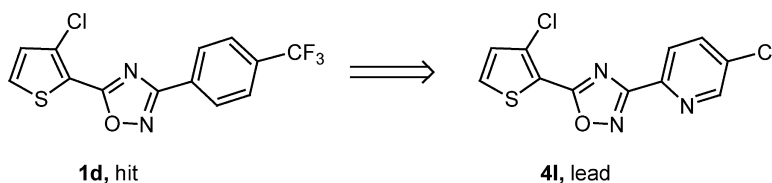


## Discovery and Structure–Activity Relationship of 3-Aryl-5-aryl-1,2,4-oxadiazoles as a New Series of Apoptosis Inducers and Potential Anticancer Agents

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## Discovery and Structure–Activity Relationship of 3-Aryl-5-aryl-1,2,4-oxadiazoles as a New Series of Apoptosis Inducers and Potential Anticancer Agents

Han-Zhong Zhang, Shailaja Kasibhatla, Jared Kuemmerle, William Kemnitzer, Kristin Ollis-Mason, Ling Qiu, Candace Crogan-Grundy, Ben Tseng, John Drewe, and Sui Xiong Cai\*

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We have identified 5-(3-chlorothiophen-2-yl)-3-(4-trifluoromethylphenyl)-1,2,4-oxadiazole (**1d**) as a novel apoptosis inducer through our caspase- and cell-based high-throughput screening assay. Compound **1d** has good activity against several breast and colorectal cancer cell lines but is inactive against several other cancer cell lines. In a flow cytometry assay, treatment of T47D cells with **1d** resulted in arrest of cells in the G<sub>1</sub> phase, followed by induction of apoptosis. SAR studies of **1d** showed that the 3-phenyl group can be replaced by a pyridyl group, and a substituted five-member ring in the 5-position is important for activity. 5-(3-Chlorothiophen-2-yl)-3-(5-chloropyridin-2-yl)-1,2,4-oxadiazole (**4l**) has been found to have in vivo activity in a MX-1 tumor model. Using a photoaffinity agent, the molecular target has been identified as TIP47, an IGF II receptor binding protein. Therefore, our cell-based chemical genetics approach for the discovery of apoptosis inducers can identify potential anticancer agents as well as their molecular targets.

### Introduction

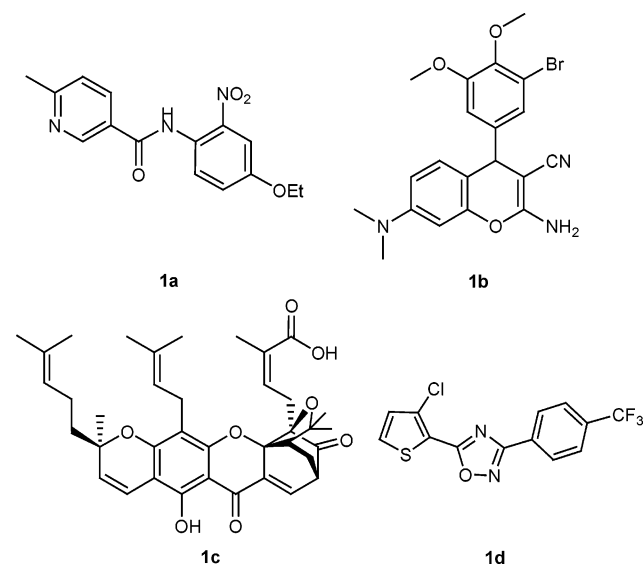
Cancer is the second leading cause of mortality in developed countries, and the discovery and development of new treatments is urgently needed due to problems with currently available treatments, such as toxicities and drug-resistance.<sup>1</sup> Many of the current clinically used anticancer agents, including paclitaxel, vinblastine,<sup>2–4</sup> and compounds under clinical evaluation, such as epothilone,<sup>5</sup> are microtubule-interfering agents that bind to tubulin. Several new anticancer agents developed recently, such as Gleevec,<sup>6</sup> are kinase inhibitors designed to be more selective, resulting in less toxicity.

It has been recently reported that the antitumor efficacy of several chemotherapeutic agents correlated with their apoptosis-inducing ability.<sup>7</sup> Therefore, the identification of apoptosis inducers represents an attractive approach for the discovery and development of potential anticancer agents. Moreover, by inducing apoptosis, these new agents may overcome tumor resistance to conventional anticancer agents.<sup>8</sup> It is known that caspases, a family of proteolytic enzymes, play a pivotal role in the apoptotic process.<sup>9</sup> Activation of these proteases, which are normally present inside cells as inactive zymogens, results in the cleavage of many protein substrates inside cells and leads to irreversible apoptotic cell death. Among the caspases, caspase-3 is one of the most downstream caspases and is called an effector caspase.<sup>10</sup>

In our effort to discover and develop apoptosis inducers as potential new anticancer agents, we have developed a cell-based, high-throughput screening technology to identify apoptosis inducers using our novel caspase-3 substrates.<sup>11,12</sup> One of the major advantages of this technology is that, by monitoring the activation of downstream caspase-3 in a cell-based assay, we can

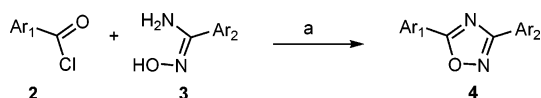
discover compounds that induce apoptosis through interaction with either known or novel targets.

Applying this HTS assay, we have identified several classes of molecules as novel apoptosis inducers. *N*-phenylnicotinamides, represented by 6-methyl-*N*-(4-ethoxy-2-nitrophenyl)pyridine-3-carboxamide (**1a**), was

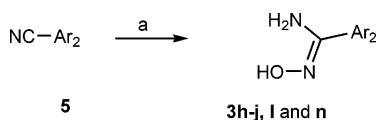


discovered as a series of potent apoptosis inducers that interact with tubulin.<sup>13</sup> 4-Aryl-4*H*-chromenes, such as 2-amino-3-cyano-7-(dimethylamino)-4-(3-bromo-4,5-dimethoxyphenyl)-4*H*-chromene (**1b**), have been identified as another class of potent apoptosis inducers.<sup>14</sup> These 4-aryl-4*H*-chromenes bind at or close to the binding site of colchicine, and some members of this class have been demonstrated to have vascular targeting activity with good efficacy in several anticancer animal models.<sup>15,16</sup> Gambogic acid (**1c**), isolated from the gamboge resin of the *Garcinia hanburyi* tree, was discovered

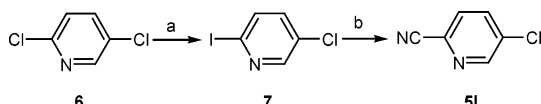
\* Corresponding author. Tel: 858-202-4006. Fax: 858-202-4000. E-mail: scai@maxim.com.

**Scheme 1<sup>a</sup>**

<sup>a</sup> Conditions: (a) dioxane/pyridine.

**Scheme 2<sup>a</sup>**

<sup>a</sup> Conditions: (a) H<sub>2</sub>NOH·HCl/EtOH/NaOH, or H<sub>2</sub>NOH/EtOH/THF.

**Scheme 3<sup>a</sup>**

<sup>a</sup> Conditions: (a) NaI/AcCl/CH<sub>3</sub>CN; (b) CuCN/pyridine.

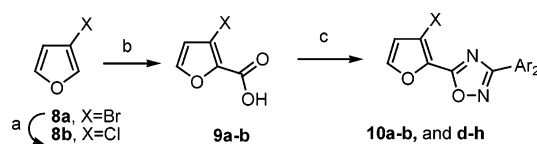
as a fast and potent apoptosis inducer with a novel mechanism of action.<sup>17</sup> Herein, we report the discovery and biological characterization of 5-(3-chlorothiophen-2-yl)-3-(4-trifluoromethylphenyl)-1,2,4-oxadiazole (**1d**) as a novel apoptosis inducer with tumor-selective properties, as well as the structure–activity relationship (SAR) of this novel series of 5-aryl-3-aryl-1,2,4-oxadiazoles. These studies led to the characterization of 5-(3-chlorothiophen-2-yl)-3-(5-chloropyridin-2-yl)-1,2,4-oxadiazole (**4l**) as a potent inducer of apoptosis with *in vivo* anticancer activity and the identification of TIP47, an insulin-like growth factor II (IGF II) receptor binding protein, as the molecular target for this class of novel apoptosis inducers.<sup>18</sup>

**Results and Discussion**

**Chemistry.** 5-(3-Chlorothiophen-2-yl)-3-(4-trifluoromethylphenyl)-1,2,4-oxadiazole (**1d**) was originally obtained from a commercial library. It was prepared from 3-chlorothiophene-2-carbonyl chloride and *N*-hydroxy-4-(trifluoromethyl)benzamide by a one-pot reaction in high yield.<sup>19,20</sup> Other 3-aryl-5-aryl-1,2,4-oxadiazoles (**4**) were synthesized similarly via reaction of substituted arylcarbonyl chloride **2** with substituted *N*-hydroxybenzamide or *N*-hydroxypyridinecarboxamide **3** (Scheme 1).

Several substituted *N*-hydroxybenzamide and *N*-hydroxypyridine-2-carboxamide (**3**) were not commercially available, and these were prepared via reaction of the corresponding substituted benzonitrile or pyridinecarbonitrile (**5**) with hydroxylamine in ethanol and THF (Scheme 2).<sup>21</sup> 5-Chloro-2-cyanopyridine (**5l**) was prepared by treatment of 2,5-dichloropyridine (**6**) with sodium iodide in the presence of acetyl chloride<sup>22</sup> to produce 5-chloro-2-iodopyridine (**7**), followed by refluxing of **7** with copper cyanide (Scheme 3).

Compounds with a furan ring as Ar<sub>1</sub> were synthesized from 3-bromofuran (Scheme 4). Treatment of 3-bromofuran (**8a**) with lithium diisopropylamide followed by quenching with dry ice generated 3-bromofuran-2-carboxylic acid (**9a**).<sup>23</sup> Compound **9a** was converted to 3-bromofuran-2-carbonyl chloride by treatment with thionyl chloride,<sup>24</sup> followed by reaction with *N*-hydroxyaryl-2-carboxamide (**3**), to produce 5-(3-bromo-

**Scheme 4<sup>a</sup>**

<sup>a</sup> Conditions: (a) *n*-BuLi, then C<sub>2</sub>Cl<sub>6</sub>; (b) (*i*-Pr)<sub>2</sub>NLi, then CO<sub>2</sub>; (c) SOCl<sub>2</sub>, then HON=C(NH<sub>2</sub>)-Ar<sub>2</sub>/pyridine.

furan-2-yl)-3-aryl-1,2,4-oxadiazoles (**10**). The corresponding 3-chlorofuran analogues were synthesized similarly from 3-chlorofuran (**8b**), which was prepared via reaction of **8a** with *n*-butyllithium, followed by treatment with hexachloroethane.<sup>25</sup>

**HTS Assay.** 5-(3-Chlorothiophen-2-yl)-3-(4-trifluoromethylphenyl)-1,2,4-oxadiazole (**1d**) was identified as an inducer of apoptosis using our cell- and caspase-3-based HTS assay as described previously.<sup>13</sup> Briefly, human breast cancer T47D cells, in the well of a microtiter plate containing 10 μM of test compound, were incubated for 24 or 48 h to induce apoptosis. Fluorogenic caspase-3 substrate *N*-(Ac-DEVD)-*N'*-ethoxycarbonyl-R110<sup>26</sup> in a caspase buffer was then added and the sample was incubated at room temperature for 3 h. Fluorescence of cleaved product ethoxycarbonyl-R110 was measured to determine the level of caspase activation. Compounds found to give a ratio of >3-fold over background were considered active and retested for confirmation and subsequently at different concentrations to produce a dose–response curve. The concentration needed to give the midpoint of the caspase activation activity was determined (EC<sub>50</sub>).

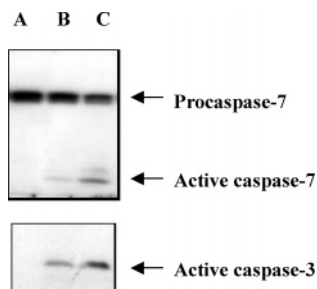
Compound **1d** was found to have an EC<sub>50</sub> of 1.2 μM with a maximal ratio of approximately 10 vs control in T47D cells. Interestingly, caspase activity was consistently observed after cells were incubated with **1d** for 48 h, but not after 24 h incubation. In comparison, other apoptosis inducers discovered from our assay, including 6-methyl-*N*-(4-ethoxy-2-nitrophenyl)pyridine-3-carboxamide (**1a**),<sup>13</sup> 2-amino-3-cyano-7-(dimethylamino)-4-(3-bromo-4,5-dimethoxyphenyl)-4*H*-chromene (**1b**),<sup>14</sup> and gambogic acid (**1c**),<sup>17</sup> all produced consistent caspase activity in the 24 h assay. These data indicate that compound **1d** is a slower inducer of apoptosis and suggest that **1d** may have a different mechanism of action than compounds **1a**, **1b**, and **1c**. Compound **1d** was tested against multiple cancer cell lines and the results are summarized in Table 1. Compound **1d** was active against several cancer cell lines, including breast cancer cell lines T47D and ZR75-1 and colorectal cancer cell lines DLD-1 and HT29, but was not active against human non-small-cell lung cancer cell line H1299, human prostate cancer cell line LnCap, or mouse leukemia cell line P388, indicating that **1d** is selective toward certain cancer types.

**Characterization of Compound 1d.** The ability of compound **1d** to induce apoptosis was further characterized by additional apoptosis assays. Cleavage of pro-caspases to active caspases is one of the hallmarks of caspase-mediated cellular apoptosis.<sup>27</sup> SKBr3 cells, which are sensitive to the compound, were treated with 0.6 or 1.2 μM of compound **1d** for 48 h, and the proteins were extracted and analyzed by Western blot for caspase proteins. Figure 1 showed the reduction in the amount of pro-caspase-7 and the appearance of active caspase-7

**Table 1.** Caspase Activation Activity of 5-(3-Chlorothiophen-2-yl)-3-(4-trifluoromethylphenyl)-1,2,4-oxadiazole (**1d**) against Cancer Cell Lines

cell line	T47D	ZR75-1	DLD	HT29	H1299	LnCap	P388
EC <sub>50</sub> (μM) <sup>a</sup>	1.21 ± 0.10	3.34 ± 0.84	0.44 ± 0.06	0.76 ± 0.15	>40	>10	>10

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

**Figure 1.** Western blots showing activation of caspase-3 and caspase-7 in SKBr3 cells treated with compound **1d** for 48 h. (A) Control cells. (B) Cells treated with 0.6 μM of **1d**. (C) Cells treated with 1.2 μM of **1d**.

protein as well as the appearance of active caspase-3, consistent with the activation of apoptosis. As expected, more active caspase-7 and active caspase-3 was observed when cells were treated with 1.2 μM of **1d** than with 0.6 μM of **1d**. In contrast, no active caspase-7 and caspase-3 was observed in the cells treated with solvent (DMSO). These results show that treatment with compound **1d** activates caspases in the cells, a key marker of cellular apoptosis.

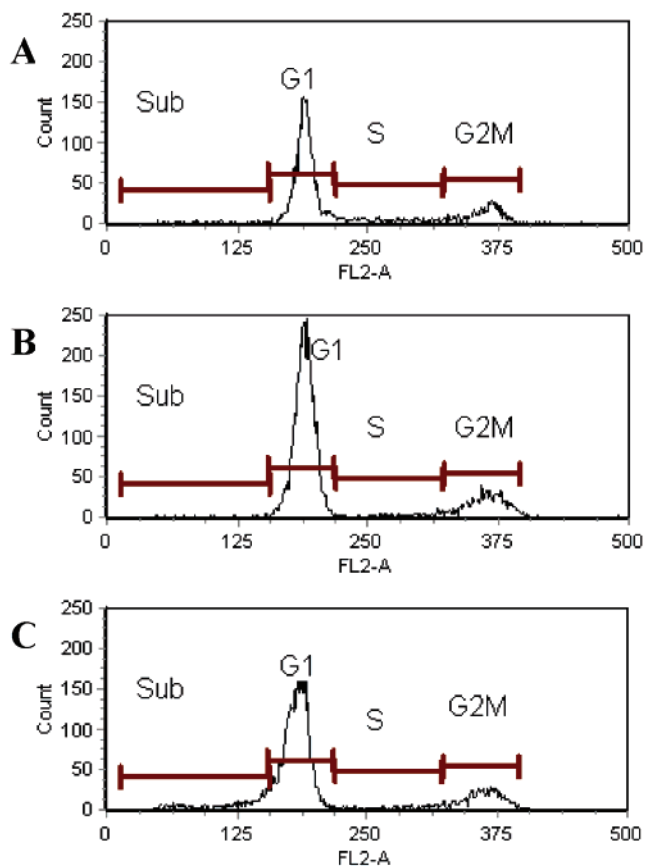
The apoptosis-inducing activity of compound **1d** was also characterized by flow cytometry. T47D cells were treated with 1.5 μM of compound **1d** for 24 or 48 h and then stained with propidium iodide and analyzed by flow cytometry. Figure 2A showed that control cells are mostly in the G<sub>1</sub> phase of the cell cycle. Cells treated with compound **1d** for 24 h showed that most of the cells are arrested in the G<sub>1</sub> phase, with a reduction of cells in the S phase, and with no increase of cells with subdiploid DNA content, a marker of apoptotic cells. Cells treated with compound **1d** for 48 h showed that most of the cells are still arrested in the G<sub>1</sub> phase, with a large increase of apoptotic cells with subdiploid DNA content. These results are consistent with the caspase activation assay, which showed that caspase activity was more consistently observed after 48-h treatment. These data also indicate that compound **1d** arrests cells in the G<sub>1</sub> phase, followed by induction of apoptosis.

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

The cell-based caspase HTS assay was also used for testing of analogues of compound **1d** for SAR studies. The caspase activation activity (EC<sub>50</sub>) of **1d** and analogues was tested against three cell lines, T47D breast cancer cells, DLD-1 colon cancer cells, and H-1299 non-small-cell lung cancer cells, and results are summarized in Table 2.

Table 2 shows that compound **1d** has an EC<sub>50</sub> of 1.2 and 0.44 μM in T47D and DLD-1, respectively, and is inactive up to 40 μM against H-1299 cell line in the caspase activation assay. For SAR studies, we maintained the Ar<sub>1</sub> as 3-chloro-thiophen-2-yl and explored the Ar<sub>2</sub> first with a series of substituted phenyl groups. Compound **4a**, with a Cl replacing the CF<sub>3</sub> of **1d**, was found to have similar activity as **1d**, which is expected, since a Cl group is known to be a good replacement for

Entry	Sub	G1	S	G <sub>2</sub> M
1A	2.2	64.8	11.9	17.5
1B	1.7	75.1	4.4	18.1
1C	11.4	67.6	5.3	16.3

**Figure 2.** Drug-induced 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 G<sub>1</sub> phase of the cell cycle. (B) Cells treated with 1.5 μM of compound **1d** for 24 h showing most of the cells arrested in G<sub>1</sub> phase and reduction of cells in the S phase. (C) Cells treated with 1.5 μM of compound **1d** for 48 h showing a progression from G<sub>1</sub> to cells with subdiploid DNA content, which are apoptotic cells with fragmented nuclei.

a CF<sub>3</sub> group. Removing the CF<sub>3</sub> group from **1d** results in **4b**, which was 2-fold less active than **1d** and **4a**, indicating that the Cl and CF<sub>3</sub> group in the 4-position is important for activity. Compounds **4c**, **4d**, **4e**, and **4v**, with an OCF<sub>3</sub>, OMe, Me, and NO<sub>2</sub> group in the 4-position, also had good activity, suggesting that the 4-position can tolerate many different groups. Compound **4f**, with a dimethylamino group in the 4-position, was not active up to 40 μM, suggesting that a basic group may not be tolerated in that position.



**Table 2.** SAR of 3-Aryl-5-aryl-1,2,4-oxadiazoles in the Caspase Activation Assay

Compound code	Ar <sub>1</sub>	Ar <sub>2</sub>	Caspase activation (EC <sub>50</sub> , μM <sup>a</sup> )		
			T47D	DLD1	HI299
<b>1d</b>			1.21 ± 0.10	0.44 ± 0.06	>40
<b>4a</b>			1.77 ± 0.20	0.79 ± 0.11	>40
<b>4b</b>			2.82 ± 0.13	0.76 ± 0.09	>40
<b>4c</b>			0.89 ± 0.14	0.43 ± 0.09	>10
<b>4d</b>			4.17 ± 0.41	1.45 ± 0.15	>10
<b>4e</b>			3.66 ± 0.39	1.42 ± 0.08	>10
<b>4f</b>			>40	>10	>40
<b>4g</b>			0.90 ± 0.13	0.28 ± 0.02	>10
<b>4h</b>			1.41 ± 0.19	0.37 ± 0.05	>10
<b>4i</b>			0.93 ± 0.18	0.56 ± 0.17	>10
<b>4j</b>			0.91 ± 0.09	0.38 ± 0.06	>40
<b>4k</b>			1.44 ± 0.05	0.41 ± 0.11	>10
<b>4l</b>			1.59 ± 0.09	0.39 ± 0.06	>40
<b>4m</b>			0.92 ± 0.14	0.24 ± 0.06	>10
<b>4n</b>			0.75 ± 0.04	0.29 ± 0.05	>10
<b>4o</b>			3.32 ± 0.56	1.32 ± 0.13	>10

Compound code	Ar <sub>1</sub>	Ar <sub>2</sub>	Caspase activation (EC <sub>50</sub> , μM <sup>a</sup> )		
			T47D	DLD1	HI299
<b>4p</b>			>40	>10	>40
<b>4q</b>			>40	>10	>40
<b>4r</b>			>40	>10	>40
<b>4s</b>			>40	>10	>40
<b>4t</b>			>40	ND <sup>b</sup>	>40
<b>4u</b>			>40	ND	>40
<b>4v</b>			1.97	ND	>40
<b>10a</b>			1.08 ± 0.14	0.83 ± 0.12	>10
<b>10b</b>			1.74 ± 0.18	1.49 ± 0.20	>10
<b>10c</b>			17.4 ± 2.1	>10	>40
<b>10d</b>			2.01 ± 0.14	1.07 ± 0.15	>10
<b>10e</b>			0.52 ± 0.01	0.48 ± 0.09	>10
<b>10f</b>			1.61 ± 0.11	0.84 ± 0.10	>10
<b>10g</b>			0.61 ± 0.15	0.70 ± 0.21	>10
<b>10h</b>			1.11 ± 0.08	0.58 ± 0.08	>10

<sup>a</sup> Data are the mean of three or more experiments and are reported as mean ± standard error of the mean (SEM). <sup>b</sup> ND, not determined.

Compound **4g**, with the CF<sub>3</sub> group in the 3-position, was about as active as **1d**, suggesting that a CF<sub>3</sub> group in the 3-position also is good for activity. Compounds **4h** and **4i** were about as potent as **4a** and **4g**, indicating that there are no additive effects via 3,4-disubstitution. Compound **4j** also was about as potent as **4a**, suggesting that a small group in the 2-position has little effect on potency.

We then explored the replacement of the phenyl group by a pyridyl group, with the goal of reducing cLogP values and improving the solubility profiles of these 3-aryl-5-aryl-1,2,4-oxadiazoles. Compound **4k** was found to have similar activity as that of compound **1d**, indicating that a 2-pyridyl group is a good replacement for the phenyl group. Similarly, compound **4l** also was as active as compound **4a**, with a reduced cLogP value (**4l** cLogP = 3.62 vs **4a** cLogP = 4.78). Compounds **4m** and **4n**, the corresponding 3-pyridyl analogues, were found to be slightly more potent than compounds **4k** and **4l**.

The SAR of the Ar<sub>1</sub> group was then explored. Compound **4o**, with a 3-Br group instead of the 3-Cl group, was found to have similar potency as **4a**. Compound **4p**, with a 3-ethoxy group, was not active up to 40 μM, suggesting that there might be a size limited pocket in

the 3-position. Compound **4q**, with an extra Me group in the 4-position, was also not active up to 40 μM, suggesting that substitution in the 4-position may not be tolerated. Compounds **4r** and **4s**, without a substitution in the 4-position, were all inactive up to 40 μM, indicating that a small group in the 3-position is important for activity. Compounds **4t** and **4u**, with a 2-chlorophenyl group instead of the 3-chlorothiophen-2-yl group, were all inactive up to 40 μM, indicating that the five-member thiophene ring as Ar<sub>1</sub> is important for these 3-aryl-5-aryl-1,2,4-oxadiazoles as caspase activators and apoptosis inducers.

Since the five-member thiophene is important for activity, we explored the replacement of the thiophene by furan. Compound **10a**, with a 3-bromofuran-2-yl group, was about 3-fold more potent than compound **4o**. Compound **10b**, with a 3-chlorofuran-2-yl group, was about as active as **4a**. Therefore, a furan ring can be used to replace the thiophene ring, with an additional benefit of reduced cLogP, which might improve the solubility profile (**10b** cLogP = 4.26 vs **4a** cLogP = 4.78). Compound **10c** was 10-fold less active than **10b**, confirming the importance of a small substitution in the 3-position. Interestingly, compound **10d** was slightly

**Table 3.** Cell Growth Inhibition of 3-Aryl-5-aryl-1,2,4-oxadiazoles

compd	growth inhibition (GI <sub>50</sub> , μM <sup>a</sup> )		
	T47D	DLD1	H1299
<b>1d</b>	0.22 ± 0.05	0.77 ± 0.29	>10
<b>4a</b>	0.19 ± 0.03	0.40 ± 0.06	>10
<b>4d</b>	0.52 ± 0.29	0.93 ± 0.53	>10
<b>4e</b>	2.20 ± 0.47	0.85 ± 0.15	>10
<b>4k</b>	0.35 ± 0.05	0.93 ± 0.29	>10
<b>4l</b>	0.20 ± 0.02	0.80 ± 0.11	>10
<b>4o</b>	0.57 ± 0.16	0.58 ± 0.15	>10
<b>10a</b>	0.12 ± 0.01	1.5 ± 0.29	>10
<b>10b</b>	0.12 ± 0.01	0.51 ± 0.10	>10
<b>10d</b>	0.32 ± 0.04	0.13 ± 0.02	>10
<b>10e</b>	0.17 ± 0.04	0.35 ± 0.05	>10
<b>10f</b>	0.38 ± 0.10	0.43 ± 0.05	>10
<b>10h</b>	0.13 ± 0.01	0.60 ± 0.16	>10

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

less potent than **10a**, while compound **10e** is slightly more potent than **10b**. Compound **10f** was about as active as **10a**, confirming that the Ar<sub>2</sub> phenyl group can be replaced by a pyridyl group. As expected, compounds **10g** and **10h** also had good activities.

The activities of these 3-aryl-5-aryl-1,2,4-oxadiazoles toward the human colorectal cancer cell line DLD-1 was roughly parallel to their activity toward T47D cells. In general, DLD-1 cells were more sensitive (about 2–4-fold as indicated by the EC<sub>50</sub> values) to the compounds than T47D cells. Similar to compound **1d**, all the 3-aryl-5-aryl-1,2,4-oxadiazoles tested are not active against non-small-cell lung cancer cell line H1299, indicating that these compounds are selective toward certain tumor types.

Selected compounds were also tested by the 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 confirm the tumor selectivity as observed from the caspase assays. The growth inhibition assays in T47D, DLD-1, and H1299 cells were run in a 96-well microtiter plate as described previously.<sup>13</sup> The GI<sub>50</sub> values are summarized in Table 3.

Table 3 shows that as expected, compound **1d**, and many of its analogues are good inhibitors of tumor cells growth. Most of these compounds have GI<sub>50</sub> in the submicromolar range against T47D and DLD-1 cells. In general, T47D is slightly more sensitive (as indicated by the GI<sub>50</sub> values) to these compounds than DLD-1. All the tested compounds are not active against H1299 up to 10 μM. This is similar to what is observed in the caspase activation assay, confirming that these compounds are selective against certain tumor types. In addition, compounds **4a** and **4l** also have been tested against three primary normal cells, HUVEC (human umbilical vascular endothelial cells), RWPE-1, and HMEC (human mammary epithelial cells). Both **4a** and **4l** are not active against these primary cells up to 10 μM.<sup>18</sup>

Compound **4l** was selected for in vivo tumor efficacy testing on the basis of solubility and pharmacokinetic profiles. Compound **4l** was found to be active in a MX-1 tumor model in mice, resulted in 83% tumor growth inhibition in combination with paclitaxel.<sup>18</sup> In addition, compounds **4a** and **4l** have been found to downregulate

cyclin D1, a key protein that is known to be critical for cell replication, by approximately 5–10-fold in T47D cells.<sup>18</sup>

On the basis of the SAR studies, which showed that the 4-position of the phenyl group can tolerate a variety of groups, we elected to prepare 5-(3-chlorothiophen-2-yl)-3-(3,5-ditritium-4-azidophenyl)-1,2,4-oxadiazole as a photoaffinity labeling agent for the identification of the molecular target. The corresponding cold 4-azido analogue was found to have good apoptosis-inducing activity in the T47D cells with EC<sub>50</sub> of 2.0 μM. Applying this photoaffinity labeling agent, TIP47, an insulin-like growth factor II (IGF II) receptor binding protein, has been identified as the molecular target.<sup>18</sup>

## Conclusion

In conclusion, 5-(3-chlorothiophen-2-yl)-3-(4-trifluoromethylphenyl)-1,2,4-oxadiazole (**1d**) was identified as a potent apoptosis inducer through our caspase- and cell-based high-throughput screening assays. Compound **1d** was further characterized in a caspase activation assay in SKBr3 breast cancer cells by Western blots, showing that treatment of cells with **1d** led to the cleavage of pro-caspases to active caspases, one of the hallmarks of apoptosis. The ability of compound **1d** to induce apoptosis also was confirmed in a flow cytometry assay in T47D cells, showing that treatment of cells with **1d** resulted in G<sub>1</sub> arrest, followed by apoptosis. This distinguishes compound **1d** from many anticancer agents, including the taxanes and vinca alkaloids, that interact with tubulin and arrest cells at the M phase of the cell cycle. Importantly, these 3-aryl-5-aryl-1,2,4-oxadiazoles were found to selectively downregulate cyclin D1 and induce apoptosis selectively in certain tumor types and were not active against primary normal cells. This selectivity might provide better toxicity profiles for this series of apoptosis inducers, in comparison with many of the current anticancer agents. In addition, the selectivity against different cancer cells could provide some guidelines for the selection of animal models as well as clinical indications. It is interesting to note that the selective COX-2 inhibitor Celecoxib, a 1,5-diarylpyrazole that structurally is quite different from **1d**, was reported recently to cause a reduction in amount of cyclin D1 in PC3 xenografts, as well as to arrest PC3 cells in G<sub>1</sub> phase after 96 h treatment.<sup>28</sup> Therefore, there is some similarity between the biological activities of **1d** and Celecoxib.

Through structure–activity relationship studies of 3-aryl-5-aryl-1,2,4-oxadiazoles, we have found that a 3-substituted thiophen-2-yl or a furan-2-yl in the 5-position of 1,2,4-oxadiazole is important for the apoptosis-inducing activity. In the 3-position of 1,2,4-oxadiazole, both substituted phenyl and pyridyl provide potent compounds. Compounds with a pyridyl group offer both good potency, more desirable cLogP values, and better solubility profiles. Compound **4l** has been found to be active in a MX-1 tumor model in mice.<sup>18</sup> Using a photoaffinity labeling agent, 5-(3-chlorothiophen-2-yl)-3-(3,5-ditritium-4-azidophenyl)-1,2,4-oxadiazole, TIP47, an insulin-like growth factor II (IGF II) receptor binding protein, has been identified as the molecular target.<sup>18</sup>

Taken together, we have developed a novel chemical genetics approach for the discovery of potential anti-

cancer agents and their molecular targets. Starting with a caspase- and cell-based HTS assay, through SAR study and biological characterization of hits, compounds with *in vivo* anticancer activity were discovered. Reagents for target identification were designed and synthesized, resulting in the identification of the molecular target for these *in vivo* active compounds. Additional SAR and animal efficacy studies of **4l** and related analogues are in progress and will be reported in the future.

## Experimental Section

**General Methods and Materials.** Commercial-grade reagents and solvents were obtained from Acros, Aldrich, Lancaster, or Butt Park and were used without further purification except as indicated. All reactions were stirred magnetically; moisture-sensitive reactions were performed under argon in oven-dried glassware. 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  $^1\text{H}$  NMR spectra were recorded at 300 MHz. All samples were prepared as dilute solutions in either deuteriochloroform ( $\text{CDCl}_3$ ) with *v/v* 0.05% tetramethylsilane (TMS), dimethyl- $d_6$ -sulfoxide ( $\text{CD}_3\text{SOCD}_3$ ) with *v/v* 0.05% TMS, or acetone- $d_6$  ( $\text{CD}_3\text{COCD}_3$ ) with *v/v* 1% 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 Numeqa Resonance Labs, Inc. (San Diego, CA). Human breast cancer cells T47D, SKBr3 and ZR75-1; human colon cancer cells DLD-1 and HT29; human non-small-cell lung cancers H1299; human prostate cancer cell line LnCap; and mouse leukemia cell line P388 were obtained from American Type Culture Collection (Manassas, VA). Compounds **1d**, **4a**, **4p**, and **4q** were obtained from Maybridge (Cornwall, England); **4r**, **4t**, and **4u** were from Chembridge (San Diego, CA); and **10c** was from Specs (Delft, Netherlands), and their structures were confirmed by  $^1\text{H}$  NMR. The structures of compounds **1d** and **4a** also were confirmed by resynthesis.

**5-Chloro-2-iodopyridine (7).** A solution of 2,5-dichloropyridine (**6**) (12.2 g, 82.2 mmol) and sodium iodide (37.0 g, 247 mmol) in acetonitrile (170 mL) was brought to reflux at 105 °C with magnetic stirring under argon. To the solution was added acetyl chloride (9.0 mL, 13 mmol) and it was refluxed for 5 h. More sodium iodide (24.9 g, 166 mmol) was added and the solution was refluxed for 16 h. More sodium iodide (12.5 g, 83.6 mmol) was added and the solution was refluxed for 4.5 h. The solution was cooled to room temperature and partitioned in a separatory funnel between 10% sodium thiosulfate/10% aqueous sodium carbonate (300 mL) and ether (400 mL). The ether layer was washed with brine (100 mL) and dried over anhydrous sodium sulfate. The solution was decanted and evaporated to give a wet solid. The wet solid was vacuum-dried overnight to yield a brown solid (16.7 g, 85% yield):  $^1\text{H}$  NMR ( $\text{CDCl}_3$ ) 8.36 (d,  $J = 2.8$ , 1H), 7.66 (d,  $J = 8.8$ , 1H), 7.32 (dd,  $J = 8.4$ , 2.6, 1H). The product contains about 5% of starting material **6** as determined by  $^1\text{H}$  NMR and was used for the next reaction without further purification.

**5-Chloro-2-cyanopyridine (5l).** A solution of 5-chloro-2-iodopyridine (16.7 g, 69.5 mmol), cuprous cyanide (8.15 g, 91.0 mmol), and pyridine (120 mL) was refluxed for 1.5 h at 130 °C with magnetic stirring under argon. The solution was cooled to room temperature and was poured into 56 g/L aqueous potassium cyanide solution (500 mL). The solution was extracted with dichloromethane (4 × 200 mL). The organic layers were dried over anhydrous sodium sulfate, decanted, and concentrated to dryness by rotary evaporation at 50 °C. The crude product was purified by column chromatography (dichloromethane) to yield **5l** as a white solid (6.76 g, 70% yield):  $^1\text{H}$  NMR ( $\text{CDCl}_3$ ) 8.69 (dd,  $J = 2.5$ , 0.6, 1H), 7.84 (dd,  $J = 8.4$ , 2.3, 1H), 7.87 (dd,  $J = 8.4$ , 0.7, 1H). The product

contains about 4% of starting material **6** as determined by  $^1\text{H}$  NMR and was used for the next reaction without further purification.

**5-Chloro-*N*-hydroxypyridine-2-carboxamide (3l).** A solution of 5-chloro-2-cyanopyridine (6.75 g, 48.7 mmol) and 50% hydroxylamine (3.5 mL, 57 mmol) in ethanol (10 mL) and tetrahydrofuran (50 mL) was magnetically stirred at room temperature for 20 min. The solution was evaporated to dryness by rotary evaporation and the solid was dried under vacuum overnight. The solid was suspended in hexane (50 mL) and sonicated for 5 min. The mixture was then refluxed for 5 min, cooled to room temperature, and filtered on a Buchner funnel, and the solid was dried to yield **3l** as a white solid (7.89 g, 94%):  $^1\text{H}$  NMR ( $\text{DMSO}-d_6$ ) 10.05 (s, 1H), 8.62 (dd,  $J = 2.3$ , 0.7, 1H), 7.94 (dd,  $J = 8.7$ , 2.3, 1H), 7.86 (dd,  $J = 8.7$ , 0.7, 1H), 5.86 (s, 2H).

**3,4-Dichloro-*N*-hydroxybenzamide (3h).** A solution of 3,4-dichlorobenzonitrile (516 mg, 3.00 mmol), 0.576 M hydroxylamine hydrochloride in 95% ethanol (5.8 mL, 3.3 mmol), 3.0 N sodium hydroxide (1.1 mL, 3.2 mmol), and ethanol (10 mL) was stirred at room temperature for 20 min and the solution was concentrated. The product was suspended in hexane, refluxed for 5 min, cooled to room temperature, and filtered to yield 276 mg (45%) of **3h** as a white solid:  $^1\text{H}$  NMR (acetone- $d_6$ ) 9.21 (s, 1H), 7.89 (d,  $J = 2.2$ , 1H), 7.70 (dd,  $J = 8.5$ , 2.2, 1H), 7.58 (d,  $J = 8.5$ , 1H), 5.62 (s, 2H).

The following compounds were prepared from the corresponding substituted aryl nitrile and hydroxylamine by a procedure similar to that described for the preparation of compound **3h**.

**4-Chloro-*N*-hydroxy-3-trifluoromethylbenzamide (3i):** white solid (74%);  $^1\text{H}$  NMR (acetone- $d_6$ ) 9.29 (s, 1H), 8.15 (d,  $J = 2.2$ , 1H), 8.00 (m, 1H), 7.69 (d,  $J = 8.2$ , 1H), 5.73 (s, 2H).

**4-Chloro-*N*-hydroxy-2-methylbenzamide (3j):** white solid (31%);  $^1\text{H}$  NMR ( $\text{DMSO}-d_6$ ) 9.41 (s, 1H), 7.32 (s, 1H), 7.27 (m, 2H), 5.78 (br s, 2H), 2.34 (s, 3H).

**6-Chloro-*N*-hydroxypyridine-3-carboxamide (3n):** white solid (53%);  $^1\text{H}$  NMR (acetone- $d_6$ ) 9.26 (s, 1H), 8.71 (dd,  $J = 2.5$ , 0.8, 1H), 8.10 (dd,  $J = 8.2$ , 2.4, 1H), 7.47 (dd,  $J = 8.4$ , 0.7, 1H), 5.69 (s, 2H).

**3-(4-Chlorophenyl)-5-(3-chlorothiophen-2-yl)-1,2,4-oxadiazole (4a).** A solution of 3-chlorothiophene-2-carbonyl chloride (1.45 g, 8.00 mmol) and 4-chloro-*N*-hydroxybenzamide (1.37 g, 8.00 mmol) in dioxane/pyridine (110 mL, 10:1) was refluxed for 12 h and cooled to room temperature. To the stirred solution was added water (200 mL) to produce precipitates. The precipitates were collected by filtration, washed with water (4 × 20 mL), and dried to yield 2.36 g of colorless solid, which was further purified by column chromatography (silica gel; ethyl acetate:hexane, 1:10) to yield 2.01 g (85%) of **4a** as white solid:  $^1\text{H}$  NMR ( $\text{CDCl}_3$ ) 8.10 (d,  $J = 8.4$ , 2H), 7.61 (d,  $J = 5.1$ , 1H), 7.49 (d,  $J = 8.4$ , 2H), 7.13 (d,  $J = 5.1$ , 1H). Anal. ( $\text{C}_{12}\text{H}_6\text{Cl}_2\text{N}_2\text{OS}$ ) C, H, N.

The following compounds were prepared from the corresponding substituted thiophen-2-carbonyl chloride and substituted *N*-hydroxybenzamide or *N*-hydroxypyridinecarboxamide by a procedure similar to that described for the preparation of compound **4a**.

**5-(3-Chlorothiophen-2-yl)-3-(4-trifluoromethylphenyl)-1,2,4-oxadiazole (1d):** white solid (82%);  $^1\text{H}$  NMR ( $\text{CDCl}_3$ ) 8.29 (d,  $J = 8.1$ , 2H), 7.78 (d,  $J = 8.4$ , 2H), 7.63 (d,  $J = 5.1$ , 1H), 7.15 (d,  $J = 5.1$ , 1H). Anal. ( $\text{C}_{13}\text{H}_6\text{ClF}_3\text{N}_2\text{OS}$ ) C, H, N.

**5-(3-Chlorothiophen-2-yl)-3-phenyl-1,2,4-oxadiazole (4b):** white solid (23%);  $^1\text{H}$  NMR ( $\text{CDCl}_3$ ) 8.18–8.15 (m, 2H), 7.60 (d,  $J = 5.4$ , 1H), 7.54–7.48 (m, 3H), 7.13 (d,  $J = 5.1$ , 1H). Anal. ( $\text{C}_{12}\text{H}_7\text{ClN}_2\text{OS}$ ) C, H, N.

**5-(3-Chlorothiophen-2-yl)-3-(4-trifluoromethoxyphenyl)-1,2,4-oxadiazole (4c):** white solid (75%);  $^1\text{H}$  NMR ( $\text{CDCl}_3$ ) 8.20 (d,  $J = 9.0$ , 2H), 7.62 (d,  $J = 5.4$ , 1H), 7.35 (d,  $J = 9.3$ , 2H), 7.14 (d,  $J = 5.1$ , 1H). Anal. ( $\text{C}_{13}\text{H}_6\text{ClF}_3\text{N}_2\text{O}_2\text{S}$ ) C, H, N.

**5-(3-Chloro-2-thienyl)-3-(4-methoxyphenyl)-1,2,4-oxadiazole (4d):** white solid (74%);  $^1\text{H}$  NMR ( $\text{CDCl}_3$ ) 8.10 (d,  $J = 9.0$ , 2H), 7.59 (d,  $J = 5.1$ , 1H), 7.35 (d,  $J = 9.0$ , 1H), 7.12 (d,  $J$



= 5.1, 1H), 7.00 (d,  $J$  = 9.0, 1H), 3.89 (s, 3H). Anal. (C<sub>13</sub>H<sub>9</sub>ClN<sub>2</sub>O<sub>2</sub>S) C, H, N.

**5-(3-Chlorothiophen-2-yl)-3-(4-methylphenyl)-1,2,4-oxadiazole (4e)**: white solid (33%); <sup>1</sup>H NMR (CDCl<sub>3</sub>) 8.04 (d,  $J$  = 8.1, 2H), 7.59 (d,  $J$  = 5.1, 1H), 7.30 (d,  $J$  = 8.0, 2H), 7.12 (d,  $J$  = 5.1, 1H), 2.42 (s, 3H). Anal. (C<sub>13</sub>H<sub>9</sub>ClN<sub>2</sub>O<sub>2</sub>S·0.5H<sub>2</sub>O) C, H, N.

**5-(3-Chlorothiophen-2-yl)-3-(4-dimethylaminophenyl)-1,2,4-oxadiazole (4f)**: white solid (14%); <sup>1</sup>H NMR (CDCl<sub>3</sub>) 8.01 (m, 2H), 7.56 (d,  $J$  = 5.2, 1H), 7.10 (d,  $J$  = 5.5, 1H), 6.76 (m, 2H), 3.04 (s, 6H). Anal. (C<sub>14</sub>H<sub>12</sub>ClN<sub>3</sub>OS) C, H, N.

**5-(3-Chlorothiophen-2-yl)-3-(3-trifluoromethylphenyl)-1,2,4-oxadiazole (4g)**: white solid (72%); <sup>1</sup>H NMR (CDCl<sub>3</sub>) 8.44 (s, 1H), 8.36 (d,  $J$  = 7.7, 1H), 7.80 (d,  $J$  = 8.5, 1H), 7.67 (d,  $J$  = 8.0, 1H), 7.64 (d,  $J$  = 5.2, 1H), 7.15 (d,  $J$  = 5.2, 1H). Anal. (C<sub>13</sub>H<sub>6</sub>ClF<sub>3</sub>N<sub>2</sub>O<sub>2</sub>S) C, H, N.

**5-(3-Chlorothiophen-2-yl)-3-(3,4-dichlorophenyl)-1,2,4-oxadiazole (4h)**: white solid (69%); <sup>1</sup>H NMR (CDCl<sub>3</sub>) 8.27 (d,  $J$  = 1.4, 1H), 8.00 (dd,  $J$  = 8.5, 1.5, 1H), 7.63 (d,  $J$  = 5.5, 1H), 7.59 (d,  $J$  = 8.2, 1H), 7.14 (d,  $J$  = 5.2, 1H). Anal. (C<sub>12</sub>H<sub>5</sub>Cl<sub>2</sub>N<sub>2</sub>O<sub>2</sub>S) C, H, N.

**5-(3-Chlorothiophen-2-yl)-3-(4-chloro-3-trifluoromethylphenyl)-1,2,4-oxadiazole (4i)**: white solid (68%); <sup>1</sup>H NMR (CDCl<sub>3</sub>) 8.49 (d,  $J$  = 1.6, 1H), 8.28 (dd,  $J$  = 8.5, 1.9, 1H), 7.66 (m, 2H), 7.15 (d,  $J$  = 5.2, 1H). Anal. (C<sub>13</sub>H<sub>5</sub>Cl<sub>2</sub>F<sub>3</sub>N<sub>2</sub>O<sub>2</sub>S) C, H, N.

**3-(4-Chloro-2-methylphenyl)-5-(3-chlorothiophen-2-yl)-1,2,4-oxadiazole (4j)**: white solid (46%); <sup>1</sup>H NMR (CDCl<sub>3</sub>) 8.04 (d,  $J$  = 8.0, 1H), 7.61 (dd,  $J$  = 5.2, 1.4, 1H), 7.35–7.28 (m, 2H), 7.14 (dd,  $J$  = 5.4, 1.2, 1H), 2.64 (s, 3H). Anal. (C<sub>13</sub>H<sub>8</sub>Cl<sub>2</sub>N<sub>2</sub>O<sub>2</sub>S) C, H, N.

**5-(3-Chlorothiophen-2-yl)-3-(5-trifluoromethylpyridin-2-yl)-1,2,4-oxadiazole (4k)**: white solid (77%); <sup>1</sup>H NMR (CDCl<sub>3</sub>) 9.09 (m, 1H), 8.36 (d,  $J$  = 8.2, 1H), 8.14 (m, 1H), 7.66 (d,  $J$  = 5.2, 1H), 7.16 (d,  $J$  = 5.2, 1H). Anal. (C<sub>12</sub>H<sub>5</sub>ClF<sub>3</sub>N<sub>3</sub>O<sub>2</sub>S) C, H, N.

**5-(3-Chlorothiophen-2-yl)-3-(5-chloropyridin-2-yl)-1,2,4-oxadiazole (4l)**: white solid (85%); <sup>1</sup>H NMR (CDCl<sub>3</sub>) 8.78 (dd,  $J$  = 2.5, 0.8, 1H), 8.18 (dd,  $J$  = 8.4, 0.7, 1H), 7.86 (dd,  $J$  = 8.5, 2.5, 1H), 7.64 (d,  $J$  = 5.2, 1H), 7.14 (d,  $J$  = 5.2, 1H). Anal. (C<sub>11</sub>H<sub>5</sub>Cl<sub>2</sub>N<sub>3</sub>O<sub>2</sub>S) C, H, N.

**5-(3-Chlorothiophen-2-yl)-3-(6-trifluoromethylpyridin-3-yl)-1,2,4-oxadiazole (4m)**: white solid (80%); <sup>1</sup>H NMR (CDCl<sub>3</sub>) 9.49 (m, 1H), 8.63 (m, 1H), 7.85 (d,  $J$  = 8.2, 1H), 7.66 (d,  $J$  = 5.5, 1H), 7.17 (d,  $J$  = 5.2, 1H). Anal. (C<sub>12</sub>H<sub>5</sub>ClF<sub>3</sub>N<sub>3</sub>O<sub>2</sub>S) C, H, N.

**5-(3-Chlorothiophen-2-yl)-3-(6-chloropyridin-3-yl)-1,2,4-oxadiazole (4n)**: white solid (27%); <sup>1</sup>H NMR (CDCl<sub>3</sub>) 9.16 (d,  $J$  = 2.2, 1H), 8.39 (dd,  $J$  = 8.2, 2.2, 1H), 7.65 (d,  $J$  = 5.2, 1H), 7.49 (d,  $J$  = 8.5, 1H), 7.15 (d,  $J$  = 5.2, 1H). Anal. (C<sub>11</sub>H<sub>5</sub>Cl<sub>2</sub>N<sub>3</sub>O<sub>2</sub>S) C, H, N.

**5-(3-Bromothiophen-2-yl)-3-(4-chlorophenyl)-1,2,4-oxadiazole (4o)**: white solid (98%); <sup>1</sup>H NMR (CDCl<sub>3</sub>) 8.11 (d,  $J$  = 8.7, 2H), 7.60 (d,  $J$  = 5.4, 1H), 7.49 (d,  $J$  = 8.4, 2H), 7.20 (d,  $J$  = 5.1, 1H). Anal. (C<sub>12</sub>H<sub>6</sub>BrClN<sub>2</sub>O<sub>2</sub>S) H, N; C: calcd, 42.19; found: C, 42.95.

**3-(4-Methylphenyl)-5-(thiophen-2-yl)-1,2,4-oxadiazole (4s)**: white solid (53%); <sup>1</sup>H NMR (CDCl<sub>3</sub>) 8.04 (d,  $J$  = 8.4, 2H), 7.95 (d,  $J$  = 3.6, 1H), 7.66 (d,  $J$  = 5.1, 1H), 7.31 (d,  $J$  = 8.7, 2H), 7.22 (m, 1H), 2.43 (s, 3H). Anal. (C<sub>13</sub>H<sub>10</sub>N<sub>2</sub>O<sub>2</sub>S) C, H, N.

**5-(3-Chlorothiophen-2-yl)-3-(4-nitrophenyl)-1,2,4-oxadiazole (4v)**: white solid (80%); <sup>1</sup>H NMR (CDCl<sub>3</sub>) 8.37 (s, 4H), 7.65 (d,  $J$  = 5.4, 1H), 7.16 (d,  $J$  = 5.4, 1H). Anal. (C<sub>12</sub>H<sub>6</sub>ClN<sub>3</sub>O<sub>3</sub>S): H, N; C: calcd, 46.84; found: C, 45.16.

**3-Chlorofuran (8b)**. A solution of freshly distilled 3-bromofuran (**8a**) (19.5 g, 132 mmol) in anhydrous THF (40 mL) and anhydrous ether (70 mL) was cooled to  $-78^{\circ}\text{C}$  and stirred for 20 min. To the solution was added *n*-butyllithium in pentane (66 mL, 2.0 M) dropwise through an addition funnel. The reaction mixture was stirred for 0.5 h at  $-78^{\circ}\text{C}$  and then hexachloroethane (31.4 g, 133 mmol) in anhydrous THF (15 mL) was added dropwise to the stirring solution. The solution was stirred for an additional 1 h at  $-78^{\circ}\text{C}$ . The ice bath was

removed and then the solution was stirred at room temperature for 2 h, and precipitates were observed. The mixture was poured into water, and the organic phase was separated. The organic phase was washed with 2 N HCl and with water, dried over MgSO<sub>4</sub>, filtered, and distilled under vacuum (bp 35–40  $^{\circ}\text{C}$ ) to yield 6.09 g (44.7%) of **8b** as light yellow liquid: <sup>1</sup>H NMR (CDCl<sub>3</sub>) 7.36 (m, 1H), 7.30 (m, 1H), 6.33 (m, 1H).

**3-Bromofuran-2-carboxylic Acid (9a)**. To a solution of lithium diisopropylamide in tetrahydrofuran (26.2 mL, 1.4 M) cooled at  $-78^{\circ}\text{C}$  was added dropwise a solution of **8a** (5.0 g, 34 mmol) in tetrahydrofuran (26 mL). The solution was stirred for 30 min at  $-78^{\circ}\text{C}$  and then poured into a solution of crushed carbon dioxide in anhydrous ether (50 mL) and the mixture was stirred for 10 min. The mixture was poured into water (**Caution**: emission of a lot of gas could produce a violent reaction) and the aqueous phase was separated. The aqueous phase was acidified to pH 3 by 2 N HCl and extracted with ethyl acetate (3  $\times$  80 mL). The combined extracts were dried over MgSO<sub>4</sub>, filtered, and concentrated to give a solid. The solid was recrystallized from hexane and ethyl acetate to yield 4.21 g (65%) of **9a** as a yellowish powder: <sup>1</sup>H NMR (CDCl<sub>3</sub>) 7.58 (m, 1H), 6.66 (m, 1H).

**3-Chlorofuran-2-carboxylic Acid (9b)**. Compound **9b** was prepared from **8b** by a procedure similar to that described for the preparation of compound **9a**: white solid (51%); <sup>1</sup>H NMR (CDCl<sub>3</sub>) 7.57 (d,  $J$  = 2.1, 1H), 6.59 (d,  $J$  = 1.8, 1H).

**5-(3-Bromofuran-2-yl)-3-(4-chlorophenyl)-1,2,4-oxadiazole (10a)**. A solution of **9a** (74 mg, 1.0 mmol) and thionyl chloride (0.5 mL) in benzene (5 mL) was stirred at 60  $^{\circ}\text{C}$  for 4 h and then evaporated to dryness. The residue was dissolved in pyridine (10 mL) and to the solution was added 4-chloro-*N*-hydroxybenzamide (73 mg, 0.43 mmol). The solution was refluxed for 10 h and then cooled to room temperature. Water (20 mL) was added to the solution to produce a precipitate, which was collected by filtration, washed with water, and then dried to give 28 mg (22%) of **10a** as white solid: <sup>1</sup>H NMR (CDCl<sub>3</sub>) 8.11 (d,  $J$  = 9.0, 2H), 7.67 (d,  $J$  = 2.1, 1H), 7.42 (d,  $J$  = 9.0, 2H), 6.75 (d,  $J$  = 1.8, 1H). Anal. (C<sub>12</sub>H<sub>6</sub>BrClN<sub>2</sub>O<sub>2</sub>) C, H, N.

The following compounds were prepared from the corresponding 3-bromo- or 3-chlorofuran-2-carboxylic acid (**9a** or **9b**) and substituted *N*-hydroxybenzamide or *N*-hydroxypyridine-carboxamide by a procedure similar to that described for the preparation of compound **10a**.

**5-(3-Chlorofuran-2-yl)-3-(4-chlorophenyl)-1,2,4-oxadiazole (10b)**: white solid (45%); <sup>1</sup>H NMR (CDCl<sub>3</sub>) 8.12 (d,  $J$  = 8.4, 2H), 7.68 (d,  $J$  = 1.8, 1H), 7.49 (d,  $J$  = 8.4, 2H), 6.69 (d,  $J$  = 1.8, 1H). Anal. (C<sub>12</sub>H<sub>6</sub>Cl<sub>2</sub>N<sub>2</sub>O<sub>2</sub>) C, H, N.

**5-(3-Bromofuran-2-yl)-3-(4-trifluoromethylphenyl)-1,2,4-oxadiazole (10d)**: white solid (15%); <sup>1</sup>H NMR (CDCl<sub>3</sub>) 8.33 (d,  $J$  = 8.7, 2H), 7.79 (d,  $J$  = 8.7, 2H), 7.68 (d,  $J$  = 2.1, 1H), 6.76 (d,  $J$  = 2.1, 1H). Anal. (C<sub>13</sub>H<sub>6</sub>BrF<sub>3</sub>N<sub>2</sub>O<sub>2</sub>) C, H, N.

**5-(3-Chlorofuran-2-yl)-3-(4-trifluoromethylphenyl)-1,2,4-oxadiazole (10e)**: white powder (36%); <sup>1</sup>H NMR (DMSO-*d*<sub>6</sub>) 8.29 (m, 3H), 7.99 (d,  $J$  = 8.7, 2H), 7.16 (d,  $J$  = 1.8, 1H). Anal. (C<sub>13</sub>H<sub>6</sub>ClF<sub>3</sub>N<sub>2</sub>O<sub>2</sub>) C, H, N.

**5-(3-Bromofuran-2-yl)-3-(5-chloropyridin-2-yl)-1,2,4-oxadiazole (10f)**: white solid (54%); <sup>1</sup>H NMR (CDCl<sub>3</sub>) 8.81 (d,  $J$  = 2.5, 1H), 8.22 (d,  $J$  = 8.2, 1H), 7.90 (dd,  $J$  = 8.3, 2.5, 1H), 7.71 (d,  $J$  = 1.9, 1H), 6.79 (d,  $J$  = 1.6, 1H). Anal. (C<sub>11</sub>H<sub>5</sub>BrClN<sub>3</sub>O<sub>2</sub>) H; C: calcd, 40.46; found: C, 41.15; N: calcd, 12.87; found: N, 12.33.

**5-(3-Bromofuran-2-yl)-3-(5-trifluoromethylpyridin-2-yl)-1,2,4-oxadiazole (10g)**: white solid (85%); <sup>1</sup>H NMR (CDCl<sub>3</sub>) 9.09 (m, 1H), 8.37 (d,  $J$  = 8.2, 1H), 8.14 (dd,  $J$  = 8.1, 2.3, 1H), 7.70 (d,  $J$  = 1.9, 1H), 6.78 (d,  $J$  = 1.9, 1H). Anal. (C<sub>12</sub>H<sub>5</sub>BrF<sub>3</sub>N<sub>3</sub>O<sub>2</sub>) C, H, N.

**5-(3-Chlorofuran-2-yl)-3-(5-chloropyridin-2-yl)-1,2,4-oxadiazole (10h)**: white solid (21%); <sup>1</sup>H NMR (DMSO-*d*<sub>6</sub>) 8.87 (m, 1H), 8.28 (d,  $J$  = 2.1, 1H), 8.19 (d,  $J$  = 2.1, 1H), 8.18 (d,  $J$  = 0.9, 1H), 7.15 (d,  $J$  = 2.1, 1H). Anal. (C<sub>11</sub>H<sub>5</sub>Cl<sub>2</sub>N<sub>3</sub>O<sub>2</sub>) C, H, N.

**Caspase Activation Assay (EC<sub>50</sub>)**. The potency of compound **1d** and its analogues as inducer of apoptosis was



measured by our caspase-based cell assay as previously described.<sup>13</sup> Briefly, human breast cancer cell lines T47D, human colon cancer cell line DLD-1, and human non-small-cell lung cancers H1299 were treated with various concentrations of compound **1d** or its analogues and incubated at 37 °C for 24 or 48 h. The samples were then treated with the fluorogenic substrate *N*-(Ac-DEVD)-*N'*-ethoxycarbonyl-R110<sup>26</sup> and incubated for 3 h. The fluorescent signal was measured using a fluorescent plate reader (Model Spectrafour Plus Tecan). The EC<sub>50</sub> (μM) was determined by a sigmoidal dose–response calculation (XLFit3, IDBS), as the concentration of compound that produces 50% maximum response. Compound **1d** also was tested against human breast cancer cell ZR75-1, human colon cancer cell line HT29, human prostate cancer cell LnCap, and mouse leukemia cell P388, and the results are summarized in Table 1. The caspase activation activity (EC<sub>50</sub>) of the analogues tested in the three cancer cell lines T47D, DLD-1, and H1299 are summarized in Table 2.

**Cell Growth Inhibition Assays (GI<sub>50</sub>).** The potency of compound **1d** and its analogues as inhibitors of cell proliferation was measured as previously described.<sup>13</sup> Briefly, T47D, DLD-1, and H1299 cells were treated with various concentrations of **1d** or analogues. The samples were incubated at 37 °C for 48 h and then treated with CellTiter-Glo reagent (Promega). 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). GI<sub>50</sub> values were calculated from dose–response curves using XLFit3 (IDBS) software.

The GI<sub>50</sub> for compound **1d** and several analogues are summarized in Table 3.

**Detection of Caspase-7 and Caspase-3 in SKBr3 cells treated with compound 1d.** SKBr3 breast cancer cells were cultured at 5 × 10<sup>6</sup>/mL and treated with DMSO or 0.6 or 1.2 μM of compound **1d** and incubated for 48 h at 37 °C. Cells were lysed in RIPA buffer, subjected to SDS/PAGE, and then blotted onto PVDF membrane. Membranes were probed with anti-caspase-7 (Cal Biochem) or anti-caspase-3 antibodies (Santa Cruz Biotechnologies) and analyzed by chemiluminescence (Figure 1A–C). Figure 1 (parts B and C) shows the appearance of active caspase-7 and caspase-3 after SKBr3 cells were treated with compound **1d**.

**Measurement of Apoptosis by Flow Cytometry.** Cell cycle analysis was performed as previously described.<sup>12</sup> Briefly, T47D cells were treated with 1.5 μM of compound **1d** and incubated for 24 or 48 h at 37 °C. Control cells were treated with the solvent (DMSO). After the 24- or 48-h incubation, cells were treated with propidium iodide and RNase A and analyzed on a flow cytometer. All flow cytometry analyses were performed on 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 G<sub>1</sub> phase of the cell cycle. After treatment with 1.5 μM of **1d** for 24 h, more cells are accumulated in the G<sub>1</sub> phase (Figure 2B), with reduction of cells in the S phase, indicating that **1d** causes G<sub>1</sub> arrest. After treatment with 1.5 μM of **1d** for 48 h, most of the cells are still arrested in the G<sub>1</sub> phase, as well as an increase of cells with subdiploid DNA (Figure 2C). The subdiploid amount of DNA is indicative of apoptotic cells that have undergone DNA degradation or fragmentation.

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**Supporting Information Available:** Elemental analysis data for targeted compounds **1d**, **4a–4o**, **4s**, **4v**, **10a**, **10b**, **10d–10h**. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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