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Discovery of *N*-aryl-9-oxo-9*H*-fluorene-1-carboxamides as a new series of apoptosis inducers using a cell- and caspase-based high-throughput screening assay. 1. Structure–activity relationships of the carboxamide group

William Kemnitzer, Nilantha Sirisoma, Bao Nguyen, Songchun Jiang, Shailaja Kasibhatla, Candace Crogan-Grundy, Ben Tseng, John Drewe, Sui Xiong Cai *

Epicept Corporation, Inc. 6650 Nancy Ridge Drive, San Diego, CA 92121, USA

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ABSTRACT

N-(2-Methylphenyl)-9-oxo-9*H*-fluorene-1-carboxamide (**2a**) was identified as a novel apoptosis inducer through our caspase- and cell-based high-throughput screening assay. Compound **2a** was found to be active with sub-micromolar potencies for both caspase induction and growth inhibition in T47D human breast cancer, HCT116 human colon cancer, and SNU398 hepatocellular carcinoma cancer cells. It arrested HCT116 cells in G_2/M followed by apoptosis as assayed by the flow cytometry. Structure–activity relationship (SAR) studies of the carboxamide group identified the lead compound *N*-(2-(1*H*-pyrazol-1-yl)phenyl)-9-oxo-9*H*-fluorene-1-carboxamide (**6s**). Compound **6s**, with increased aqueous solubility, was found to retain the broad activity in the caspase activation assay and in the cell growth inhibition assay with sub-micromolar EC_{50} and GI_{50} values in T47D, HCT116, and SNU398 cells, respectively.

The ultimate goal of cancer therapy is to expose the body to anticancer agents that selectively target and kill cancer cells. Over the past decades it has been established that defects in the apoptosis machinery are intimately involved in the proliferation and metathesis of malignant tumor cells.[1,2] As a result, chemotherapeutic agents that promote or induce apoptosis could be potential therapies for the treatment of cancer.[3–5] Apoptosis, or programed cell death, involves a series of well-regulated events that lead to the removal of damaged or excessive cells.[6–9] During apoptosis, characteristic processes occur including condensation of the nucleoplasm and cytoplasm, chromosomal DNA fragmentation, and the formation of apoptotic bodies, which are rapidly recognized and removed by phagocytes. The mechanism of apoptosis involves a cascade of initiator and effector caspases. Cell death occurs when caspase-3 is activated leading to the cleavages of multiple different substrates in cells.[10] We have sought to exploit the role of apoptosis in tumor growth by discovering and developing apoptosis inducers. Toward this end we have developed a cell- and caspase-based Anti-cancer Screening Apoptosis Program (ASAP) HTS assay.

By applying this HTS assay, we have identified and reported the discovery and structure–activity relationship (SAR) of several series of apoptosis inducers. 4-Aryl-4*H*-chromenes, such as 2-amino-3-cyano-7-dimethylamino-4-(3-bromo-4,5-dimethoxyphenyl)-4*H*-chromene (**1a**), have been identified as a class of potent apoptosis inducers (Chart 1) binding to tubulin,[11] and several of them have demonstrated vascular disrupting activity (VDA) with good efficacy in several anticancer animal models.[12,13] Gambogic acid (**1b**) was discovered as a fast and potent apoptosis inducer,[14] and the transferrin receptor has been identified as the molecular target.[15] 3-Aryl-5-aryl-1,2,4-oxadiazoles, exemplified by 5-(3-chlorothiophen-2-yl)-3-(4-trifluoromethylphenyl)-1,2,4-oxadiazole (**1c**) were found to induce apoptosis selectively in certain tumor types,[16] and TIP47, an insulin-like growth factor II (IGF II) receptor binding protein, has been identified as the molecular target.[17] More recently, we reported the discovery of 1-benzoyl-3-cyanopyrrolo[1,2-*a*]quinolines (**1d**)[18] as potent apoptosis inducers, as well as of 4-aryl-3-(3-aryl-1-oxo-2-propenyl)-2(1*H*)-quinolinones (**1e**) that activated apoptosis in cancer cell lines with deregulated Myc.[19] We also reported the discovery of 2-chloro-*N*-(4-methoxyphenyl)-*N*-methylquinazolin-4-amine (**1f**)[20] and *N*-(4-methoxyphenyl)-*N*,2-dimethylquinazolin-4-amine (**1g**)[21] as potent apoptosis inducers with high BBB penetration and high efficacies in in vivo anticancer xenograft models. Herein, we report the discovery of *N*-(2-methylphenyl)-9-oxo-9*H*-fluorene-1-carboxamide (**2a**) as a potent apoptosis inducer and the SAR study of the carboxamide group.

The original hit **2a**, as well as **2c–2e**, **3b**, **5a**, **6j**, and **6p** was obtained from a commercial library, structures verified by ^{1}H NMR. Compounds **2b**, **2f**, **3a**, **4a–4b**, **5b**, **6a–6c**, **6e–6i**, **6k**, **6n–6o**, **6q–6w**, **7a–7b**, and **8a** (Tables 1 and 2) were prepared starting from

* Corresponding author. Tel.: +1 858 202 4006; fax: +1 858 202 4000.
E-mail address: scai@epicept.com (S.X. Cai).

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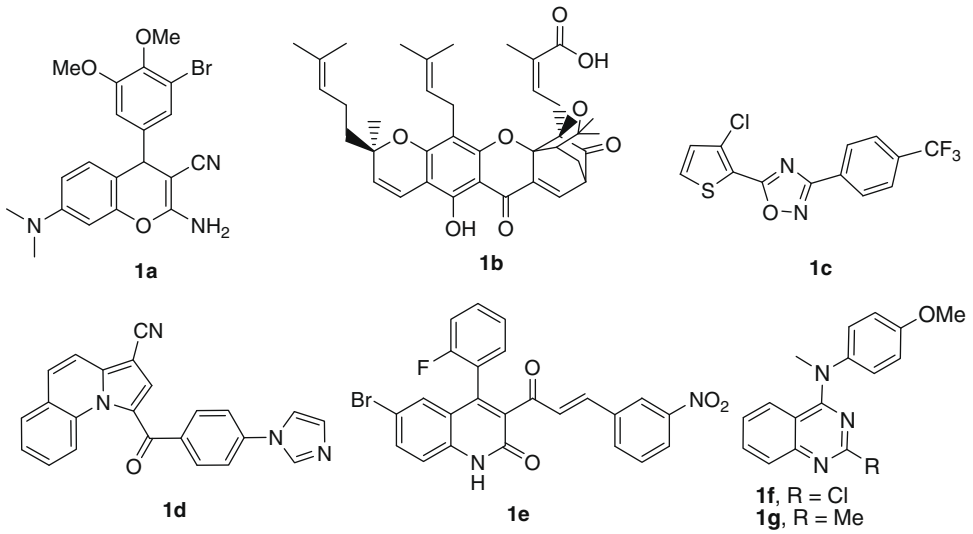


Chart 1.

the commercially available 9-oxo-9*H*-fluorene-1-carboxylic acid (**10**), which was converted to 9-oxo-9*H*-fluorene-1-carbonyl chloride (**11**) by reaction with oxalyl chloride and DMF. Subsequent reaction of **11** with the appropriate aniline, benzylamine, pyridylamine or 2-methylcyclohexylamine produced the target compounds (Scheme 1). The anilines and amines were either commercially available or prepared according to the literature procedures.[22,23] The reverse amide linkage analog **9** was prepared similarly from 1-amino-9*H*-fluoren-9-one and 2-methylbenzoyl chloride. The bromomethyl analog **6d** was prepared from bromination of **2a** using *N*-bromosuccinimide. Hydrogenation of the nitro analog **6j** produced the amino analog **6l**, which was converted to the azido analog **6m** via diazotization according to reported procedures (Scheme 2).[17] Methylation of **6s** using methyl iodide gave the *N*-methyl analog **8b** (Scheme 3).

The apoptosis-inducing activity of these *N*-aryl-9-oxo-9*H*-fluorene-1-carboxamides was measured by our proprietary cell- and caspase-based HTS assay as previously described[11] in T47D human breast cancer cells, HCT116 human colon cancer cells, and SNU398 hepatocellular carcinoma cancer cells, and the results are summarized in Tables 1 and 2. The original hit **2a** had EC_{50} values of 0.98, 1.1, and 0.64 μM for caspase activation in T47D, HCT116, and SNU398 cells, respectively. By maintaining the 9-oxo-9*H*-fluorene structure of **2a**, the SAR of the *N*-aryl ring at the carboxamide group was explored first (Table 1). Compound **2b**, without the methyl group at the 2-position of the phenyl group, was >4-fold less active than **2a** against T47D cells, suggesting that substitution at the 2-position might be important for apoptosis-inducing activity. The 3-methyl analog **2c** was inactive up to 10 μM, and the 4-methyl (**2d**) and 4-methoxy (**2e**) analogs were >4-fold less active than **2a**, confirming that substitution at the 2-position is important for activity. Interestingly, the 2,6-dimethyl analog **2f** was also inactive up to 10 μM in T47D cells. The 2-pyridyl (**3a**) and 3-pyridyl (**3b**) analogs, methyl-substituted 2-pyridyl (**4a**) and 3-pyridyl (**4b**) analogs, as well as thiazolyl (**5a**) and cyclohexyl (**5b)** analogs, were all inactive up to 10 μM in T47D cells. These data indicate that the phenyl ring is important for activity and its replacement by other heterocycles or a saturated ring is not tolerated.

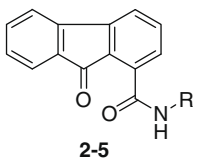
Based on the above-mentioned data, we decided to explore the SAR further by introducing other substituents at the 2-position of the phenyl ring. The 2-methoxy (**6a**) and 2-fluoromethoxy (**6b**) analogs were slightly more active than **2a**, indicating that small and electron-donating groups at the 2-position are preferred. The 2-ethoxy (**6c**) and 2-bromomethyl (**6d**) analogs however, were 3–5-fold less active than **2a**. Among the 2-halogen analogs, 2-chloro analog (**6f**) was the most potent one and was about twofold more potent than **2a**. Interestingly, the 2-cyano analog (**6h**) was inactive up to 10 μM, and the 2-methyl ester (**6i**) and 2-nitro (**6j**) analogs were both >2-fold less active than **2a**, suggesting that a strong electron-withdrawing group is not desirable. Both the 2-hydroxy (**6k**) and 2-amino (**6l**) analogs were inactive up to 10 μM, indicating that a hydrophilic group is not tolerated at the 2-position. The 2-azido analog (**6m**) had activity across all three cell lines in the 3–4 μM range, providing the opportunity to use the photoactivable azido group for target identification.[17]

In an effort to address the solubility of *N*-aryl-9-oxo-9*H*-fluorene-1-carboxamides we explored the synthesis of a *N,N*-dimethylamino analog (**6n**). Significantly, unlike the 2-amino analog **6l**, **6n** showed activity across all three cell lines similar to **2a**. Compound **6n** was found to have a solubility of 6.1 mg/mL in a solution of ethanol: Cremophor: D5W of 12.5:12.5:75, a formulation suitable for administration to mice. The more basic *N,N*-dimethylmethanamine analog (**6o**) was found to be <1.5-fold less potent than **2a** in T47D cells and about equipotent in the HCT116 and SNU398 cells, suggesting that there might be a pocket of reasonable size at the 2-position.

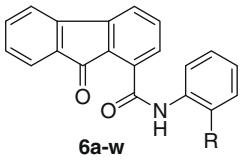
We then explored the introduction of a ring structure at the 2-position. The 2-phenyl analog (**6p**) was about twofold less potent than **2a** in T47D cells. Several compounds with five-membered rings at the 2-position were prepared. A pyrrolyl analog (**6q**) was >5-fold less active than **2a** in the three cell lines, while the imidazolyl analog (**6r**) and tetrazolyl analog (**6t**) were inactive up to 10 μM. Importantly, the pyrazolyl analog (**6s**) was found to maintain potency similar to **2a** and **6o**. Compound **6s** was also found to have solubility of 6.1 mg/mL in a solution of ethanol: Cremophor: D5W of 12.5:12.5:75. We further explored the introduction of non-aromatic heterocycles at the 2-position which might improve the solubility profile. The piperidyl analog (**6u**) was about twofold less active than **6s** in T47D cells. The morpholino analog (**6v**) was found to be >3-fold less potent than **6s** in T47D cells, while *N*-methyl-piperazine was inactive up to 10 μM in all three cell lines.

The SAR at the amide linker was also investigated. Both the benzyl analogs **7a** and **7b**, with the linker extended by a methylene group, were inactive up to 10 μM in all three cell lines. In comparison, the corresponding phenyl analogs **6a** and **6f** both were active

Table 1
Activity of *N*-aryl-9-oxo-9*H*-fluorene-1-carboxamides (**2**–**6**) in the caspase activation assay



2-5



6a-w

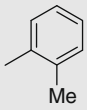
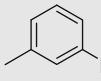
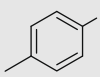
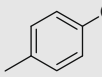
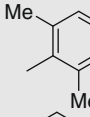
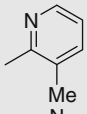
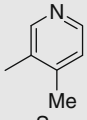
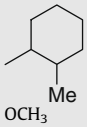
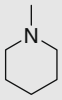
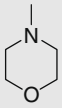
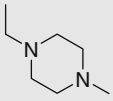
Compound	R	EC_{50} (μM)[a]		
		T47D	HCT116	SNU398
2a		0.98 ± 0.13	1.1 ± 0.2	0.64 ± 0.04
2b		4.4 ± 0.3	>10	>10
2c		>10	>10	>10
2d		4.6 ± 0.7	>10	>10
2e		3.7 ± 0.8	>10	>10
2f		>10	>10	>10
3a		>10	>10	>10
3b		>10	>10	>10
4a		>10	>10	> 10
4b		>10	>10	>10
5a		>10	>10	>10
5b		>10	>10	>10
6a	OCH_3	0.62 ± 0.07	7.5 ± 0.8	4.9 ± 0.5
6b	OCH_2F	0.48 ± 0.07	2.3 ± 0.2	1.5 ± 0.2
6c	OCH_2CH_3	2.9 ± 1.1	1.6 ± 0.5	0.96 ± 0.29
6d	CH_2Br	4.7 ± 0.3	10.7 ± 0.2	7.2 ± 1.1
6e	F	1.8 ± 0.1	>10	>10
6f	Cl	0.47 ± 0.03	0.56 ± 0.08	0.41 ± 0.04
6g	Br	2.3 ± 0.3	2.7 ± 0.4	2.2 ± 0.1
6h	CN	>10	>10	> 10
6i	CO_2Me	2.4 ± 0.3	0.52 ± 0.17	0.34 ± 0.08
6j	NO_2	2.5 ± 0.3	5.5 ± 1.0	2.6 ± 0.3
6k	OH	>10	>10	>10
6l	NH_2	> 10	>10	>10
6m	N_3	3.5 ± 0.3	3.6 ± 0.7	4.4 ± 1.0
6n	NMe_2	0.98 ± 0.11	1.2 ± 0.2	0.83 ± 0.06
6o		1.4 ± 0.1	0.94 ± 0.09	0.71 ± 0.03

Table 1 (*continued*)

Compound	R	EC_{50} (μM)[a]		
		T47D	HCT116	SNU398
6p		1.7 ± 0.3	1.8 ± 0.2	1.7 ± 0.3
6q		5.9 ± 1.1	6.3 ± 0.5	5.5 ± 1.1
6r		>10	>10	>10
6s		1.4 ± 0.3	1.3 ± 0.1	1.1 ± 0.1
6t		>10	>10	>10
6u		2.8 ± 0.02	2.6 ± 0.1	2.1 ± 0.2
6v		5.3 ± 0.1	5.2 ± 0.2	3.9 ± 0.7
6w		>10	>10	> 10

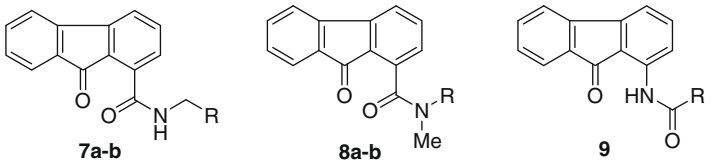
[a] Cells were treated with the test compounds for 48 h, data are the mean of three or more experiments and are reported as mean ± standard error of the mean (SEM).

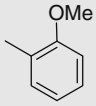
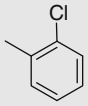
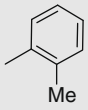
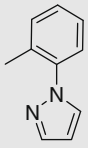
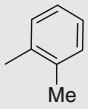
with EC_{50} values of less than 1 μM in T47D cells, indicating that the length of the linker is important for activity. Methylation of the nitrogen at the amide linker of **2a** and **6s** led to inactive analogs **8a** and **8b**, indicating that the NH group might play important function for activity. Interestingly, analog **9** was only about twofold less active in T47D cells than that of **2a**, indicating that reversing the amide bond is tolerated.

Selected compounds were also tested by the traditional cell growth inhibition assay (GI_{50}) to confirm that the active compounds can inhibit tumor cell growth. The growth inhibition assays in T47D, HCT116, and SNU398 cells were run in a 96-well microtiter plate as previously described.[11] The GI_{50} values are summarized in Table 3. Compound **2a** was found to have high activity in the T47D, HCT116, and SNU398 cells, with GI_{50} values of 0.18, 0.36, and 0.30 μM, respectively. In general, these compounds were more active in T47D cells than in HCT116 and SNU398 cells. Compound **6s** had GI_{50} value 0.16 μM in T47D cells and was slightly less active in HCT116 and SNU398 cells.

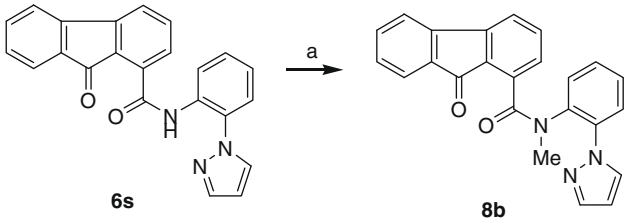
Characterization of compound **2a**. The apoptosis-inducing activity of compound **2a** was characterized by cell cycle analysis. HCT116 cells were treated with 2 μM of compound **2a** for 24 h or 48 h at 37 °C. Cells were then stained with propidium iodide and analyzed by flow cytometry. Figure 1A shows that control cells (treated with solvent DMSO) were mostly in the G_1 phase of the cell cycle. A large increase in G_2/M content (from 12% to 48%) was observed after 24 h of treatment (Fig. 1A vs 1B). After 48 h of treatment, sub-G_1 population increased to 41% with an equivalent decrease in G_2/M content, indicating transition of the cells to

Table 2
Activity of compounds (**7**–**9**) with modified linkers in the caspase activation assay



Compound	R	EC_{50} (μM)[a]		
		T47D	HCT116	SNU398
7a		>10	>10	>10
7b		>10	>10	>10
8a		>10	>10	>10
8b		>10	>10	>10
9		2.1 ± 0.2	2.2 ± 0.02	1.3 ± 0.02

[a] Cells were treated with the test compounds for 48 h, data are the mean of three or more experiments and are reported as mean ± standard error of the mean (SEM).



Scheme 3. Reagents and conditions: (a) (1) NaH, THF, **6s**, 0 °C, (2) MeI, 2 h.

Table 3
Inhibition of cell growth of *N*-aryl-9-oxo-9*H*-fluorene-1-carboxamides

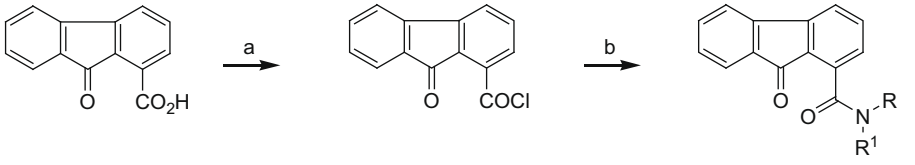
Compound	GI_{50} (μM)[a]		
	T47D	HCT116	SNU398
2a	0.18 ± 0.03	0.36 ± 0.09	0.30 ± 0.07
6c	0.45 ± 0.17	0.40 ± 0.13	0.27 ± 0.12
6f	0.23 ± 0.06	0.54 ± 0.10	0.53 ± 0.10
6i	0.18 ± 0.06	0.48 ± 0.09	0.31 ± 0.06
6p	0.21 ± 0.08	0.58 ± 0.14	0.35 ± 0.07
6s	0.16 ± 0.05	1.1 ± 0.2	0.78 ± 0.22

[a] Cells were treated with the test compounds for 48 h, data are the mean of three or more experiments and are reported as mean ± standard error of the mean (SEM).

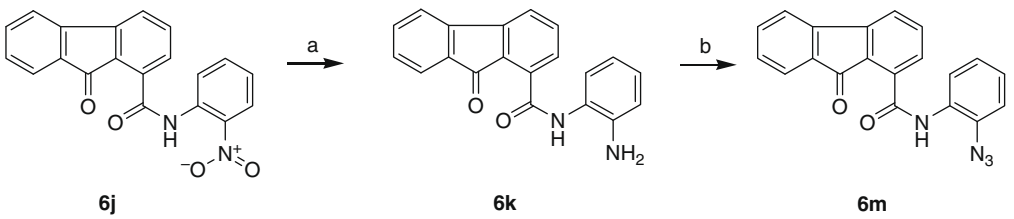
apoptosis (Fig. 1C). A similar profile was found for lead compound **6s** (data not shown).

Since cell cycle analysis shows that treatment with **2a** and **6s** 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 using previously reported procedure.[24] In brief, lyophilized tubulin (Cytoskeleton) was assayed for the effect of **2a** and **6s** on tubulin polymerization, measured as the concentration of compound to decrease the fluorescence of 4,6-diamidino-2-phenylindole (DAPI) by 50%. Polymerized tubulin (DMSO treated sample) gives a higher DAPI fluorescence than non-polymerized tubulin. Neither compound was found to inhibit tubulin polymerization at up to 50 μM, indicating that they induce apoptosis through some unknown mechanism. In comparison, the IC_{50} values for control compounds vinblastine and colchicine were 0.5 μM.

In conclusion, we have discovered and explored the SAR of the carboxamide group of apoptosis-inducing *N*-aryl-9-oxo-9*H*-fluorene-1-carboxamides. It was found that small groups at the 2-position of the phenyl group such as chloro or methoxy were preferred for apoptosis-inducing activity. Substitution at the 3- or 4-position of the phenyl group was not well tolerated. In addition, replacing the phenyl group with heterocycles such as 2- or 3-pyridyl groups led to inactive compounds. Significantly, substi-



Scheme 1. Reagents and conditions: (a) oxalyl chloride, CH_2Cl_2, DMF, 0 °C; (b) aniline, benzylamine, pyridylamine or 2-methylcyclohexylamine, CH_2Cl_2, Et_3N, 0 °C, then **11**, or aniline, THF, NaH, 0 °C, then **11**, or aniline/pyridine, 60 °C, 1 h.



Scheme 2. Reagents and conditions: (a) Pd/C, EtOH, EtOAc, H_2(g), 40 psi, rt; (b) (1) **6k**, 1 N HCl, MeOH,0 °C; (2) $NaNO_2$, H_2O, 0 °C; (3) NaN_3, H_2O, 0 °C.

Entry	Sub (%)	G_1 (%)	S (%)	G_2M (%)
1A	4	78	6	12
1B	8	32	10	48
1C	41	29	11	16

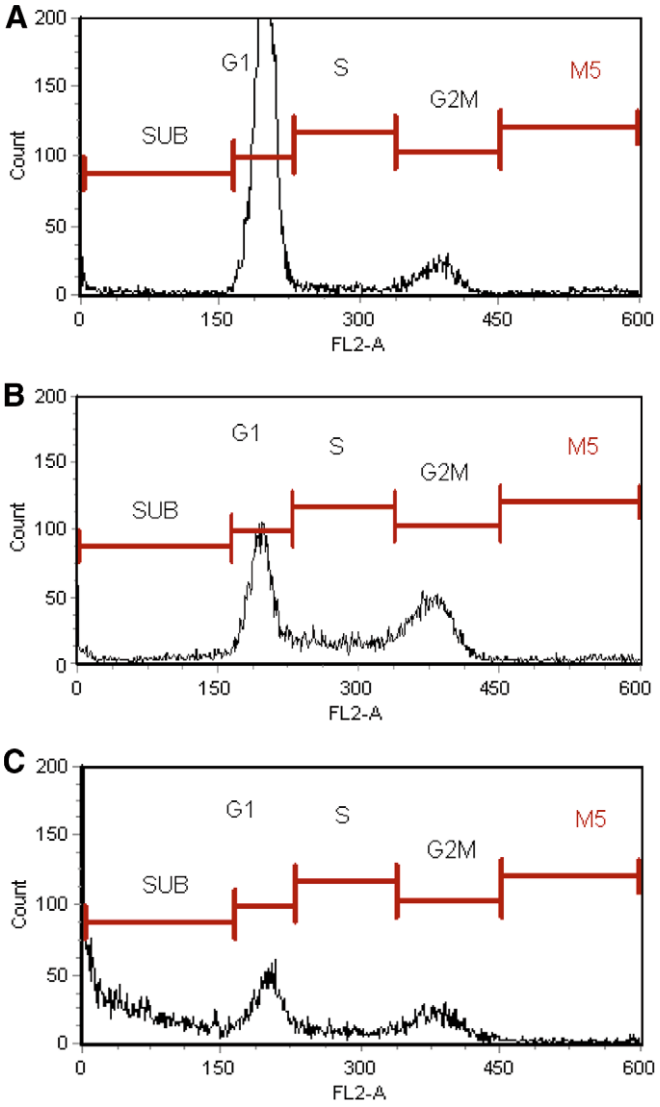


Figure 1. Drug-induced apoptosis in HCT116 cells as measured by flow cytometric analysis. The *x*-axis is the fluorescence intensity and the *y*-axis is the number of cells with that fluorescence intensity. (A) Control cells showing most of the cells in G_1 phase of the cell cycle. (B) Cells treated with 2 μM of compound **2a** for 24 h showing most of the cells arrested in G_2/M phase. (C) Cells treated with 2 μM of compound **2a** for 48 h progressing to subG_1.

tuting at the 2-position of the phenyl group with *N*,*N*-dimethylamino or *N*,*N*-dimethylmethanamine led to potent compounds with enhanced solubility properties. SAR studies also revealed that substitution at the 2-position with a phenyl or pyrazolyl group resulted in potent compounds. The amide linker was found to be important for activity. Through SAR studies, compound **6s** was identified, which had sub-micromolar potency in both the caspase activation assay and growth inhibition assay and was significantly more soluble than **2a**. Additional SAR studies via modifications of other positions of the *N*-aryl-9-oxo-9*H*-fluorene ring will be reported in future publications.

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