

## Discovery of Substituted *N*-Phenyl Nicotinamides as Potent Inducers of Apoptosis Using a Cell- and Caspase-Based High Throughput Screening Assay

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Received November 15, 2002

By applying a novel cell- and caspase-based HTS assay, a series of *N*-phenyl nicotinamides has been identified as a new class of potent inducers of apoptosis. Through SAR studies, a 20-fold increase in potency was achieved from a screening hit *N*-(4-methoxy-2-nitrophenyl)pyridine-3-carboxamide (**1**) to lead compound 6-methyl-*N*-(4-ethoxy-2-nitrophenyl)pyridine-3-carboxamide (**10**), with an EC<sub>50</sub> of 0.082 μM in the caspase activation assay in T47D breast cancer cells. The *N*-phenyl nicotinamides also were found to be active in the growth inhibition assay where compound **10** had a GI<sub>50</sub> value of 0.21 μM in T47D cells. More importantly, compound **10** was found to be equipotent in MES-SA cells and paclitaxel-resistant, *p*-glycoprotein overexpressed MES-SA/DX5 cells. Compounds **1** and 6-chloro-*N*-(4-ethoxy-2-nitrophenyl)pyridine-3-carboxamide (**8**), a more potent analogue, were found to arrest T47D cells in G<sub>2</sub>/M phase of the cell cycle followed by induction of apoptosis as measured by flow cytometry. Compound **8**, which was more potent than **1** in the caspase activation assay, also was found to be more potent in G<sub>2</sub>/M arrest and apoptosis assay. These data confirm that the cell-based caspase activation assay is useful for screening for inducers of apoptosis, as well as for SAR studies and lead optimization. Upon further characterization, *N*-phenyl nicotinamides were found to be inhibitors of microtubule polymerization in vitro. The identification of *N*-phenyl nicotinamides as a novel series of inducers of apoptosis demonstrates that our cell- and caspase-based HTS assay is useful for the discovery and optimization of potentially novel anticancer agents.

### Introduction

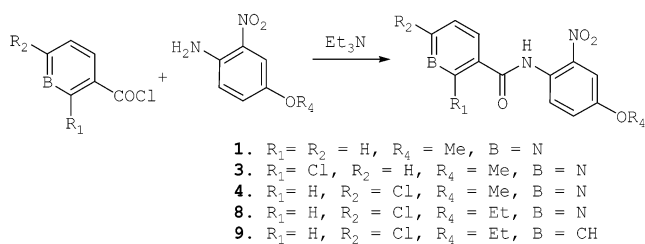
Apoptosis or programmed cell death is a normal process that organisms use to eliminate unwanted and excessive cells and is important in animal development, as well as in tissue homeostasis.<sup>1</sup> However, improperly regulated apoptosis can lead to many pathological conditions. Inadequate or abnormal inhibition of apoptosis, which leads to unchecked cell proliferation, results in cell accumulation and is a hallmark of cancer.<sup>2</sup> Excessive apoptosis, on the other hand, could lead to organ failure and neurodegenerative diseases.<sup>3</sup> Apoptosis can be initiated by many agents, including TNF-α and FAS ligand, as well as by chemotherapy and radiation. The mechanism of apoptosis involves a cascade of initiator and effector caspases that are activated sequentially. Caspases are a family of cysteine proteases that require aspartic acid residues at the P<sub>1</sub> position of substrates for cleavage.<sup>4</sup> Among these caspases, caspase-3, -6, and -7 are key effector caspases that cleave multiple protein substrates in cells, leading irreversibly to cell death.<sup>5</sup>

Since many of the current anticancer agents are known to kill tumor cells through the induction of apoptosis,<sup>6,7</sup> we are interested in the discovery and development of inducers of apoptosis as potential anticancer agents. Toward this goal, we developed a cell-

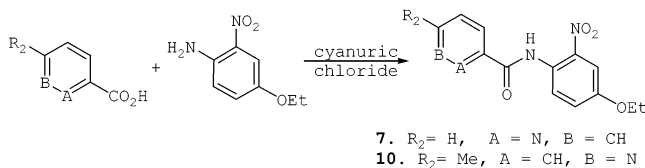
based high throughput screening (HTS) system for inducers of apoptosis<sup>8</sup> using our novel fluorescent caspase substrates.<sup>9,10</sup> The screening uses a mono-DEVD-tetrapeptide rhodamine 110-based caspase-3 substrate *N*-(Ac-DEVD)-*N*-ethoxycarbonyl-R110, which is a profluorescent molecule that exhibits little background signal before cleavage.<sup>10</sup> The substrate, after cleavage by caspases, fluoresces at a long wavelength (525 nm), thereby avoiding interference from cellular fluorescence and from the approximately 1% of test compounds found to fluoresce at short wavelengths (<500 nm, data not shown). The screening assay is extremely flexible: it can be applied to any cell type or cell line that expresses caspases and can be adapted to screen for either apoptosis inducers or inhibitors. Furthermore, the assay can detect any apoptosis inducer as long as it acts by a caspase-mediated mechanism and triggers apoptosis at a point upstream of caspase-3 activation. In addition, since a compound active in this assay must interact either with cell surface or intracellular targets in order to induce apoptosis and activate caspases, the assay also avoids the false positives observed with some enzyme screening assays due to precipitation or crystallization of screening compounds on the enzymes as reported recently by McGovern et al.<sup>11</sup> Herein we report the discovery, characterization, and preliminary SAR of substituted *N*-phenyl nicotinamides as potent inducers of apoptosis using our cell- and caspase-based apoptosis HTS assay.

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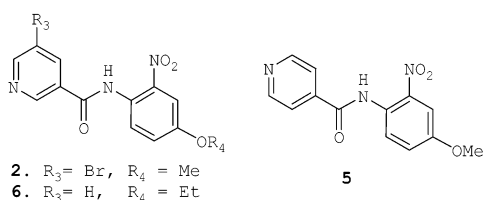
## Scheme 1



## Scheme 2



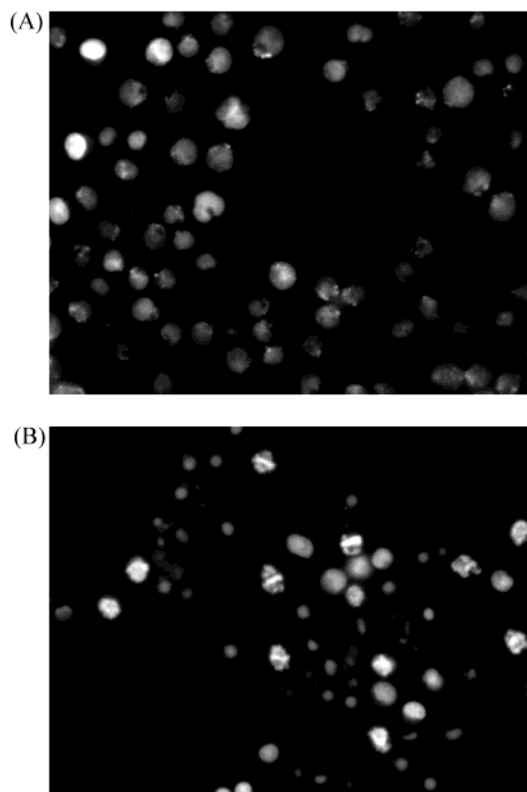
## Chart 1



## Results and Discussion

**Chemistry.** *N*-(4-Methoxy-2-nitrophenyl)pyridine-3-carboxamide **1** was obtained originally from a commercial compound library. It was prepared by reaction of nicotinoyl chloride with 4-methoxy-2-nitroaniline and was isolated as an orange solid. Compounds **3**, **4**, **5**, **8**, and **9** were prepared similar to **1** from the corresponding substituted nicotinoyl chlorides or acyl chloride and anilines (Scheme 1). Compound **7** and **10** were prepared from the corresponding acids and anilines in the presence of cyanuric chloride (Scheme 2). Compounds **2** and **6** were obtained from commercial sources, and their structures were confirmed by  $^1\text{H}$  NMR spectroscopy (Chart 1).

**HTS Assays.** *N*-(4-Methoxy-2-nitrophenyl)pyridine-3-carboxamide **1** was identified as an inducer of apoptosis from a commercial compound library using our cell-based apoptosis induction HTS assay in T47D breast cancer cells. Briefly, human breast cancer T47D cells, in a 96-well microtiter plate containing  $10\ \mu\text{M}$  of test compound, were incubated for 24 h for the induction of apoptosis. Caspase-3 fluorogenic substrate *N*-(Ac-DEVD)-*N*-ethoxycarbonyl-R110<sup>10</sup> 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 confirmation. Compounds confirmed to be active are then tested at several concentrations to provide a dose response and the caspase activation activity ( $\text{EC}_{50}$ ) calculated. Compound **1** was found to



**Figure 1.** Fluorescent micrographs of Jurkat cells treated with compound **1** and stained with a fluorescent DNA probe, Syto16. (A) Control cells. (B) Cells treated with  $5\ \mu\text{M}$  of compound **1** for 24 h, showing shrunken and fragmented nuclei, as well as condensed chromatin spindles.

induce apoptosis and activate caspase in T47D cells with a ratio of 6 over untreated cells, and an  $\text{EC}_{50}$  value of  $1.6\ \mu\text{M}$ .

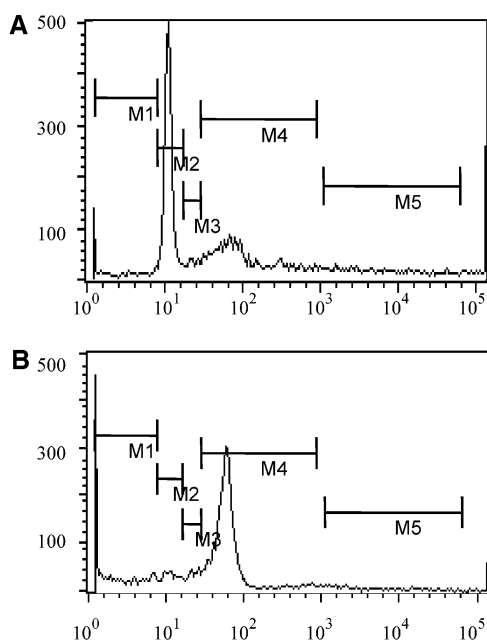
**Characterization of Compound 1.** The ability of compound **1** to induce apoptosis was confirmed in a nuclear fragmentation assay. Jurkat cells were treated with  $5\ \mu\text{M}$  of compound **1** for 24 h followed by staining with Syto16, a DNA stain which allows the visualization of nuclear morphology. The compound-treated cells were characterized by shrunken and fragmented nuclei with condensed chromatin (Figure 1B), which is a hallmark of caspase-mediated apoptosis, as well as by condensed mitotic spindles crucial to the transition to metaphase, similar to the effect of vinca alkaloids.<sup>12</sup> In contrast, the nuclei of Jurkat cells treated with vehicle control (DMSO) appeared to be normal with dispersed chromatin that is moderately stained with Syto16 (Figure 1A).

The apoptosis-inducing activities of compound **1** were also characterized by treatment of T47D cells with  $10\ \mu\text{M}$  of compound **1** for 48 h, staining with propidium iodide, and analysis by flow cytometry. An increase in the  $G_2/M$  DNA content (M4) in cells treated with compound **1** was observed, as shown in Figure 2B. The sub-diploid DNA content (M1, apoptotic sub- $G_1$  area) of cells increased from 2.6% to 25.1% upon compound treatment, indicating the presence of apoptotic cells which have undergone DNA degradation and nuclear fragmentation. The results showed that treatment of T47D cells by compound **1** for 48 h results in cell cycle arrest in the  $G_2/M$  phase, as well as induction of apoptosis.

**Table 1.** Caspase Activation Activity of Substituted *N*-Phenyl Nicotinamides and Analogues

	A	B	D	R <sub>1</sub>	R <sub>2</sub>	R <sub>3</sub>	R <sub>4</sub>	EC <sub>50</sub> (μM) <sup>a</sup>		
								T47D	ZR75-1	DLD-1
1	C	N	C	H	H	H	Me	1.6 ± 0.1	0.88 ± 0.02	2.9 ± 0.1
2	C	N	C	H	H	Br	Me	>10	5.4 ± 0.1	>10
3	C	N	C	Cl	H	H	Me	>10	>10	>10
4	C	N	C	H	Cl	H	Me	0.69 ± 0.03	0.30 ± 0.01	0.69 ± 0.20
5	C	CH	N	H	NA	H	Me	>10	>10	>10
6	C	N	C	H	H	H	Et	0.20 ± 0.01	0.11 ± 0.02	0.19 ± 0.02
7	N	CH	C	NA	H	H	Et	>10	>10	>10
8	C	N	C	H	Cl	H	Et	0.24 ± 0.02	0.11 ± 0.01	0.38 ± 0.01
9	C	CH	C	H	Cl	H	Et	5.7 ± 0.3	3.0 ± 0.3	>10
10	C	N	C	H	Me	H	Et	0.082 ± 0.005	0.052 ± 0.006	0.11 ± 0.01
11	NA	NA	NA	NA	NA	NA	NA	0.17 ± 0.01	0.13 ± 0.01	0.15 ± 0.01
12	NA	NA	NA	NA	NA	NA	NA	0.014 ± 0.001	0.006 ± 0.001	0.19 ± 0.01

<sup>a</sup> Data are the mean of three or more experiments and are reported as mean ± standard error of the mean (SEM). NA = not applied.



	M1 (%)	M2 (%)	M3 (%)	M4 (%)	M5 (%)
2A	2.6	42.4	3.8	34.8	9.8
2B	25.1	7.4	5.3	57.2	3.8

**Figure 2.** Drug-induced cell cycle arrest and apoptosis in T47D cells as measured by flow cytometric analysis. The *x*-axis is the fluorescence intensity and the *y*-axis is the number of cells with that fluorescence intensity. (A) Control cells showing most of the cells in G1 (M2). (B) Cells treated with 10 μM of compound **1** for 48 h showing a reduction in the G1 (M2), an increase in the G2/M (M4) and sub-diploid DNA content of cells (M1).

#### Structure Activity Relationship (SAR) Studies.

The cell-based caspase HTS assay was used to test analogues of compound **1** for SAR studies. A panel of three different cell lines was used for these experiments. Briefly, human breast cancer T47D, ZR75-1, and a colorectal DLD-1 cell line were placed in a 384-well

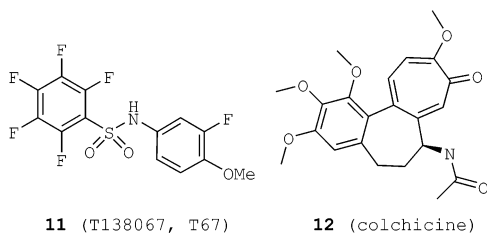
microtiter plate containing various concentration of test compound and incubated at 37 °C for 24 h. After incubation, the samples were treated with the *N*-(Ac-DEVD)-*N*-ethoxycarbonyl-R110 fluorogenic substrate. The caspase activation activity (EC<sub>50</sub>) of compound **1** and its analogues in the three cancer cell lines is summarized in Table 1.

Table 1 shows that compound **1** has an EC<sub>50</sub> for caspase activation of 1.6, 0.88, and 2.9 μM in T47D, ZR75-1 and DLD-1 cells, respectively. Substitution by a bromine in the 5-position (**2**) or a chlorine in the 2-position (**3**) of pyridine ring resulted in reduction of potency by >6-fold in the T47D cells, suggesting a space limited pocket around the 2 and 5-position. Substitution at the 6-position of the pyridine ring by a chlorine (**4**) increased the potency by >2-fold, suggesting the presence of a binding pocket in that position. Interestingly, the 4-pyridyl analogue (**5**) was found to be at least 6 times less active than **1**, suggesting that the position of nitrogen in the 3-pyridyl group of **1** plays an important role for interaction with its target, probably by hydrogen bonding. Replacing the methoxy group in the 4-position of the phenyl ring by an ethoxy group (**6**) resulted in a 8-fold increase in potency, suggesting that there is a hydrophobic binding pocket in that position. The 2-pyridyl analogue (**7**) was >50 times less active than **6**, again suggesting the importance of the position of nitrogen in the pyridyl group. A combination of 6-chlorine in the pyridine ring with 4-ethoxy group in the phenyl ring yielded compound **8**, which was >6-fold more potent than **1**. The corresponding phenyl analogue (**9**) was >20 times less active than **8**, confirming the importance of the nitrogen in the 3-pyridyl group. Finally, replacement of the 6-chloro group in **8** by a 6-methyl group yielded compound **10**, which was approximately 20-fold more potent than **1**, the original screening hit. Compared with reference compounds, compound **10** is approximately 2-fold more potent in caspase activation assays than T67 (**11**), a pentafluorophenyl sulfonamide compound developed by Tularik and currently in Phase II clinical trials,<sup>13</sup> and 6-fold less

**Table 2.** Comparison of Caspase Activation Activity and Inhibition of Cell Proliferation Activity of Substituted N-Phenyl Nicotinamides

	T47D			DLD-1		
	EC <sub>50</sub> <sup>a</sup> (μM)	GI <sub>50</sub> <sup>b</sup> (μM)	GI <sub>50</sub> /EC <sub>50</sub>	EC <sub>50</sub> <sup>a</sup> (μM)	GI <sub>50</sub> <sup>b</sup> (μM)	GI <sub>50</sub> /EC <sub>50</sub>
<b>1</b>	1.6 ± 0.1	7.0 ± 0.5	4.4	2.9 ± 0.1	10.0 ± 0.1	3.4
<b>6</b>	0.20 ± 0.01	0.56 ± 0.05	2.8	0.19 ± 0.02	0.90 ± 0.02	4.7
<b>10</b>	0.082 ± 0.005	0.21 ± 0.03	2.6	0.11 ± 0.01	0.53 ± 0.05	4.8

<sup>a</sup> From Table 1. <sup>b</sup> Data are the mean of three experiments and are reported as mean ± standard error of the mean (SEM).

**Chart 2**

potent than the well-known antimetabolic agent, colchicine (**12**) (Chart 2).

The activities of these compounds toward the breast cancer cell line ZR75-1 and colon cancer cell line DLD-1 was roughly the same as their activity toward T47D cells. ZR75-1 cells were slightly more sensitive (about 2-fold more as measured by the EC<sub>50</sub> value) to the compounds than T47D cells in this assay. DLD-1 cells were slightly less sensitive (about 1.5-fold less as measured by the EC<sub>50</sub> value) to the compounds than T47D cells.

Selected compounds were also tested by a more traditional inhibition of cell proliferation (GI<sub>50</sub>) 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. Briefly, T47D and DLD-1 cells in a 96-well microtiter plate were treated with serial concentrations of test compound. The survival profiles of the cells were quantitated using the CellTiter-Glo (Promega, Madison, WI) according to the manufacturer's protocol. This assay is a homogeneous method of determining the number of viable cells in culture based on the quantitation of ATP, which represents the presence of metabolically active cells. GI<sub>50</sub> values were calculated from dose-response curves using XLFit3 (IDBS) software. The GI<sub>50</sub> (μM), along with the EC<sub>50</sub> data and the ratio of GI<sub>50</sub>/EC<sub>50</sub>, are summarized in Table 2.

Table 2 shows that compounds **1**, **6**, and **10** all inhibited the growth of tumor cells. Compound **10** has a GI<sub>50</sub> value of 0.21 μM and 0.53 μM in T47D and DLD-1 cells, respectively. The compound that is more active in the apoptosis induction assay, as measured by caspase activation, also is more potent in the growth inhibition assay. The ratio of GI<sub>50</sub>/EC<sub>50</sub> for the three compounds in T47D and DLD-1 cells has a value of around 3.5, suggesting a correlation between the caspase activation activity and inhibition of cell proliferation activity for these compounds. Overall, these data indicate that the cell-based caspase activation HTS assay not only is useful for the identification of apoptosis inducers, but also can be used for subsequent optimization and SAR studies of screening hits.

The success of microtubule interacting antimetabolic agents in the clinic, such as the vinca alkaloids and the

**Table 3.** GI<sub>50</sub> Assay of Compound **10** in Comparison with Paclitaxel in MES-SA and MES-SA/DX5 Cells

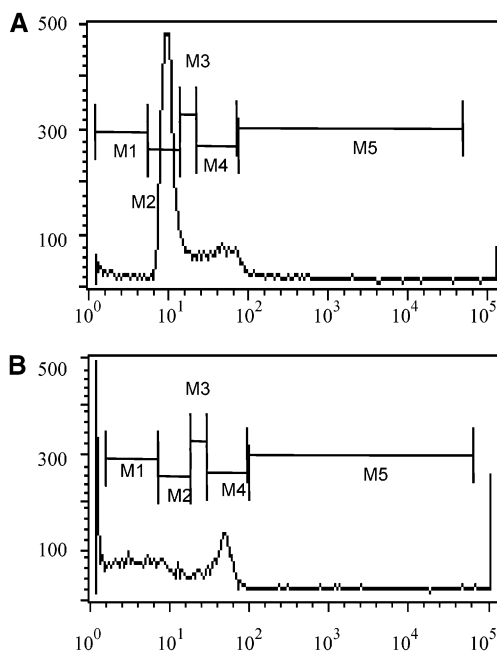
compound	GI <sub>50</sub> (μM) <sup>a</sup>	
	MES-SA	MES-SA/DX5
<b>10</b>	0.073 ± 0.008	0.074 ± 0.008
paclitaxel	0.063 ± 0.012	> 10

<sup>a</sup> Data are the mean of three or more experiments and are reported as mean ± standard error of the mean (SEM).

taxanes, has made microtubules the single best cancer drug target identified to date.<sup>14</sup> However, one of the most serious problems of currently used anticancer agents is multidrug resistance. Many tumor cells overexpress the *p*-glycoprotein, a plasma membrane transporter with a broad substrate specificity that includes many of the anticancer agents.<sup>15</sup> This transporter efficiently removes the anticancer agents from the interior of the cell, thus reducing or eliminating the effects of these agents. Therefore, it is important to discover and develop novel anticancer agents that are active against multidrug resistant cancers. Using the cell proliferation assay, compound **10** was tested against both the human uterus sarcoma MES-SA cell and its doxorubicin-resistant variant, MES-SA/DX5. As shown in Table 3, compound **10** had similar GI<sub>50</sub> values in MES-SA (0.073 μM) and MES-SA/DX5 (0.074 μM) cells. Therefore, the growth inhibition activity of compound **10** is not affected by the overexpression of *mdr1* *p*-glycoprotein in the MES-SA/DX5 cells. In comparison, the activity of paclitaxel in the MES-SA/DX5 was reduced by > 160-fold. These data suggest that compound **10** should be effective in tumors that are resistant to paclitaxel due to overexpression of *mdr1*.

The apoptosis-inducing activity of compound **8** was also tested by flow cytometry similar to that of compound **1**. T47D cells treated with 1 μM of compound **8** for 48 h also showed an increase in G<sub>2</sub>/M DNA content (M4) (Figure 3B). The sub-diploid DNA content of cells increased from 3.3% to 56.1% with compound treatment, indicating that >50% of the cells were apoptotic. In comparison, compound **1** at 10 μM increased the sub-diploid DNA content of cells from 2.6% to 25.1%. These results show that compound **8** is about 10-fold more potent than compound **1** as an inducer of apoptosis as measured by the flow cytometry, similar to the results obtained in the caspase activation assay, confirming that the cell-based caspase activation assay is useful for SAR studies.

Since tubulin inhibitors are known to arrest cells in the G<sub>2</sub>/M phase, we hypothesized that compound **1** and its analogues might interact with tubulin. In addition, we also observed some structural similarity between compound **1** and the known tubulin inhibitor sulfonamide **11**,<sup>16</sup> which was reported to covalently modify β-tubulin due to the chemically reactive pentafluorophenylsulfonyl group. Therefore, we tested the ability of



	M1 (%)	M2 (%)	M3 (%)	M4 (%)	M5 (%)
3A	3.3	68.4	6.1	16.5	5.5
3B	56.2	13.8	4.4	18.5	7.3

**Figure 3.** Drug-induced cell cycle arrest and apoptosis in T47D cells as measured by flow cytometric analysis. The *x*-axis is the fluorescence intensity and the *y*-axis is the number of cells with that fluorescence intensity. (A) Control cells showing most of the cells in G1 (M2). (B) Cells treated with 1  $\mu$ M of compound **8** for 48 h showing a reduction in the G1 (M2), an increase in the G2/M (M4) and sub-diploid DNA content of cells (M1).

compound **8** as an inhibitor of tubulin polymerization in a tubulin polymerization assay. Compound **8** was found to be a potent inhibitor of tubulin polymerization with an  $IC_{50}$  value of 0.5  $\mu$ M. In comparison, vinblastine, a well-known tubulin polymerization inhibitor, was found to have an  $IC_{50}$  value of 0.3  $\mu$ M. Therefore, compound **8** is about as potent as vinblastine for inhibition of tubulin polymerization. Compound **8**, and other compounds in this series, most probably induces apoptosis through the inhibition of tubulin polymerization similar to that of vinblastine and pentafluorophenyl sulfonamide **11**.<sup>16</sup>

Although the *N*-phenyl nicotinamides and the sulfonamide **11** series of compounds are both tubulin inhibitors and share some similar structural features, suggesting that they might bind to tubulin in a similar manner or at the same site, the SAR of *N*-phenyl nicotinamides and sulfonamide **11** are very different. The pentafluorophenyl group is essential for the activity of **11**, and it has been reported that the thiol group in Cys-239 of  $\beta$ -tubulin can displace the *p*-F in the pentafluorophenyl group, resulting in the covalent attachment of **11** to  $\beta$ -tubulin.<sup>16</sup> The importance of the pentafluorophenyl group was confirmed from SAR studies of sulfonamide **11**, and it has been reported that replacement of the *p*-F in **11** by a chlorine or other group eliminates activity.<sup>17</sup> In comparison, the activity of *N*-phenyl nicotinamides is not dependent on a chemi-

cally reactive group. For instance, there is no potentially reactive group in the phenyl group and the pyridyl group in compound **1**. The chloro group in compound **8** might be considered potentially reactive. However, replacement of the chloro group by a methyl group resulted in **10**, which was 3-fold more active than **8**, indicating that the activity is not dependent on potential reactivity of the chloro group. Since tubulin is an important component of every cell, a tubulin inhibitor that does not covalently modify and bind to tubulin might be expected to have fewer side effects and toxicity.

## Conclusions

In conclusion, using a cell- and caspase-based HTS assay, a series of substituted *N*-phenyl nicotinamides has been identified as potent inducers of apoptosis. SAR studies of *N*-phenyl nicotinamides indicate that the 3-pyridyl group is very important for its activity and substitution in the 6-position by a chloro or methyl group resulted in increased potency. Substitution at the 4-position of the phenyl group also is important, and changing the methoxy group to an ethoxy group resulted in increased potency. Through the SAR studies, a 20-fold increase in potency was obtained from compound **1** to compound **10**. The *N*-phenyl nicotinamides were also found to be active in the traditional growth inhibition assays. More importantly, compound **10**, the most potent compound in this series, was found to be active in paclitaxel-resistant, *p*-glycoprotein overexpressed MES-SA/DX5 cells.

*N*-Phenyl nicotinamides were found to arrest cells in G<sub>2</sub>/M and induce apoptosis. Compound **8**, which is more potent than **1** in the caspase activation assay, also was found to be more potent as an inducer of apoptosis in the flow cytometric assay. These data, together with the growth inhibition data, confirm that the cell-based caspase activation assay is useful for screening for inducers of apoptosis, as well as for SAR studies and lead optimization. The *N*-phenyl nicotinamides were found to be inhibitors of microtubule polymerization similar to vinblastine. The identification of *N*-phenyl nicotinamides as a new class of inducers of apoptosis demonstrates that our cell- and caspase-based HTS assay is useful for the discovery of potentially novel anticancer agents. Since the *N*-phenyl nicotinamides are active in paclitaxel-resistant cells and are not chemically reactive, they might offer advantages over tubulin-interacting drugs currently used in the clinic. Compounds **8** and **10** are interesting leads for additional lead expansion and in vivo studies and the results will be reported in due course.

## Experimental Sections

**General Methods and Materials.** The <sup>1</sup>H NMR spectra were recorded at 300 MHz. Chemical shifts are reported in ppm ( $\delta$ ) and *J* coupling constants are reported in hertz. Elemental analyses were performed by Numega Resonance Labs, Inc. (San Diego, CA). Substituted anilines, nicotinic acids, nicotinoyl chloride, and acyl chloride were obtained from Aldrich or Lancaster and used as received. Reagent grade solvents were used without further purification unless otherwise specified. *N*-(Ac-DEVD)-*N*-ethoxycarbonyl-R110 was prepared as described in US patent 6,335,429.<sup>10</sup> Colchicine (**12**) was obtained from Sigma-Aldrich (St. Louis, MO). Sulfonamide **11** was prepared from pentafluorobenzenesulfonyl chloride and 3-fluoro-4-methoxyaniline as reported.<sup>17</sup> Syto16 was obtained

from Molecular Probes (Eugene, OR). Human leukemia cancer cells (Jurkat cells), human breast cancer cells T47D and ZR75-1, human colon cancer cells DLD-1, human uterus sarcoma MES-SA cell, and human uterus sarcoma doxorubicin-resistant MES-SA/DX5 cells were obtained from American Type Culture Collection (Manassas, VA). Tubulin was obtained from Cytoskeleton (Boulder, CO).

**2-Chloro-N-(4-methoxy-2-nitrophenyl)pyridine-3-carboxamide (3).** A mixture of 2-chloronicotinoyl chloride (200 mg, 1.14 mmol), 4-methoxy-2-nitroaniline (191 mg, 1.14 mmol), and triethylamine (160  $\mu$ L) in THF (10 mL) was refluxed for 20 h. The mixture was cooled to room temperature, and the solid was filtered. The filtrate was rotary evaporated to dryness, and the resulting residue was purified by flash column chromatography (40% ethyl acetate in hexanes) to obtain a yellow solid (192 mg, 55%).  $^1\text{H NMR}$  ( $\text{CDCl}_3$ ): 10.76 (s, 1H), 8.78 (d,  $J = 9.3$ , 1H), 8.58–8.55 (m, 1H), 8.13–8.09 (m, 1H), 7.73 (d,  $J = 3.0$ , 1H), 7.45–7.40 (m, 1H), 7.34–7.28 (m, 1H), 3.90 (s, 3H). Anal. ( $\text{C}_{13}\text{H}_{10}\text{ClN}_3\text{O}_4$ ) C, H, N.

The following compounds were prepared by a procedure similar to that described for the preparation of compound 3.

**N-(4-Methoxy-2-nitrophenyl)pyridine-3-carboxamide (1).** The title compound was prepared from nicotinoyl chloride and 4-methoxy-2-nitroaniline.  $^1\text{H NMR}$  ( $\text{CDCl}_3$ ): 11.13 (s, 1H), 9.24 (d,  $J = 2.1$ , 1H), 8.86 (d,  $J = 9.3$ , 1H), 8.84 (dd,  $J = 1.5$ , 5.1, 1H), 8.29–8.25 (m, 1H), 7.76 (d,  $J = 3.0$ , 1H), 7.49 (dd,  $J = 4.8$ , 8.1, 1H), 7.33 (dd,  $J = 3.0$ , 9.3, 1H), 3.90 (s, 3H). Anal. ( $\text{C}_{13}\text{H}_{11}\text{N}_3\text{O}_4$ ) C, H, N.

**6-Chloro-N-(4-methoxy-2-nitrophenyl)pyridine-3-carboxamide (4).** The title compound was prepared from 6-chloronicotinoyl chloride and 4-methoxy-2-nitroaniline (54%).  $^1\text{H NMR}$  ( $\text{CDCl}_3$ ): 11.13 (s, 1H), 9.01 (d,  $J = 2.7$ , 1H), 8.83 (d,  $J = 9.3$ , 1H), 8.24–8.20 (m, 1H), 7.76 (d,  $J = 2.7$ , 1H), 7.52 (d,  $J = 8.7$ , 1H), 7.35–7.31 (m, 1H), 3.90 (s, 3H). Anal. ( $\text{C}_{13}\text{H}_{10}\text{ClN}_3\text{O}_4$ ) C, H, N.

**N-(4-Methoxy-2-nitrophenyl)pyridine-4-carboxamide (5).** The title compound was prepared from isonicotinoyl chloride and 4-methoxy-2-nitroaniline (40%).  $^1\text{H NMR}$  ( $\text{CDCl}_3$ ): 10.79 (s, 1H), 8.83–8.81 (m, 2H), 7.85–7.83 (m, 2H), 7.61–7.55 (m, 2H), 7.40–7.36 (m, 1H), 3.87 (s, 3H). Anal. ( $\text{C}_{13}\text{H}_{11}\text{N}_3\text{O}_4$ ) H, N, C, calcd: 57.14; found: 57.93.

**6-Chloro-N-(4-ethoxy-2-nitrophenyl)pyridine-3-carboxamide (8).** The title compound was prepared from 6-chloronicotinoyl chloride and 4-ethoxy-2-nitroaniline (34%).  $^1\text{H NMR}$  ( $\text{CDCl}_3$ ): 11.13 (s, 1H), 9.01 (d,  $J = 2.4$ , 1H), 8.82 (d,  $J = 9.3$ , 1H), 8.24–8.20 (m, 1H), 7.74 (d,  $J = 3.0$ , 1H), 7.51 (d,  $J = 7.8$ , 1H), 7.34–7.30 (m, 1H), 4.11 (q,  $J = 6.9$ , 2H), 1.47 (t,  $J = 6.9$ , 3H). Anal. ( $\text{C}_{14}\text{H}_{12}\text{ClN}_3\text{O}_4$ ) C, H, N.

**4-Chloro-N-(4-ethoxy-2-nitrophenyl)benzamide (9).** The title compound was prepared from 4-chlorobenzoyl chloride and 4-ethoxy-2-nitroaniline (57%).  $^1\text{H NMR}$  ( $\text{CDCl}_3$ ): 11.10 (brs, 1H), 8.86 (d,  $J = 9.3$ , 1H), 7.93 (d,  $J = 8.4$ , 2H), 7.73 (d,  $J = 3.0$ , 1H), 7.51 (d,  $J = 8.4$ , 2H), 7.32–7.27 (m, 1H), 4.11 (q,  $J = 6.9$ , 2H), 1.46 (t,  $J = 6.9$ , 3H). Anal. ( $\text{C}_{15}\text{H}_{13}\text{ClN}_2\text{O}_4$ ) C, H, N.

**6-Methyl-N-(4-ethoxy-2-nitrophenyl)pyridine-3-carboxamide (10).** A mixture of 6-methylnicotinic acid (1.01 g, 7.29 mmol) and cyanuric chloride (1.34 g, 7.29 mmol) in THF (25 mL) was stirred at room temperature for 30 min, then a solution of 4-ethoxy-2-nitroaniline (1.33 g, 7.29 mmol) in THF (25 mL) and triethylamine (2 mL) was added. The mixture was refluxed for 20 h, cooled to room temperature, and then diluted with 1:1 of hexane:ethyl acetate (200 mL). The solution was washed with water (2  $\times$  100 mL) and brine (100 mL), dried over  $\text{Na}_2\text{SO}_4$ , and evaporated. The residue was purified by flash column chromatography (33% ethyl acetate in hexanes) to obtain a yellow solid (707 mg, 32%).  $^1\text{H NMR}$  ( $\text{CDCl}_3$ ): 11.09 (s, 1H), 9.12 (d,  $J = 2.1$ , 1H), 8.84 (d,  $J = 9.6$ , 1H), 8.14 (dd,  $J = 2.7$ , 8.1, 1H), 7.73 (d,  $J = 3.3$ , 1H), 7.34–7.28 (m, 2H), 4.11 (q,  $J = 6.9$ , 2H), 2.67 (s, 3H), 1.46 (t,  $J = 6.9$ , 3H). Anal. ( $\text{C}_{15}\text{H}_{15}\text{N}_3\text{O}_4$ ) C, H, N.

**N-(4-Ethoxy-2-nitrophenyl)pyridine-2-carboxamide (7).** The title compound was prepared from picolinic acid and 4-ethoxy-2-nitroaniline by a procedure similar to that described

for preparation of compound 10 (52%).  $^1\text{H NMR}$  ( $\text{CDCl}_3$ ): 12.54 (s, 1H), 8.92 (d,  $J = 9.3$ , 1H), 8.73 (d,  $J = 3.9$ , 1H), 8.28 (d,  $J = 6.9$ , 1H), 7.95–7.90 (m, 1H), 7.74 (d,  $J = 3.0$ , 1H), 7.54–7.50 (m, 1H), 7.31–7.27 (m, 1H), 4.11 (q,  $J = 6.9$ , 2H), 1.46 (t,  $J = 6.9$ , 3H). Anal. ( $\text{C}_{14}\text{H}_{13}\text{N}_3\text{O}_4$ ) C, H, N.

Compound 2 and 6 were obtained from Chembridge (San Diego, CA):

**5-Bromo-N-(4-methoxy-2-nitrophenyl)pyridine-3-carboxamide (2).**  $^1\text{H NMR}$  ( $\text{CDCl}_3$ ): 11.12 (s, 1H), 9.11 (d,  $J = 1.8$ , 1H), 8.89 (d,  $J = 2.4$ , 1H), 8.14 (d,  $J = 9.6$ , 1H), 8.42 (t,  $J = 2.1$ , 1H), 7.76 (d,  $J = 3.0$ , 1H), 7.33 (dd,  $J = 3.0$ , 9.3, 1H), 3.90 (s, 3H).

**N-(4-Ethoxy-2-nitrophenyl)pyridine-3-carboxamide (6).**  $^1\text{H NMR}$  ( $\text{CDCl}_3$ ): 11.12 (s, 1H), 9.24 (d,  $J = 1.8$ , 1H), 8.85 (d,  $J = 9.3$ , 1H), 8.83 (dd,  $J = 1.2$ , 5.1, 1H), 8.29–8.24 (m, 1H), 7.74 (d,  $J = 3.0$ , 1H), 7.51–7.47 (m, 1H), 7.31 (dd,  $J = 3.0$ , 9.3, 1H), 4.11 (q,  $J = 6.9$ , 2H), 1.47 (t,  $J = 6.9$ , 3H).

**Caspase Activation Assay ( $\text{EC}_{50}$ ).** Human breast cancer cell lines T47D, ZR75-1, and the human colon cancer cell line (DLD-1) were grown according to media component mixtures designated by American Type Culture Collection in RPMI-1640 + 10% FCS in a 5%  $\text{CO}_2$ –95% humidity incubator at 37  $^\circ\text{C}$ . Cells were harvested using trypsin and washed at 600g and resuspended at  $0.65 \times 10^6$  cells/mL into RPMI media + 10% FCS. An aliquot of 22.5  $\mu\text{L}$  of cells was added to a well of a 384-well microtiter plate containing 2.5  $\mu\text{L}$  of 0.05 to 100  $\mu\text{M}$  of test compound in RPMI-1640 containing 25 mM HEPES media solution with 10% DMSO (0.005 to 10  $\mu\text{M}$  final). An aliquot of 22.5  $\mu\text{L}$  of cells was added to a well of a 384-well microtiter plate containing 2.5  $\mu\text{L}$  of RPMI-1640 media solution with 10% DMSO and without test compound as the control sample. The samples were then incubated at 37 $^\circ\text{C}$  for 24 h in a 5%  $\text{CO}_2$ –95% humidity incubator. After incubation, the samples were removed from the incubator and 25  $\mu\text{L}$  of a solution containing 14  $\mu\text{M}$  of *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 500  $\mu\text{g}/\text{mL}$  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).

**Calculation.** The relative fluorescence unit values (RFU) were used to calculate the sample readings as follows:

$$\text{RFU}_{(T=3\text{h})} - \text{control RFU}_{(T=0)} = \text{net RFU}_{(T=3\text{h})}$$

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  $\text{EC}_{50}$  ( $\mu\text{M}$ ) 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 ( $\text{EC}_{50}$ ) in three cancer cell lines, T47D, ZR75-1, and DLD-1, are summarized in Table 1.

**Morphological Assessment of Nuclear Fragmentation of Apoptotic Cells.** Jurkat cells, grown and harvested as above, were treated with 5  $\mu\text{M}$  of compound 1 for 24 h followed by staining of the nucleus with Syto16, a fluorescent DNA dye. The cells were then observed under a fluorescent microscope for chromosomal condensation and nuclear fragmentation (490 nm). The nuclei of Jurkat cells treated with vehicle control (DMSO) are seen to be round with dispersed chromatin that is moderately stained with Syto16 (Figure 1A). In contrast, Jurkat cells treated with 5  $\mu\text{M}$  of compound 1 have shrunken and fragmented nuclei (Figure 1B), which is a hallmark of caspase-mediated apoptosis, as well as condensed mitotic spindles. These results corroborate the caspase activation assays, showing that compound 1 can induce a key cellular marker of apoptosis.

**Cell Cycle Analysis and Measurement of Apoptosis.** T47D cells were maintained and harvested as described above.

Cells ( $1 \times 10^6$ ) were treated with  $10 \mu\text{M}$  of compound **1** for 48 h at  $37^\circ\text{C}$ . As a control, cells were also incubated with an equivalent amount of solvent (DMSO). Cells were harvested at 1200 rpm and washed twice with 5 mM EDTA/PBS. Cells were then resuspended in  $300 \mu\text{L}$  EDTA/PBS and  $700 \mu\text{L}$  of 100% ethanol, vortexed, and incubated at room temperature for 1 h. Samples were centrifuged at 1200 rpm for 5 min, and the supernatant was removed. A solution containing  $100 \mu\text{g}/\text{mL}$  of propidium iodide and  $1 \text{ mg}/\text{mL}$  of RNase A (fresh) was added to the samples and incubated for 1 h at room temperature. Samples were then transferred to  $12 \times 75 \text{ mm}$  polystyrene tubes and analyzed on a flow cytometer. All flow cytometry analyses were performed on a FACScalibur (Becton Dickinson) using Cell Quest 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 2A with most of the cells in the  $\text{M}_2$  phase. An increase in the  $\text{G}_2/\text{M}$  DNA content ( $\text{M}_4$ ) of cells from 34.8% to 57.2% was observed when cells were treated with compound **1** at  $10 \mu\text{M}$  for 48 h (Figure 2B). Concurrently, an increase in the sub-diploid DNA content of cells (marker  $\text{M}_1$  region, Figure 2B) from 2.6% to 25.1% was observed with compound treatment. The sub-diploid amount of DNA ( $\text{M}_1$ ) is indicative of apoptotic cells which have undergone DNA degradation and nuclear fragmentation.

In a separate experiment, T47D cells were treated with  $1 \mu\text{M}$  of compound **8** and measured in a similar manner as that of compound **1**. Cells treated with compound **8** at  $1 \mu\text{M}$  for 48 h (Figure 3B) were accumulated in the  $\text{G}_2/\text{M}$  phase ( $\text{M}_4$ ), from 16.5% to 18.5%. In addition, the sub- $\text{G}_1$  population of cells with reduced DNA content ( $\text{M}_1$ ) was increased substantially over control cells (Figure 3A), from 3.3% to 56.1%. These data indicate that treatment of T47D cells with compound **1**, and its more potent analogue **8**, results in cell cycle arrest in the  $\text{G}_2/\text{M}$  phase and induction of apoptosis.

**Cell Growth Inhibition Assays ( $\text{GI}_{50}$ ).** Cells were grown and harvested as described above. An aliquot of  $45 \mu\text{L}$  of cells ( $4.4 \times 10^4$  cells/mL) were added to a well of a 96-well microtiter plate, then  $5 \mu\text{L}$  of 0.01 to  $100 \mu\text{M}$  of test compound (0.001 to  $10 \mu\text{M}$  final concentration) in RPMI-1640 media solution with 10% DMSO was added. An aliquot of  $45 \mu\text{L}$  of cells were added to a well of a 96-well microtiter plate containing  $5 \mu\text{L}$  of RPMI-1640 media solution with 10% DMSO and without test compound as the control sample for maximal cell proliferation ( $A_{\text{max}}$ ). The samples were then incubated at  $37^\circ\text{C}$  for 48 h in a 5%  $\text{CO}_2$ -95% humidity incubator. After incubation, the samples were removed from the incubator and  $25 \mu\text{L}$  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  $A_{\text{test}}$  value.

Baseline for  $\text{GI}_{50}$  (dose for 50% inhibition of cell proliferation) of initial cell numbers was determined by adding an aliquot of  $45 \mu\text{L}$  of cells and  $5 \mu\text{L}$  of RPMI-1640 media solution with 10% DMSO to wells of a 96-well microtiter plate. The samples were then incubated at  $37^\circ\text{C}$  for 0.5 h in a 5%  $\text{CO}_2$ -95% humidity incubator. After incubation, the samples were removed from the incubator and  $25 \mu\text{L}$  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  $A_{\text{start}}$  value, defining luminescence for initial cell number used as baseline in  $\text{GI}_{50}$  determinations.

**Calculation.**  $\text{GI}_{50}$  (dose for 50% inhibition of cell proliferation) is the concentration where  $[(A_{\text{test}} - A_{\text{start}})/(A_{\text{max}} - A_{\text{start}})] = 0.5$ . The  $\text{GI}_{50}$  ( $\mu\text{M}$ ) of compound **1**, **6**, and **10** in T47D and DLD-1 cells are summarized in Table 2 in comparison with the caspase activation activity ( $\text{EC}_{50}$ ). The  $\text{GI}_{50}$  ( $\mu\text{M}$ ) of compound **10** in MES-SA and MES-SA/DX5 cells are summarized in Table 3 in comparison with that of paclitaxel.

**Tubulin Polymerization Assay.** Lyophilized tubulin (Cytoskeleton #ML113, 1 mg, MAP-rich) was assayed for the effect of the test compound on tubulin polymerization according to the recommended procedure of the manufacturer. To  $1 \mu\text{L}$  of

each experimental compound (from a  $100\times$  stock) in a 96-well incubation was added  $99 \mu\text{L}$  of supplemented tubulin supernatant. Incubation was done in a Molecular Devices plate reader at  $37^\circ\text{C}$ , and absorbance readings at 340 nm were recorded every minute for an hour. The  $\text{IC}_{50}$  for tubulin inhibition was the concentration found to decrease the initial rate of tubulin polymerization by 50% as calculated with Prism 2.0.

**Acknowledgment.** The authors acknowledge the fine technical support given by Regina Brand and Ryan Yoshimura.

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JM0205200