[15] The GI_{50}, along with the EC_{50} data and the ratio of GI_{50}/EC_{50}, are summarized in Table 2.

Table 2 shows that compounds **6o** and **5l** are potent inhibitors of tumor cell growth. Compound **6o** has a GI_{50} value of 0.21 μM and 0.74 μM in T47D and H1299 cells, respectively. Similarly, compound **5l** has a GI_{50} value of 0.20 μM and 0.12 μM in T47D and H1299 cells, respectively. Compounds **5a**, **6a**, and **5c**, which are active against T47D cells and not active against H1299 cells in the apoptosis induction assay, also are active against T47D and not active against H1299 in the growth inhibition assay, confirming the selectivity of these compounds. Compounds **6o**, **5l**, and **5m** are active against both T47D and H1299 cells, confirming that these compounds are not selective against certain cells. In general, the compounds that are more active in the apoptosis induction assay, as measured by caspase activation, also are more potent in the growth inhibition assay. For T47D cells, most of the compounds are more potent in the apoptosis induction assay (as measured by EC_{50} values) than in the growth inhibition assay (as measured by GI_{50} values), except for compound **5c**. This is also true for H1299 cells except for compound **5l**.

Since cell cycle analysis shows that treatment of T47D cells with **5a** and **5l** led to G_2/M arrest followed by induction of apoptosis, a characteristic that is similar to tubulin inhibitors, we decided to test inhibition of tubulin polymerization as a possible mechanism for this series of compounds. Compound **5a** was found not to inhibit tubulin polymerization at up to 50 μM, indicating that **5a** is unlikely to be a tubulin inhibitor and suggesting that **5a** might induce apoptosis through some unknown mechanism other than tubulin inhibition. In a MDS Panlabs screen against nine kinases tested at 10, 100 or 300 μM, compound **5a** was found to inhibit 62% of PKC at a concentration of 100 μM, and no significant inhibition was found for the other kinases tested.[31] Interestingly, compound **5l** was found to inhibit tubulin polymerization completely at concentration at or above 0.5 μM, a potency that is similar to other inhibitors of tubulin such as the 4-aryl-chromenes,[17] indicating that inhibition of tubulin polymerization, at least partially, should be the mechanism of action for the apoptosis-inducing activity of **5l**. Compound **5l** is active against all cancer cells tested both in the apoptosis induction assay and growth inhibition assay. This is similar to other tubulin inhibitors we identified which are active against all cancer cells tested.[15–18] On the other hand, **5a** is selective against certain cancer cells both in the apoptosis induction assay and growth inhibition assay. Therefore, the tubulin inhibition data are in agreement with results from other assays, which suggests that compounds **5a** and **5l** should have a different mechanism of action in the induction of apoptosis.

3. Conclusion

In conclusion, we have discovered a series of substituted 4-anilino-2-(2-pyridyl)pyrimidines as potent inducers of apoptosis using our cell- and caspase-based HTS assay. SAR studies of the anilino group showed that a *meta*-methoxy is critical for apoptosis-inducing activity. A 2,5-disubstituted anilino group was found to increase the potency >10-fold versus the mono-*meta*-substituted

analogs. Compound **5l**, 4-(2,5-dimethoxyanilino)-2-(2-pyridinyl)-6-(trifluoromethyl)pyrimidine, was found to be the most potent compound, with an EC_{50} value of 18 nM in the apoptosis induction assay. Interestingly, the mono-*meta*-substituted-anilino analogs such as **5a** and **6a** were found to be selective for T47D cells and are not active against H1299 and HT29 cells, while the 2,5-disubstituted-anilino analogs such as **6o** and **5l** were found to be active against all the three cell lines (T47D, H1299 and HT29) in the apoptosis induction assay. The selectivity of these compounds was confirmed in the traditional growth inhibition assay (T47D and H1299), suggesting that these compounds should have a different mechanism of action.

The screening hit **5a** was found to arrest cells in G_2/M, followed by induction of apoptosis, as measured by flow cytometry assays in T47D cells. The more potent analog **5l** also was found to be more potent in the cell cycle analysis assay. Compound **5a** did not inhibit tubulin polymerization at up to 50 μM, while compound **5l** inhibited tubulin polymerization completely at concentration at or above 0.5 μM. These data indicated that the extra 2-methoxy group in **5l** has changed the mechanism of action, as suggested from the differential selectivity for both apoptosis induction assay and the growth inhibition assay. Additional SAR studies of the 4-anilino-2-(2-pyridyl)pyrimidines series of apoptosis inducers are in progress and will be reported in future publications.

4. Experimental

4.1. General methods and materials

Commercial-grade reagents and solvents from Acros, Aldrich, Lancaster, or TCI were used without further purification except as indicated. 4-Chloro-2-(2-pyridinyl)-6-(trifluoromethyl)pyrimidine (**7**) and 4-chloro-6-methyl-2-(2-pyridinyl)pyrimidine (**8**) were obtained from Key Organics (UK). All reaction mixtures were stirred magnetically. Thin-layer chromatography (TLC), usually using ethyl acetate/hexane as the solvent system, was used to monitor reactions. Solvents were removed by rotary evaporation under reduced pressure; where appropriate, the compound was further dried using a vacuum pump. The ^{1}H NMR spectra were recorded at 300 MHz. All samples were prepared as dilute solutions in either deuteriochloroform ($CDCl_3$) with v/v 0.05% tetramethylsilane (TMS) or dimethyl-d_6-sulfoxide (CD_3SOCD_3) with v/v 0.05% TMS. Chemical shifts are reported in parts per million (ppm) downfield from TMS (0.00 ppm) and *J* coupling constants are reported in hertz. Elemental analyses were performed by Numega Resonance Labs, Inc. (San Diego, CA). Human breast cancer cells T47D, non-small cell lung cancer cells H1299, and human colon cancer cells HT29 were obtained from American Type Culture Collection (Manassas, VA). Tubulin was obtained from Cytoskeleton (Boulder, CO). Compounds **5a**, **5b**, **5g**, **5h**, **5i**, and **5j** were obtained from Key Organics (UK) and their structures were confirmed by ^{1}H NMR.

4.1.1. 4-(4-Methoxyanilino)-6-methyl-2-(2-pyridinyl)pyrimidine (6d). A mixture of 4-chloro-6-methyl-2-(2-pyridinyl)pyrimidine (115 mg, 0.56 mmol), *p*-anisidine (68 mg, 0.55 mmol) in water (10 mL) and 2 N HCl (0.3 mL) was refluxed for 6 h, cooled to room temperature, and neutralized with 2 N NaOH to pH 10. The resulting mixture was then extracted with 1:1 hexane/ethyl acetate (50 mL). The organic phase was washed with water and brine, dried over Na_2SO_4, and concentrated in vacuo. The residue was purified by chromatography (hexane/ethyl acetate, 1:1) to yield the product as a tan solid (72 mg, 45%). ^{1}H NMR ($CDCl_3$) 8.80 (d, J = 4.5 Hz, 1H), 8.45 (d, J = 8.1 Hz, 1H), 7.81 (m, 1H), 7.35 (m, 1H), 7.23 (d, J = 8.4 Hz, 2H), 7.12 (s, 1H), 6.93 (d, J = 8.4 Hz, 2H), 6.41 (s, 1H), 3.83 (s, 3H), 2.45 (s, 3H). Anal. Calcd for $C_{17}H_{16}N_4O$: C, 69.85; H, 5.52; N, 19.17. Found: C, 70.06; H, 5.05; N, 19.05.

4.1.2. 4-Anilino-6-methyl-2-(2-pyridinyl)pyrimidine (6b). Compound **6b** was prepared as a solid from reaction of 4-chloro-6-methyl-2-(2-pyridinyl)pyrimidine with aniline by a procedure similar to that described for the preparation of compound **6d**. ^{1}H NMR (DMSO-d_6) 9.64 (s, 1H), 8.70 (d, J = 4.5 Hz, 1H), 8.28 (d, J = 7.5 Hz, 1H), 7.94 (m, 1H), 7.78 (s, 1H), 7.75 (s, 1H), 7.47 (m, 1H), 7.31 (m, 1H), 7.02 (m, 1H), 6.66 (s, 1H), 2.38 (s, 3H). Anal. Calcd for $C_{16}H_{14}N_4$: C, 73.26; H, 5.38; N, 21.36. Found: C, 74.37; H, 5.23; N, 21.48.

4.1.3. 2-(2-Pyridinyl)-4-(3-trifluoromethoxyanilino)-6-(trifluoromethyl)pyrimidine (5f). A mixture of 4-chloro-2-(2-pyridinyl)-6-(trifluoromethyl)pyrimidine (25 mg, 0.096 mmol), 3-trifluoromethoxyaniline (19 μL, 0.144 mmol), and 2 N HCl (75 μL) in water/ethanol (2:1, 5 mL) was refluxed for 24 h. The mixture was cooled to room temperature and the resulting precipitate was filtered, washed with water (0.5 mL), water/ethanol (2:1, 0.5 mL), and dried to give a yellow solid (23 mg, 59%). ^{1}H NMR ($CDCl_3$) 8.88–8.85 (m, 1H), 8.59–8.55 (m, 1H), 7.93–7.87 (m, 1H), 7.59 (s, 1H), 7.48 (t, J = 8.3 Hz, 1H), 7.45 (dd, J = 1.2, 7.5 Hz, 1H), 7.36 (s, 1H), 7.30–7.29 (m, 1H), 7.16–7.10 (m, 1H), 7.05 (s, 1H). Anal. Calcd for $C_{17}H_{10}F_6N_4O{\cdot}0.4H_2O$: C, 50.11; H, 2.67; N, 13.75. Found: C, 50.13; H, 2.51; N, 13.82.

The following compounds were prepared from 4-chloro-6-methyl-2-(2-pyridinyl)pyrimidine or 4-chloro-2-(2-pyridinyl)-6-(trifluoromethyl)pyrimidine and the corresponding substituted aniline by a procedure similar to that described for the preparation of compound **5f**.

4.1.4. 4-(3,5-Dimethoxyanilino)-2-(2-pyridinyl)-6-(trifluoromethyl)pyrimidine (5k). Light yellow solid (37%); ^{1}H NMR ($CDCl_3$) 8.85 (dd, J = 4.8, 1.5 Hz, 1H), 8.56 (d, J = 6.9 Hz, 1H), 7.89 (ddd, J = 9.3, 7.5, 1.5 Hz, 1H), 7.65 (br s, 1H), 7.45 (dd, J = 9.3, 4.5 Hz, 1H), 7.09 (s, 1H), 6.51 (d, J = 1.8 Hz, 1H), 6.37 (t, J = 1.8 Hz, 1H), 3.93 (s, 6H). Anal. Calcd for $C_{18}H_{15}F_3N_4O_2{\cdot}0.4H_2O$: C, 56.37; H, 4.15; N, 14.60. Found: C, 56.12; H, 3.94; N, 14.37.

4.1.5. 4-(4,5-Dimethoxy-2-methylanilino)-2-(2-pyridinyl)-6-(trifluoromethyl)pyrimidine (5p). Yellow solid (79%); ^{1}H NMR ($CDCl_3$) 8.82–8.80 (m, 1H), 8.55 (d, J = 7.8 Hz, 1H), 7.88–7.82 (m, 1H), 7.51 (s, 1H), 7.43–7.38 (m, 1H), 6.80 (s, 1H), 6.76 (s, 1H), 6.50 (s, 1H), 3.92 (s, 3H), 3.84 (s, 3H), 2.19 (s, 3H). Anal. Calcd for $C_{19}H_{17}F_3N_4O_2 \cdot 1.3H_2O$: C, 55.15; H, 4.77; N, 13.54. Found: C, 55.11; H, 4.37; N, 13.68

4.1.6. 4-(4-Chloro-2,5-dimethoxyanilino)-2-(2-pyridinyl)-6-(trifluoromethyl)pyrimidine (5q). Brown solid (73%); ^{1}H NMR ($CDCl_3$) 8.84–8.82 (m, 1H), 8.61–8.57 (m, 1H), 8.50 (br s, 1H), 7.93–7.87 (m, 1H), 7.66 (s, 1H), 7.49–7.45 (m, 1H), 7.02 (s, 1H), 7.01 (s, 1H), 4.07 (s, 3H), 3.92 (s, 3H). Anal. Calcd for $C_{18}H_{14}ClF_3N_4O_2$: C, 52.63; H, 3.44; N, 13.64. Found: C, 52.15; H, 2.99; N, 13.57.

4.1.7. 4-(3-Ethoxyanilino)-6-methyl-2-(2-pyridinyl)pyrimidine (6e). Light tan solid (78%); ^{1}H NMR ($CDCl_3$) 8.86–8.83 (m, 1H), 8.50 (d, J = 8.1 Hz, 1H), 7.89–7.83 (m, 1H), 7.42–7.38 (m, 1H), 7.35–7.30 (m, 1H), 7.09 (s, 1H), 6.96–6.84 (m, 2H), 6.76 (d, J = 3.3 Hz, 1H), 6.71 (s, 1H), 4.09 (q, J = 7.2 Hz, 2H), 2.53 (s, 3H), 1.47 (t, J = 7.1 Hz, 3H). Anal. Calcd for $C_{18}H_{18}N_4O$: C, 70.57; H, 5.92; N, 18.24. Found: C, 70.20; H, 5.79; N, 18.07.

4.1.8. 6-Methyl-2-(2-pyridinyl)-4-(3-trifluoromethoxyanilino)pyrimidine (6g). Pale white solid (93%); ^{1}H NMR (DMSO-d_6) 9.95 (s, 1H), 8.74 (s, 1H), 8.31 (d, J = 9.3 Hz, 2H), 7.95 (t, J = 7.7 Hz, 1H), 7.65 (d, J = 8.4 Hz, 1H), 7.53–7.43 (m, 2H), 6.98 (d, J = 8.4 Hz, 1H), 6.71 (s, 1H), 2.44 (s, 3H). Anal. Calcd for $C_{17}H_{13}F_3N_4O$: C, 58.96; H, 3.78; N, 16.18. Found: C, 58.59; H, 3.93; N, 16.33.

4.1.9. 4-(3-Fluoroanilino)-6-methyl-2-(2-pyridinyl)pyrimidine (6h). Light brown solid (54%); ^{1}H NMR ($CDCl_3$) 8.83 (d, J = 4.8 Hz, 1H), 8.47 (d, J = 9.0 Hz, 1H), 7.88 (m, 1H), 7.44–7.30 (m, 4H), 7.18–7.14 (m, 1H), 6.92–6.86 (m, 1H), 6.80 (s, 1H), 2.55 (s, 3H). Anal. Calcd for $C_{16}H_{13}FN_4 \cdot 1H_2O$: C, 64.42; H, 5.07; N, 18.78. Found: C, 64.29; H, 4.74; N, 18.64.

4.1.10. 4-(3-Cyanoanilino)-6-methyl-2-(2-pyridinyl)pyrimidine (6i). Pale white solid (76%); ^{1}H NMR (DMSO-d_6) 10.14 (s, 1H), 8.78–8.75 (m, 1H), 8.56 (s, 1H), 8.32 (d, J = 7.8 Hz, 1H), 8.04–7.96 (m, 2H), 7.60–7.47 (m, 3H), 6.75 (s, 1H), 2.47 (s, 3H). Anal. Calcd for $C_{17}H_{13}N_5 \cdot 0.4H_2O$: C, 69.33; H, 4.72; N, 23.78. Found : C, 68.92; H, 4.71; N, 24.09

4.1.11. 6-Methyl-2-(2-pyridinyl)-4-(3-trifluoromethylanilino)pyrimidine (6j). Pale white solid (93%); ^{1}H NMR ($CDCl_3$) 8.84–8.82 (m, 1H), 8.49–8.45 (m, 1H), 7.95 (s, 1H), 7.90–7.85 (m, 1H), 7.62 (d, J = 7.8 Hz, 1H), 7.52 (t, J = 7.8 Hz, 1H), 7.45–7.40 (m, 2H), 6.78 (s, 1H), 2.55 (s, 3H). Anal. Calcd for $C_{17}H_{13}F_3N_4$: C, 60.48; H, 3.94; N, 16.59. Found: C, 60.26; H, 3.76; N, 16.58.

4.1.12. 4-(3-Acetylanilino)-6-methyl-2-(2-pyridinyl)pyrimidine (6k). White solid (81%); ^{1}H NMR ($CDCl_3$) 8.83 (d, J = 4.5 Hz, 1H), 8.50 (d, J = 8.1 Hz, 1H), 8.13 (s, 1H), 7.89–7.83 (m, 1H), 7.75 (d, J = 7.8 Hz, 1H), 7.61–7.48 (m, 2H), 7.41–7.37 (m, 1H), 7.12 (s, 1H), 6.61 (s, 1H), 2.65 (s, 3H), 2.53 (s, 3H). Anal. Calcd for $C_{18}H_{16}N_4O \cdot 0.8H_2O$: C, 67.82; H, 5.57; N, 17.58. Found: C, 67.77; H, 5.42; N, 17.52.

4.1.13. 4-(3-Benzoylanilino)-6-methyl-2-(2-pyridinyl)pyrimidine (6l). Light yellow solid (77%); ^{1}H NMR (DMSO-d_6) 10.76 (s, 1H), 8.78 (d, J = 3.9 Hz, 1H), 8.31 (s, 1H), 8.22 (d, J = 8.7 Hz, 1H), 8.08 (d, J = 9.3 Hz, 1H), 8.02–7.97 (m, 1H), 7.81 (d, J = 7.5 Hz, 2H), 7.68–7.51 (m, 6H), 6.85 (s, 1H), 2.5 (s, 3H). Anal. Calcd for $C_{23}H_{18}N_4O \cdot 1.7H_2O$: C, 69.58; H, 5.43; N, 14.11. Found: C, 69.22; H, 5.02; N, 14.36.

4.1.14. 4-(2,3-Dimethoxyanilino)-6-methyl-2-(2-pyridinyl)pyrimidine (6p). White solid (80%). ^{1}H NMR ($CDCl_3$) 8.85–8.82 (m, 1H), 8.48 (d, J = 7.8 Hz, 1H), 7.87–7.82 (m, 1H), 7.54 (d, J = 7.5 Hz, 1H), 7.40–7.35 (m, 2H), 7.10 (t, J = 8.4 Hz, 1H), 6.73 (d, J = 1.5 Hz, 1H), 7.00 (s, 1H), 3.90 (s, 3H), 3.86 (s, 3H), 2.53 (s, 3H). Anal. Calcd for $C_{18}H_{18}N_4O_2$: C, 67.07; H, 5.63; N, 17.38. Found: C, 66.82; H, 5.53; N, 17.56.

4.1.15. 4-(3-Methoxyanilino)-6-methyl-2-(2-pyridinyl)pyrimidine (6a). A mixture of 4-chloro-6-methyl-2-(2-pyridinyl)pyrimidine (100 mg, 0.486 mmol) and *m*-anisidine (55 μL, 0.486 mmol) in water (5 mL) and 2 N HCl (300 μL) was refluxed for 6 h. The mixture was diluted with ethyl acetate (50 mL) and then washed with water and saturated NaCl. The ethyl acetate solution was dried over anhydrous sodium sulfate, concentrated in vacuo, and purified by column chromatography (hexane/ethyl acetate, 3:1) to yield n tan oil (139 mg, 98%). ^{1}H NMR ($CDCl_3$) 8.78–8.75 (m, 1H), 8.45 (dt, J = 1.2, 8.1 Hz, 1H), 7.78 (td, J = 1.9, 7.8 Hz, 1H), 7.73 (s, 1H), 7.34–7.30 (m, 1H), 7.23 (t, J = 8.1 Hz, 1H), 7.03 (t, J = 2.1 Hz, 1H), 6.92 (dd, J = 2.0, 7.7 Hz, 1H), 6.67 (dd, J = 2.4, 8.1 Hz, 1H), 6.61 (s, 1H), 3.77 (s, 3H), 2.44 (s, 3H). Anal. Calcd for $C_{17}H_{16}N_4O \cdot 0.6H_2O$: C, 67.35; H, 5.72; N, 18.48. Found: C, 67.72; H, 5.32; N, 18.18.

The following compounds were prepared from 4-chloro-6-methyl-2-(2-pyridinyl)pyrimidine and the corresponding substituted aniline by a procedure similar to that described for the preparation of compound **6a**.

4.1.16. 4-(2-Methoxyanilino)-6-methyl-2-(2-pyridinyl)pyrimidine (6c). Pale white solid (56%); ^{1}H NMR ($CDCl_3$) 8.82–8.80 (m, 1H), 8.46 (d, J = 8.1 Hz, 1H), 7.90 (dd, J = 1.4, 7.7 Hz, 1H), 7.84–7.78 (m, 1H), 7.36–7.32 (m, 1H), 7.28 (s, 1H), 7.18–6.98 (m, 2H), 6.92 (dd, J = 1.8, 8.0 Hz, 1H), 6.63 (s, 1H), 3.85 (s, 3H), 2.50 (s, 3H). Anal. Calcd for $C_{17}H_{16}N_4O \cdot 0.3H_2O$: C, 68.58; H, 5.62; N, 18.82. Found: C, 68.70; H, 5.66; N, 18.45.

4.1.17. 4-(3-Benzyloxyanilino)-6-methyl-2-(2-pyridinyl)-pyrimidine (6f). Yellow oil (90%); ^{1}H NMR ($CDCl_3$) 8.78–8.76 (m, 1H), 8.48–8.45 (m, 1H), 7.79–7.74 (m, 1H), 7.68 (s, 1H), 7.42–7.29 (m, 6H), 7.24 (t, J = 8.1 Hz, 1H), 7.10 (s, 1H), 6.92 (dd, J = 0.8, 8.0 Hz, 1H), 6.77–6.74 (m, 1H), 6.58 (s, 1H), 5.04 (s, 2H), 2.42 (s, 3H). Anal. Calcd for $C_{23}H_{20}N_4O \cdot 1H_2O$: C, 71.49; H, 5.74; N, 14.50. Found: C, 71.55; H, 4.96; N, 14.15.

4.1.18. 4-(2,5-Dimethoxyanilino)-6-methyl-2-(2-pyridinyl)pyrimidine (6o). Tan oil (74%); ^{1}H NMR ($CDCl_3$) 8.78–8.76 (m, 1H), 8.44 (d, J = 7.8 Hz, 1H), 7.81 (d, J = 2.7 Hz, 1H), 7.79–7.74 (m, 1H). 7.32–7.28 (m, 2H), 6.78 (d, J = 9.3 Hz, 1H), 6.60 (s, 1H), 6.53 (dd, J = 3.0, 8.7 Hz, 1H), 3.77 (d, J = 3.3 Hz, 6H), 2.48 (s, 3H). Anal. Calcd for $C_{18}H_{18}N_4O_2$: C, 67.07; H, 5.63; N, 17.38. Found: C 66.69; H, 6.01; N 17.40.

4.1.19. 4-(3-Methoxyphenoxy)-6-methyl-2-(2-pyridinyl)pyrimidine (10). White oil (34%). ^{1}H NMR ($CDCl_3$): 8.79–8.76 (m, 1H), 8.26–8.23 (m, 1H), 7.75–7.69 (m, 1H), 7.33–7.28 (m, 2H), 6.82–6.72 (m, 3H), 6.58 (s, 1H), 3.77 (s, 3H), 2.58 (s, 3H). Anal. Calcd for $C_{17}H_{15}N_3O_2$: C, 69.61; H, 5.15; N, 14.33. Found: C, 68.79; H, 5.32; N, 14.17

4.1.20. 4-(3-Ethoxyanilino)-2-(2-pyridinyl)-6-(trifluoromethyl)pyrimidine (5c). A mixture of 4-chloro-2-(2-pyridinyl)-6-(trifluoromethyl)pyrimidine (25 mg, 0.096 mmol), 3-ethoxyaniline (19 μL, 0.144 mmol), and 2 N HCl (75 μL) in water/ethanol (2:1, 5 mL) was refluxed for 24 h. The mixture was cooled to room temperature and basified with aqueous 2 N NaOH to pH 10–12. The resulting precipitate was filtered, washed with water (0.5 mL), water/ethanol (2:1, 0.5 mL), and dried to give a yellow solid (22 mg, 63%). ^{1}H NMR ($CDCl_3$) 8.88–8.85 (m, 1H), 8.58 (d, J = 8.1 Hz, 1H), 7.93–7.88 (m, 1H), 7.61 (s, 1H), 7.48–7.44 (m, 1H), 7.35 (t, J = 8.4 Hz, 1H), 7.05 (s, 1H), 6.89–6.81 (m, 3H), 4.07 (q, J = 7.1 Hz, 2H), 1.45 (t, J = 7.1 Hz, 3H). Anal. Calcd for $C_{18}H_{15}F_3N_4O$: C, 60.00; H, 4.20; N, 15.55. Found: C, 59.60; H, 4.15; N, 15.47.

The following compounds were prepared from 4-chloro-6-methyl-2-(2-pyridinyl)pyrimidine or 4-chloro-2-(2-pyridinyl)-6-(trifluoromethyl)pyrimidine and the corresponding substituted aniline by a procedure similar to that described for the preparation of compound **5c**.

4.1.21. 4-(3-Methylmercaptoanilino)-2-(2-pyridinyl)-6-(trifluoromethyl)pyrimidine (5e). Yellow solid (58%); ^{1}H NMR ($CDCl_3$) 8.87–8.85 (m, 1H), 8.58 (d, J = 7.8 Hz, 1H), 7.93–7.88 (m, 1H), 7.61 (s, 1H), 7.48–7.44 (m, 1H), 7.36 (t, J = 7.8 Hz, 1H), 7.25 (s, 1H), 7.17–7.14 (m, 1H), 7.10–7.06 (m, 1H), 7.01 (s, 1H), 2.52 (s, 3H). Anal. Calcd for $C_{17}H_{13}F_3N_4S$: C, 56.35; H, 3.62; N, 15.46. Found: C, 55.84; H, 3.72; N, 15.73.

4.1.22. 4-(5-Methoxy-2-methylanilino)-2-(2-pyridinyl)-6-(trifluoromethyl)pyrimidine (5m). Yellow solid (52%); ^{1}H NMR ($CDCl_3$) 8.85–8.82 (m, 1H), 8.59–8.56 (m, 1H), 7.90–7.84 (m, 1H), 7.45–7.40 (m, 2H), 7.24 (d, J = 8.1 Hz, 1H), 6.87 (s, 1H), 6.83 (dd, J = 2.6, 8.3 Hz, 1H), 6.69 (s, 1H), 3.81 (s, 3H), 2.20 (s, 3H). Anal. Calcd for $C_{18}H_{15}F_3N_4O$: C, 60.00; H, 4.20; N, 15.55. Found: C, 59.63; H, 4.41; N, 15.69.

4.1.23. 4-(2-Chloro-5-methoxyanilino)-2-(2-pyridinyl)-6-(trifluoromethyl)pyrimidine (5n). White solid (48%); ^{1}H NMR ($CDCl_3$) 8.84–8.82 (m, 1H), 8.58–8.54 (m, 1H), 7.90–7.85 (m, 1H), 7.66 (s, 1H), 7.60 (s, 1H), 7.46–7.41 (m, 1H), 7.37 (d, J = 8.7 Hz, 1H), 7.01 (s, 1H), 6.75 (dd, J = 3.0, 8.7 Hz, 1H), 3.88 (s, 3H). Anal. Calcd for $C_{17}H_{12}ClF_3N_4O$: C, 53.63; H, 3.18; N, 14.71. Found: C, 53.26; H, 3.32; N, 14.37.

4.1.24. 4-(3,5-Dimethoxyanilino)-6-methyl-2-(2-pyridinyl)pyrimidine (6m). Tan solid (26%); ^{1}H NMR ($CDCl_3$) 8.83–8.81 (m, 1H), 8.49–8.46 (m, 1H), 7.86–7.80 (m, 1H), 7.39–7.35 (m, 1H), 6.99 (s, 1H), 6.70 (s, 1H), 6.53 (d, J = 2.4 Hz, 2H), 6.30 (t, J = 2.3 Hz, 1H), 3.82 (s, 6H), 2.51 (s, 3H). Anal. Calcd for $C_{18}H_{18}N_4O_2 \cdot 0.3H_2O$: C, 65.96; H, 5.72; N, 17.09. Found: C, 65.89; H, 6.00; N, 17.10.

4.1.25. 4-(2,4-Dimethoxyanilino)-6-methyl-2-(2-pyridinyl)pyrimidine (6n). Purple crystal (64%); ^{1}H NMR ($CDCl_3$) 8.84–8.81 (m, 1H), 8.48–8.44 (m, 1H), 7.86–7.80 (m, 1H), 7.60 (d, J = 8.4 Hz, 1H), 7.38–7.34 (m, 1H), 6.93 (s, 1H), 6.56–6.53 (m, 2H), 6.46 (s, 1H), 3.84 (d, J = 0.6 Hz, 3H), 3.83 (d, J = 0.9 Hz, 3H), 2.48 (s, 3H). Anal. Calcd for $C_{18}H_{18}N_4O_2$: C, 67.07; H, 5.63; N, 17.38. Found: C, 66.69; H, 5.97; N, 17.47.

4.1.26. 4-(2,5-Dimethoxyanilino)-2-(2-pyridinyl)-6-(trifluoromethyl)pyrimidine (5l). A mixture of 4-chloro-2-(2-pyridinyl)-6-(trifluoromethyl)pyrimidine (50 mg, 0.193 mmol) and 2,5-dimethoxyaniline (59 mg, 0.386 mmol) in aqueous 2 N HCl (150 μL) and water/ethanol (1:1, 5 mL) was refluxed for 24 h. The mixture was cooled to room temperature, extracted with ethyl acetate (25 mL), and washed with water (12 mL). The ethyl acetate solution was acidified with aqueous 2 N HCl (12 mL). The resulting precipitate was collected by filtration, washed with water (0.5 mL), and dried to give a light brown solid (36 mg, 50%). ^{1}H NMR (DMSO-d_6) 9.65 (s, 1H), 8.76–8.73 (m, 1H), 8.32 (d, J = 7.8 Hz, 2H), 8.03–7.97 (m, 1H), 7.58–7.54 (m, 2H), 7.05 (d, J = 9.0 Hz, 1H), 6.70 (dd, J = 3.0, 8.7 Hz, 1H), 3.84 (s, 3H), 3.82 (s, 3H). Anal. Calcd for $C_{18}H_{15}F_3N_4O_2$: C, 57.45; H, 4.02; N, 14.89. Found: C, 56.88; H, 4.14; N, 14.62.

4.1.27. 4-(3-Hydroxyanilino)-2-(2-pyridinyl)-6-(trifluoromethyl)pyrimidine (5d). Compound **5f** was prepared from reaction of 4-chloro-2-(2-pyridinyl)-6-(trifluoromethyl)pyrimidine with 3-aminophenol by a procedure similar to that described for the preparation of compound **5l**, and isolated as a yellow solid (67%). ^{1}H NMR (DMSO-d_6) 10.40 (s, 1H), 8.86 (dd, J = 0.9, 5.1 Hz, 1H), 8.51 (d, J = 7.8 Hz, 1H), 8.32–8.26 (m, 1H), 7.82–7.78 (m, 1H), 7.29 (s, 1H), 7.26 (s, 1H), 7.23 (s, 1H), 7.19 (d, J = 7.5 Hz, 1H), 6.57 (dd, J = 1.8, 9.0 Hz, 1H). Anal. Calcd for $C_{16}H_{11}F_3N_4O$: C, 44.62; H, 3.21; N, 13.01. Found: C, 44.71; H, 3.57; N, 13.06.

4.1.28. 4-(2,5-Dichloroanilino)-2-(2-pyridinyl)-6-(trifluoromethyl)pyrimidine (5o). A mixture of 4-chloro-2-(2-pyridinyl)-6-(trifluoromethyl)pyrimidine (25 mg, 0.096 mmol), 2,5-dichloroaniline (23 mg, 0.14 mmol), and 2 N HCl (75 μL) in water/ethanol (2:1, 5 mL) was refluxed for 24 h. The mixture was cooled to room temperature and the resulting precipitate was filtered, washed with water, water/ethanol (2:1), and dried. The crude product was purified by chromatography

(hexane/ethyl acetate, 3:1) to give a light yellow solid (14 mg, 38% yield). ^{1}H NMR ($CDCl_3$) 8.85–8.85 (m, 1H), 8.54 (d, J = 8.1 Hz, 1H), 8.16 (d, J = 2.7 Hz, 1H), 7.92–7.86 (m, 1H), 7.64 (s, 1H), 7.47–7.42 (m, 1H), 7.41 (d, J = 8.4 Hz, 1H), 7.16 (dd, J = 2.4, 8.7 Hz, 1H), 7.01 (s, 1H). Anal. Calcd for $C_{16}H_9Cl_2F_3N_4$: C, 49.89; H, 2.36; N, 14.55. Found: C, 50.26; H, 2.53; N, 14.42.

4.1.29. 4-(*N*-Methyl-3-methoxyanilino)-2-(2-pyridinyl)-6-(trifluoromethyl)pyrimidine (9). A mixture of 4-chloro-2-(2-pyridinyl)-6-(trifluoromethyl)pyrimidine (180 mg, 0.693 mmol) and *N*-methyl-3-methoxyaniline (112 mg, 0.816 mmol) in 3 mL of solvent (ethanol/water, 2:1) was refluxed for 20 h. The solvent was removed under reduced pressure and the residue was dissolved in 25 mL of ethyl acetate. The ethyl acetate solution was washed with 25 mL of 1 M NaOH, and the aqueous layer was re-extracted with ethyl acetate (25 mL). The combined organic extracts were washed with saturated NaCl and dried over Na_2SO_4, filtered, and concentrated. The crude product was purified by flash column chromatography (40–45% ethyl acetate/hexane) to give an oil (250 mg, 0.693 mmol, 100%). ^{1}H NMR ($CDCl_3$) 8.86 (dd, J = 0.6, 4.5 Hz, 1H), 8.50 (d, J = 7.5 Hz, 1H), 7.85 (dt, J = 1.8, 7.8 Hz, 1H), 7.42 (m, 2H), 6.95 (dd, J = 1.8, 8.4 Hz, 1H), 6.86 (m, 1H), 6.82 (t, J = 2.1 Hz, 1H), 6.62 (s, 1H), 3.85 (s, 3H), 3.68 (s, 3H). Anal. Calcd for $C_{18}H_{15}F_3N_4O$: C, 60.00; H, 4.20; N, 15.55. Found: C, 59.23; H, 4.39; N 15.21.

4.2. Caspase activation assay (EC_{50})

T47D, H1299, and HT29 cells were grown according to media component mixtures designated by American Type Culture Collection in RPMI-1640 + 10% FCS in a 5% CO_2–95% humidity incubator at 37 °C. Cells were harvested using trypsin and washed at 600*g* and resuspended at 0.65×10^6 cells/mL into RPMI media + 10% FCS. An aliquot of 22.5 μL cells was added to a well of a 384-well microtiter plate containing 2.5 μL of 0.05–100 μM of test compound in RPMI-1640 containing 25 mM HEPES media solution with 10% DMSO (0.005–10 μM final). An aliquot of 22.5 μL of cells was added to a well of a 384-well microtiter plate containing 2.5 μL of RPMI-1640 media solution with 10% DMSO and without test compound as the control sample. The samples were then incubated at 37 °C for 48 h in a 5% CO_2–95% humidity incubator. After incubation, the samples were removed from the incubator and 25 μL of a solution containing 14 μM *N*-(Ac-DEVD)-*N*′-ethoxycarbonyl-R110 fluorogenic substrate, 20% sucrose, 20 mM DTT, 200 mM NaCl, 40 mM Na PIPES buffer, pH 7.2, and 250 μg/mL lysolecithin was added. The samples were incubated at room temperature. Using a fluorescent plate reader (Model Spectrafour Plus Tecan), an initial reading (T = 0) was made approximately 1–2 min after addition of the substrate solution employing excitation at 485 nm and emission at 525 nm, to determine the background fluorescence of the control sample. After the 3-h incubation, the samples were read for fluorescence as above (T = 3 h).

4.2.1. Calculation. The relative fluorescence unit values (RFU) were used to calculate the sample readings as follows: The activity of caspase activation was determined by the ratio of the net RFU value for the test compound to that of control samples. The EC_{50} (μM) was determined by a sigmoidal dose–response calculation (XLFit3, IDBS), as the concentration of compound that produces the 50% maximum response. The caspase activation activity (EC_{50}) in three cancer cell lines, T47D, H1299, and HT29, is summarized in Table 1.

4.3. Cell cycle analysis and measurement of apoptosis

T47D cells were maintained and harvested as described above. Cells (2×10^5) were seeded into a 6-well plate in RPMI/10% FBS (Invitrogen, Carlsbad, CA) and incubated for 24 h at 37 °C in a 5% CO_2 atmosphere. Cells were then treated with the compound (700 nM of **5a** or 50 nM of **5l**), or the cells were treated with 10 μM of caspase inhibitor **13** 30 min before the addition of compound **5a** or **5l**, or the solvent DMSO (control cells), and incubated for 24 h or 48 h. At the end of treatment cells were collected and re-suspended in 500 μL staining buffer (0.1% sodium citrate, 0.37% NP-40, and 50 μg/mL propidium iodide) and incubated for 30 min. The samples were analyzed by flow cytometry (FACS Calibur; BD Biosciences, San Jose, CA) and data analyzed using FCSExpress analysis software. On the *x*-axis is plotted the fluorescence intensity and on the *y*-axis is plotted the number of cells with that fluorescence intensity.

The T47D control cell population profile is shown in Figure 1A with most of the cells in the M2 phase. An increase in the G_2/M DNA content (M4) of cells from 16.9% to 66.5% was observed when cells were treated with 250 nM of compound **5a** for 24 h (Fig. 1B). Concurrently, an increase in the sub-diploid DNA content of cells (marker M1 region, Fig. 1B) from 3.4% to 13.6% was observed. The sub-diploid amount of DNA (M1) is indicative of apoptotic cells that have undergone DNA degradation and nuclear fragmentation. Most of the cells (73.6%) were apoptotic after treatment with 250 nM of compound **5a** for 48 h, together with 13.4% of the cells in M4 (Fig. 1C). In the presence of caspase inhibitor **13**, apoptotic cells were reduced from 13.6% (Fig. 1B) to 8.7% (Fig. 1D) after 24 h treatment, with most of the cells in M4 (72.8%); and from 73.6% (Fig. 1C) to 29.9% (Fig. 1E) after 48 h treatment, with most of the cells in M4 (53.9%).

When cells were treated with 50 nM of compound **5l** for 24 h, the G_2/M DNA content (M4) of cells increased from 16.9% (Fig. 1A) to 40.4% (Fig. 1F), accompanied by an increase of apoptotic cells from 3.4% to 26.3%. When cells were treated with compound **5l** for 48 h, most of the cells (58.5%) were apoptotic, together with 17.8% of the cells in M4 and 17.5% in M2 (Fig. 1G). In the presence of caspase inhibitor **13**, apoptotic cells were reduced from 26.3% (Fig. 1F) to 19.2% (Fig. 1H) after 24 h treatment, with 40.5% of the cells in M4 and 18.3% in M2; and apoptotic cells were reduced from 58.5% (Fig. 1G) to 28.0% (Fig. 1I) after 48 h treatment, with 20.5% of the cells in M4 and 37.9% in M2.

4.4. Cell growth inhibition assays (GI_{50})

Cells were grown and harvested as described above. An aliquot of 45 μL of cells (4.4×10^4 cells/mL) was added to a well of a 96-well microtiter plate, then 5 μL of 0.01–100 μM of test compound (0.001–10 μM final concentration) in RPMI-1640 media solution with 10% DMSO was added. An aliquot of 45 μL cells was added to a well of a 96-well microtiter plate containing 5 μL of RPMI-1640 media solution with 10% DMSO and without test compound as the control sample for maximal cell proliferation (L_{max}). The samples were then incubated at 37 °C for 48 h in a 5% CO_2–95% humidity incubator. After incubation, the samples were removed from the incubator and 25 μL of CellTiter-Glo reagent (Promega) was added. The samples were mixed by agitation and incubated at room temperature for 10–15 min. Plates were then read using a luminescent plate reader (Model Spectrafluor Plus Tecan Instrument) to give L_{test} value.

Baseline for GI_{50} (dose for 50% inhibition of cell proliferation) of initial cell numbers was determined by adding an aliquot of 45 μL of cells and 5 μL of RPMI-1640 media solution with 10% DMSO to wells of a 96-well microtiter plate. The samples were then incubated at 37 °C for 0.5 h in a 5% CO_2–95% humidity incubator. After incubation, the samples were removed from the incubator and 25 μL of CellTiter-Glo reagent (Promega) was added. The samples were mixed by agitation and incubated at room temperature for 10–15 min. Luminescence was read as above to give L_{start} value, defining luminescence for initial cell number used as baseline in GI_{50} determinations.

4.4.1. Calculation. GI_{50} (dose for 50% inhibition of cell proliferation) is the concentration where $[(L_{test} - L_{start})/(L_{max} - L_{start})] = 0.5$. The GI_{50} (μM) values in T47D and H1299 cells are summarized in Table 2 in comparison with the caspase activation activity (EC_{50}).

4.5. Tubulin inhibition

Lyophilized MAP-rich tubulin (Cytoskeleton, Boulder, CO) was assayed for the effect of the test compound on tubulin polymerization according to the recommended procedure of the manufacturer. To 1 μL of each experimental compound (from a 100× stock) in a 96-well microtitre plate was added 99 μL of supplemented tubulin supernatant. Incubation was done in a Molecular Devices plate reader at 37 °C, and absorbance readings at 340 nm were recorded every minute for 1 h. The IC_{50} for tubulin inhibition was the concentration found to decrease the initial rate of tubulin polymerization by 50% as calculated with Prism 4.0.

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