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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. 2. Structure–activity relationships of the 9-oxo-9*H*-fluorene ring

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ABSTRACT

As a continuation of our studies of apoptosis inducing 9-oxo-9*H*-fluorene-1-carboxamides as potential anticancer agents, we explored modification of the 9-oxo-9*H*-fluorene ring. SAR studies showed that most changes to the 9-oxo-9*H*-fluorene ring were not well tolerated, except the 9*H*-fluorene (**2b**) and dibenzothiophene (**2d**) analogs, which were about twofold less active than the 9-oxo-9*H*-fluorene analog **2a**. Significantly, introduction of substitutions at the 7-position of the 9-oxo-9*H*-fluorene ring led to compounds **5a**–**5c** with improved activity. Compound **5a** was found to have EC_{50} values of 0.15–0.29 μM against T47D, HCT116, and SNU398 cells, about fivefold more potent than the original lead **2a**. As opposed to the original lead compound **2a**, compounds **5a**–**5b** were active in a tubulin inhibition assay, indicating a change of mechanism of action. The potent azido analog **5c** could be utilized for target identification.

Apoptosis is a highly regulated process for the elimination of excessive or damaged cells and plays an important role in normal cell development and tissue homeostasis.[1] It is well known that inadequate apoptosis can lead to the over proliferation of cells resulting in cancer, as well as cause resistance to cancer treatment.[2] The mechanism of apoptosis involves a cascade of initiator and effector caspases that are activated sequentially. Among them, caspase-3 has been identified as a key effector caspase that cleaves multiple protein substrates in cells leading to cell death.[3]

Since it has been known that many clinically used anticancer chemotherapeutic agents kill cancer cells at least partially through induction of apoptosis,[4] discovery of compounds that interact with apoptosis regulators and promote or induce apoptosis could lead to the development of novel anticancer agents.[5,6] We therefore have developed a cell- and caspase-based high-throughput screening (HTS) technology called Anticancer Screening Apoptosis Program (ASAP) using our novel fluorescent caspase-3 substrates for the discovery of apoptosis inducers.[7] The screening assay can be applied to any cell type or cell line that expresses caspases. It can detect any apoptosis inducer that triggers apoptosis at a point upstream of caspase-3 activation by a caspase-mediated mechanism either through known targets or novel targets.

Applying this assay, we have discovered and reported the structure–activity relationship (SAR) studies of several novel series of apoptosis inducers, as well as the identification of novel molecular targets.[8] These include *N*-phenyl nicotinamides (**1a**),[9] gambogic acid (**1b**),[10] 4-aryl-4*H*-chromenes (**1c**),[11] 3-aryl-5-aryl-1,2,4-oxadiazoles (**1d**),[12] 4-anilino-2-arylpyrimidines (**1e**),[13] 4-anilinoquinazolines (**1f**),[14] *N*-phenyl-1*H*-pyrazolo[3,4-*b*]quinolin-4-amines (**1g**),[15] *N*-methyl-*N*-phenylnaphthalen-1-amines (**1h**),[16] and 1-benzoyl-3-cyanopyrrolo[1,2-*a*]quinolines (**1i**)[17] (Chart 1). More recently, we have reported the discovery and SAR study of *N*-aryl-9-oxo-9*H*-fluorene-1-carboxamides such as **2a** as a novel series of apoptosis inducers.[18] SAR studies of the 1-carboxamide group showed that substitution at the 2-position of the phenyl group are preferred for apoptosis-inducing activity. Substitution at the 3- or 4-positions of the phenyl group, or replacement of the phenyl group with heterocycles was not tolerated. Importantly, substitution at the 2-position of the phenyl group with an *N,N*-dimethylamino, *N,N*-dimethylmethanamine or pyrazolyl group led to potent compounds with enhanced aqueous solubility properties.[18] Herein, we wish to report modification of the 9-oxo-9*H*-fluorene ring of *N*-aryl-9-oxo-9*H*-fluorene-1-carboxamides as apoptosis inducers.

Compounds **2b**–**2d**, **2g**–**2i**, and **5a** (Tables 1 and 3) were prepared in two steps using previously reported procedures[18] starting from the corresponding carboxylic acids (**6a**–**6g**), which was converted to the carbonyl chlorides (**7a**–**7g**) by reaction with oxalyl

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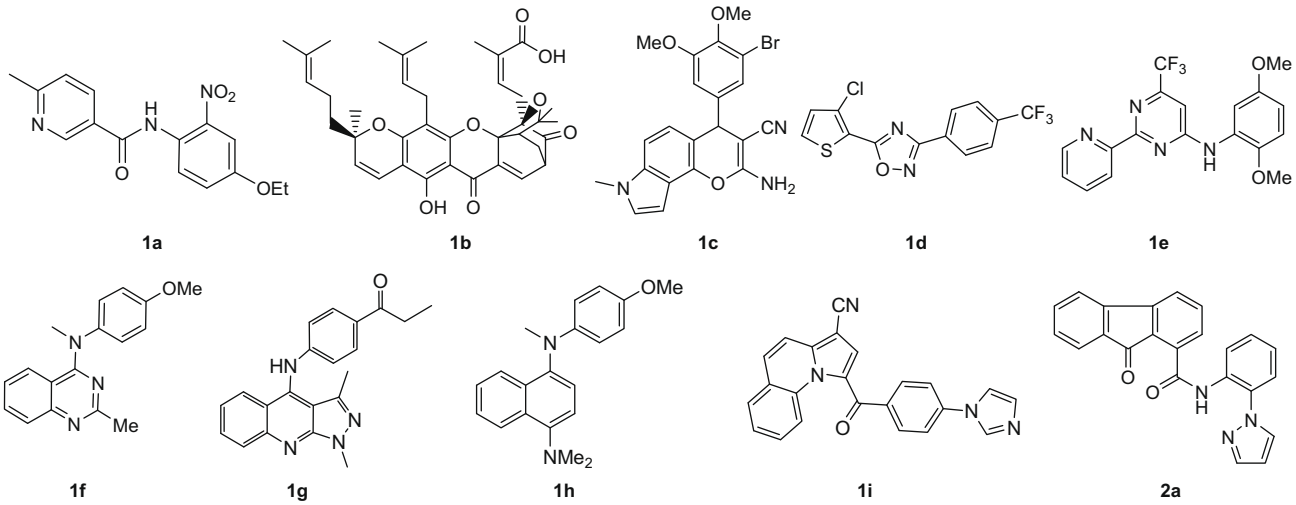


Chart 1.

chloride in DMF. Reaction of **7a**–**7g** with 2-(1*H*-pyrazol-1-yl)benzenamine produced the target compounds (Scheme 1). Among the carboxylic acids, 9*H*-fluorene-1-carboxylic acid (**6a**), 6,7,8,9-tetrahydro-5*H*-carbazole-1-carboxylic acid (**6d**), 2-benzoylbenzoic acid (**6e**), and 2,3-dihydrobenzofuran-7-carboxylic acid (**6f**) were obtained commercially. Dibenzofuran-1-carboxylic acid (**6b**) and dibenzothiophene-1-carboxylic acid (**6c**) were prepared from lithiations of dibenzofuran and dibenzothiophene as reported previously,[19] while 7-nitro-9-oxo-9*H*-fluorene-1-carboxylic acid (**6g**) was prepared from nitration of 9-oxo-9*H*-fluorene-1-carboxylic acid according to literature procedures.[20,21] Compounds **4a**–**4d** were synthesized similarly from reaction of **7a** with substituted anilines (Scheme 2).

Compounds **2e** and **2f** were prepared from oxidation of dibenzothiophene analog **2d** using mCPBA (Scheme 3). Compound **3b** was synthesized from reduction of **3a** using sodium borohydride (Scheme 4). 7-Amino analog **5b** was prepared from hydrogenation of 7-nitro analog **5a**. Diazotization of **5b** followed by treatment with sodium azide produced the azido analog **5c** (Scheme 5). The bromo analog **5d** was prepared from bromination of **5a** (Scheme 6).

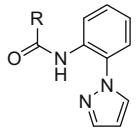
The apoptosis-inducing activity of these *N*-aryl-9-oxo-9H-fluorene-1-carboxamide analogs was measured by our cell- and caspase-based HTS assay[9] against three cell lines, T47D breast cancer cells, HCT116 human colon cancer cells and SNU398 hepatocellular carcinoma cancer cells, and the results are summarized in Tables 1–3. Since we have found previously that a pyrazolyl group at the 2-position of the phenyl group leads to potent compounds with enhanced aqueous solubility properties,[18] we decided to maintain this group and explore the replacement of the 9-oxo-9*H*-fluorene ring by other structures. 9*H*-Fluorene analog **2b** was about 2–3-folds less active than **2a**, suggesting that the 9-oxo group might contribute to activity. The dibenzofuran analog **2c** was not active up to 10 μM, supporting the notion that the 9-oxo group is important for activity. Interestingly, the dibenzothiophene analog **2d**, with the slightly larger thiophene ring compared to that of the furan in **2c**, was only about twofold less active than that of **2a** and at least >5-fold more active than **2c**. However, the corresponding oxo analog **2e** was not active up to 10 μM, while the dioxo analog **2f** was active only in SNU398 cells. The loss of activity for **2e** suggests that the oxo group might have to be in a planar position to maintain potency. The 6,7,8,9-tetrahydro-5*H*-carbazole analog **2g**, benzophenone analog **2h** and 2,3-dihydrobenzofuran analog **2i** were all inactive, indicating that the tricyclic and aromatic 9-oxo-9*H*-fluorene ring is important for activity. Compound **3b**, with a hydroxyl group at the 9-position, was >3-fold less active than the oxo analog **3a** against T47D cells, and was not active in the other two cell lines up to 10 μM, confirming the importance of the 9-oxo-9*H*-fluorene ring. Overall, except for the 9*H*-fluorene (**2b**) and dibenzothiophene (**2d**) analogs, most of the modifications to the 9-oxo-9*H*-fluorene ring led to inactive compounds.

Since the 9*H*-fluorene analog **2b** showed reasonable activity, we explored the synthesis of several analogs of **2b** with different substitutions in the phenyl group (Table 2). The 2-methyl analog **4a** was slightly more active than **2b** against T47D cells, but was not active in HCT116 and SNU398 cells up to 10 μM. Compounds **4b** and **4c**, with an additional methyl group at the 3- or 4-position, was not active up to 10 μM in all three cell lines, confirmed our previous observation that except for the 2-position, substitution at the other positions of the phenyl group is not preferred.[18] Interestingly, the 2,5-dimethyl analog **4d** was about as active as **2b** against T47D cells but was not active in HCT116 and SNU398 cells up to 10 μM.

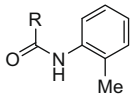
To explore SAR further and prepare a reagent for potential target identification, we synthesized compounds with substitution at the 7-position of the 9-oxo-9*H*-fluorene ring (Table 3). Interestingly, the 7-nitro analog **5a** was >5-fold more potent than **2a** in all three cell lines. Compound **5a** was about 10-fold less active than reference compounds vinblastine and paclitaxel and all were broadly active against the three cell lines tested. The 7-amino analog **5b** and 7-azido analog **5c** also were more active than **2a**. The azido analog **5c** was synthesized as a potential photoaffinity labeling agent. The bromo analog **5d**, prepared as an intermediate for the preparation of a tritium labeled photoaffinity labeling agent, also had good activity. Applying methods that we have successfully used to identify the molecular targets of the 3-aryl-5-aryl-1,2,4-oxadiazoles[12] and 4-anilinoquinazolines[14] series of apoptosis inducers, treatment of **5d** with tritium gas under hydrogenation conditions would result in the replacement of the bromo by a tritium, as well as reduction of the nitro group to an amino group. The amino group can then be converted to an azido group, which would give a radioactive version of **5c** that could be used for target identification.

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 according to previously described procedures[9] and the GI_{50} values are summarized in Table 4. The 7-nitro analog **5a** was

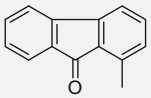
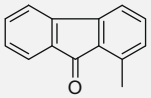
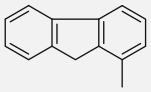
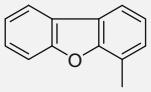
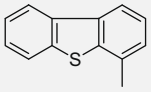
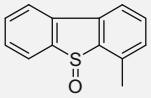
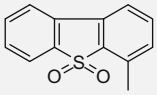
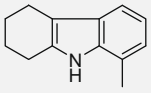
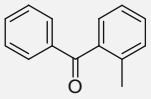
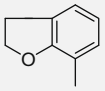
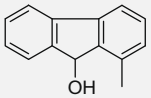
Table 1
Activity of analogs of *N*-aryl-9-oxo-9*H*-fluorene carboxamide with modified ring in the caspase activation assay



2

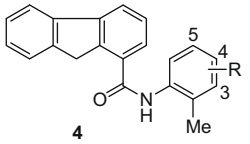


3

Compound	R	EC_{50}[a] (μM)		
		T47D	HCT116	SNU398
2a[b]		1.4 ± 0.3	1.3 ± 0.1	1.1 ± 0.1
3a[b]		0.98 ± 0.13	1.1 ± 0.2	0.64 ± 0.04
2b		3.2 ± 0.7	6.2 ± 1.3	6.9 ± 1.1
2c		>10	>10	>10
2d		2.1 ± 0.2	2.2 ± 0.2	1.5 ± 0.2
2e		>10	>10	>10
2f		>10	>10	0.48 ± 0.08
2g		>10	>10	>10
2h		>10	>10	>10
2i		>10	>10	>10
3b		3.0 ± 0.3	>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).
[b] Data from Ref. 18.

Table 2
Activity of *N*-aryl-9*H*-fluorene-1-carboxamides in the caspase activation assay

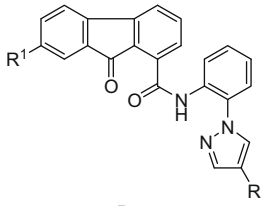


4

Compound	R	EC_{50}[a] (μM)		
		T47D	HCT116	SNU398
4a	H	1.7 ± 0.2	>10	>10
4b	3-Me	>10	>10	>10
4c	4-Me	>10	>10	>10
4d	5-Me	2.4 ± 0.2	>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).

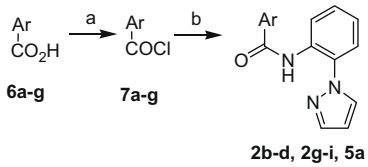
Table 3
Activity of 7-substituted *N*-aryl-9-oxo-9*H*-fluorene-1-carboxamides in the caspase activation assay



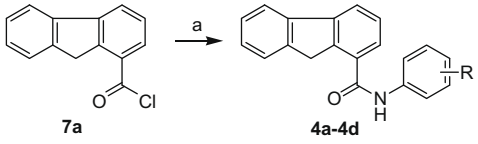
5

Compound	R	R^1	EC_{50}[a] (μM)		
			T47D	HCT116	SNU398
5a	H	NO_2	0.29 ± 0.03	0.22 ± 0.03	0.15 ± 0.02
5b	H	NH_2	0.97 ± 0.03	0.37 ± 0.15	0.15 ± 0.01
5c	H	N_3	0.47 ± 0.04	0.29 ± 0.03	0.15 ± 0.02
5d	Br	NO_2	1.3 ± 0.05	1.5 ± 0.03	1.4 ± 0.1
Vinblastine	NA[b]	NA	0.032 ± 0.006	0.036 ± 0.009	0.026 ± 0.008
Paclitaxel	NA	NA	0.037 ± 0.003	0.018 ± 0.001	0.009 ± 0.001

[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).
[b] NA, not applied.



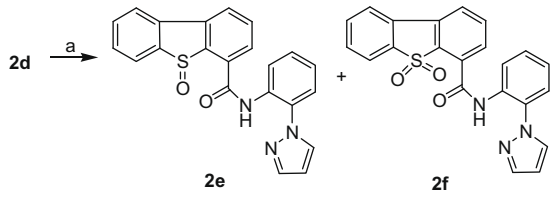
Scheme 1. Reagents and conditions: (a) oxalyl chloride, CH_2Cl_2, DMF, 0 °C; (b) 2-(1*H*-pyrazol-1-yl)benzenamine, CH_2Cl_2, Et_3N, 0 °C.



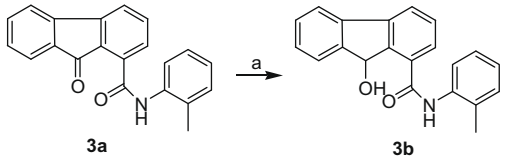
Scheme 2. Reagents and conditions: (a) aniline, CH_2Cl_2, Et_3N, 0 °C.

found to have good activity in T47D, HCT116, and SNU398 cells, with GI_{50} values of 0.11, 0.42, and 0.072 μM, respectively. Compound **5a** was more active than **2a**, especially in SNU398 cells. The 7-amino analog **5b** also had good activity and was about as active as that of **5a**. Compounds **5a** and **5b** were about 10-fold less active than the reference compounds vinblastine and paclitaxel in the three cell lines.

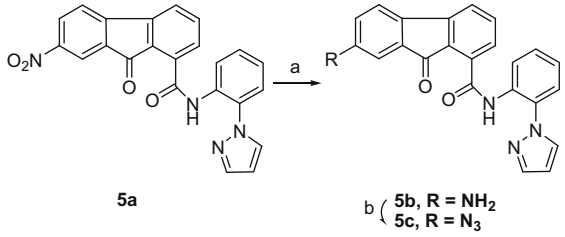
The apoptosis-inducing activity of compound **5b** was characterized by cell cycle analysis. T47D cells were treated with 2 μM of compound **5b** for 24 h or 48 h at 37 °C. Cells were then stained with propidium iodide and analyzed by flow cytometry. Figure 1A shows that control cells (treated with solvent DMSO) were mostly in the G_1 phase of the cell cycle. A large increase in G_2/M content (from 15% to 60%) was observed after 24 h of treatment (Fig. 1A vs 1B). After 48 h of treatment, sub-G_1 population increased to 50% with an equivalent decrease in G_2/M content, indicating transition of the cells to apoptosis (Fig. 1C).



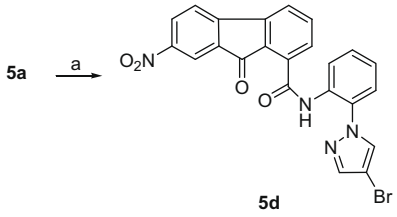
Scheme 3. Reagents and conditions: (a) mCPBA, CH_2Cl_2, 0 °C.



Scheme 4. Reagents: (a) $NaBH_4$, MeOH.



Scheme 5. Reagents and conditions: (a) Pd/C, EtOH, EtOAc, H_2(g), 40 psi, rt; (b) (1) 1 N HCl, MeOH, 0 °C; (2) $NaNO_2$, H_2O, 0 °C, then NaN_3.



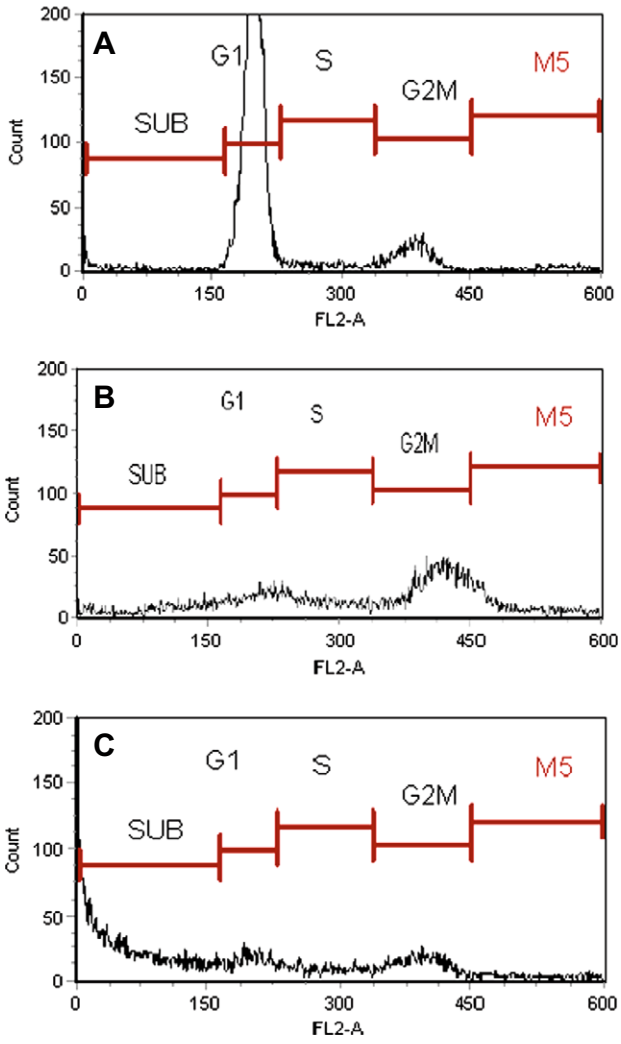
Scheme 6. Reagents: (a) Br_2, acetic acid.

Table 4
Inhibition of cell growth of *N*-aryl-9-oxo-9*H*-fluorene-1-carboxamides

Compound	GI_{50} (μM)[a]		
	T47D	HCT116	SNU398
2a[b]	0.16 ± 0.05	1.1 ± 0.2	0.78 ± 0.22
5a	0.11 ± 0.05	0.42 ± 0.19	0.072 ± 0.011
5b	0.15 ± 0.03	0.51 ± 0.04	0.084 ± 0.014
Vinblastine	0.007 ± 0.001	0.010 ± 0.003	0.005 ± 0.001
Paclitaxel	0.026 ± 0.003	0.060 ± 0.007	0.061 ± 0.005

[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).
[b] Data from Ref. 18.

Since cell cycle analysis showed that treatment with **5b** 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.[22] Compounds **5a** and **5b** inhibited 50% of tubulin polymerization at 2 and 3 μM, respectively, while vinblastine and colchicine, two known potent



Entry	Sub (%)	G_1 (%)	S (%)	G_2M (%)
1A	5	68	10	15
1B	12	11	14	60
1C	50	10	10	24

Figure 1. 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_1 phase of the cell cycle. (B) Cells treated with 2 μM of compound **5b** for 24 h showing a shift to G_2/M. (C) Cells treated with 2 μM of compound **5b** for 48 h showing a shift to sub-diploid DNA content indicative of apoptotic cells with fragmented nuclei.

tubulin inhibitors, inhibited polymerization fully at 0.5 μM, suggesting that part of the apoptotic effect of compounds **5a** and **5b** might be through inhibition of tubulin polymerization. This is different from the original hit **3a** and lead compound **2a**, which were not active in the tubulin assay up to 50 μM,[18] indicating a change of mechanism of action with substitution at the 7-position. Similar changes of mechanism of action due to simple structural variations have been reported for other series of apoptosis inducers[13,17] and DNA minor groove binders.[23]

In conclusion, we have explored modifications of the 9-oxo-9*H*-fluorene ring of the apoptosis inducing 9-oxo-9*H*-fluorene-1-carboxamides. SAR studies using the pyrazolyl group at the 2-position of the phenyl ring of the 1-carboxamide revealed that most changes to the 9-oxo-9*H*-fluorene ring were not well tolerated.

The 9*H*-fluorene (**2b**) and dibenzothiophene (**2d**) analogs were about twofold less active than the 9-oxo-9*H*-fluorene analog **2a**. Significantly, SAR studies of the 7-position of the 9-oxo-9*H*-fluorene ring led to compounds **5a**–**5c**, which were more potent than **2a**. Compounds **5a** and **5b** were found to be active in the tubulin inhibition assay, suggesting a change of mechanism of action from that of the original lead **2a**. Compound **5b** in the future will be evaluated for antitumor efficacy. Utilizing a radio labeled version of the azido compound **5c**, the proposed tubulin target will be validated and secondary proapoptotic targets will be determined.

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