Discovery of 4-Aryl-4*H*-chromenes as a New Series of Apoptosis Inducers Using a Cell- and Caspase-based High-Throughput Screening Assay. 1. Structure−Activity Relationships of the 4-Aryl Group

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By applying a novel cell- and caspase-based HTS assay, 2-amino-3-cyano-7-(dimethylamino)-4-(3-methoxy-4,5-methylenedioxyphenyl)-4*H*-chromene (**1a**) has been identified as a potent apoptosis inducer. Compound **1a** was found to induce nuclear fragmentation and PARP cleavage, as well as to arrest cells at the G_2/M stage and to induce apoptosis as determined by the flow cytometry analysis assay in multiple human cell lines (e.g. Jurkat, T47D). Through structure−activity relationship (SAR) studies of the 4-aryl group, a 4- and 7-fold increase in potency was obtained from the screening hit **1a** to the lead compounds 2-amino-4-(3-bromo-4,5-dimethoxyphenyl)-3-cyano-7-(dimethylamino)-4*H*-chromene (**1c**) and 2-amino-3-cyano-7-(dimethylamino)-4-(5-methyl-3-pyridyl)-4*H*-chromene (**4e**), with an EC_{50} of 19 and 11 nM in the caspase activation assay in T47D breast cancer cells, respectively. The 2-amino-4-aryl-3-cyano-7-(dimethylamino)-4*H*-chromenes also were found to be highly active in the growth inhibition MTT assay, with GI_{50} values in the low nanomolar range for compound **1c**. Significantly, compound **1c** was found to have a GI_{50} value of 2 nM in the paclitaxel resistant, *p*-glycoprotein overexpressed, MES-SA/DX5 tumor cells. Functionally, compound **1c** was found to be a potent inhibitor of tubulin polymerization and to effectively inhibit the binding of colchicine to tubulin. These results confirm that the cell-based caspase activation assay is a powerful tool for the discovery of potent apoptosis inducers and suggest that the 4-aryl-4*H*-chromenes have the potential to be developed into future anticancer agents.

Introduction

Apoptosis, or programmed cell death, is the normal process of cellular suicide that proceeds with characteristic biochemical and cytological features, including nuclear condensation and fragmentation. Apoptosis enables organisms to control their cell numbers and to eliminate unneeded cells that may threaten their survival.[1] The correct balance between apoptosis and inhibition of apoptosis is important in preserving tissue homeostasis and organ morphogenesis.[2] However, aberrations of this process underlie some pathological conditions, with abnormal inhibition of apoptosis as one of the hallmarks of cancer,[3] while excessive apoptosis is implicated in neurodegenerative disorders such as Alzheimer's disease.[4] Apoptosis can be initiated by many agents, including TNF-α and FAS ligand, as well as by chemotherapy and radiation.[5] The mechanism of apoptosis involves a cascade of initiator and effector caspases that are activated sequentially. Caspases are a family of cysteine proteases that require aspartic acid residues at the P_1 position of substrates for cleavage.[6] Within the caspase family, caspase-3, -6, and -7 have been identified as key effector caspases that cleave multiple protein substrates in cells, leading irreversibly to cell death.[7]

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Since many cancer cells exhibit abnormal inhibition of apoptosis,[3] we are interested in the discovery and development of inducers of apoptosis as potential anticancer agents. Toward this goal, we have developed a cell-based high-throughput screening (HTS) system for inducers of apoptosis[8] using our novel fluorescent caspase substrates.[9,10] We have reported recently the discovery and SAR studies of substituted *N*-phenylnicotinamides, exemplified by 6-methyl-*N*-(4-ethoxy-2-nitrophenyl)pyridine-3-carboxamide (A),[11] gambogic acid (B),[12] and substituted indole-2-carboxylic acid benzylidenehydrazides, exemplified by 5-methyl-3-phenylindole-2-carboxylic acid (4-methylbenzylidene)hydrazide (C),[13] as potent apoptosis inducers (Chart 1). Herein we report the discovery and characterization of 2-amino-3-cyano-7-(dimethylamino)-4-(3-methoxy-4,5-methylenedioxyphenyl)-4 *H*-chromene (**1a**) as a potent inducer of apoptosis using our cell- and caspase-based apoptosis HTS assay, as well as optimization of the screening hit **1a** via SAR studies of the 4-aryl group.

Results and Discussion

Chemistry. 2-Amino-3-cyano-7-(dimethylamino)-4-(3-methoxy-4,5-methylenedioxyphenyl)-4*H*-chromene **1a** was obtained originally from a commercial compound library. It was resynthesized by a one-pot reaction of 5-methoxypiperonal, 3-dimethylaminophenol, and malononitrile in good yield as a brown solid. Compounds

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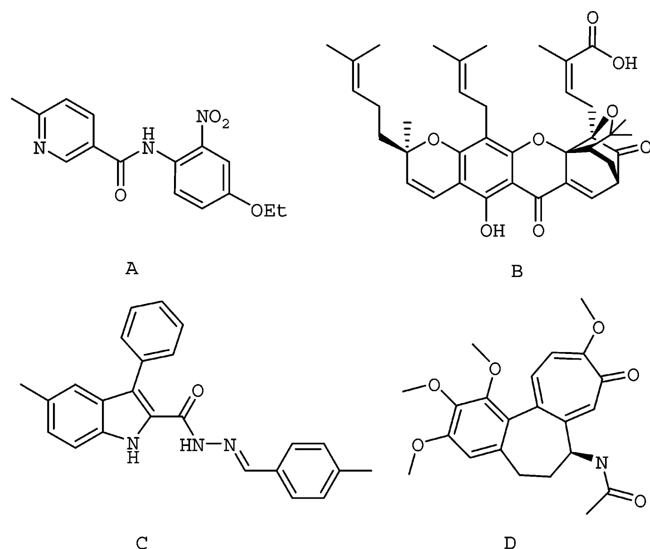
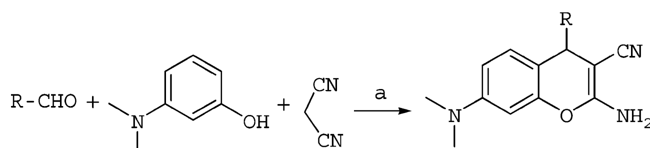
Chart 1

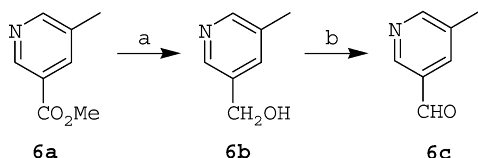


Scheme 1[a]



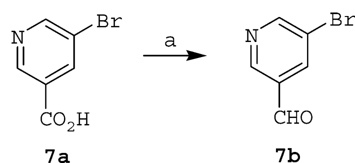
[a] Conditions: (a) EtOH, piperidine, rt.

Scheme 2[a]



[a] Conditions: (a) LAH, THF, −78 °C, 1 h, then rt, 20 h; (b) MnO_2, CH_2Cl_2, reflux, 18 h.

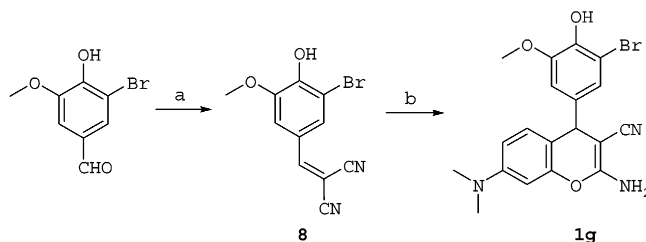
Scheme 3[a]



[a] Conditions: (a) (1) oxalyl chloride MnO_2, CH_2Cl_2, 0 °C, DMF; (2) CH_3CN, THF, −78 °C, **7a**, pyridine; (3) warm to −40 °C then cooled to −78 °C; (4) $LiAl(O^tBu)_3H$, CuI.

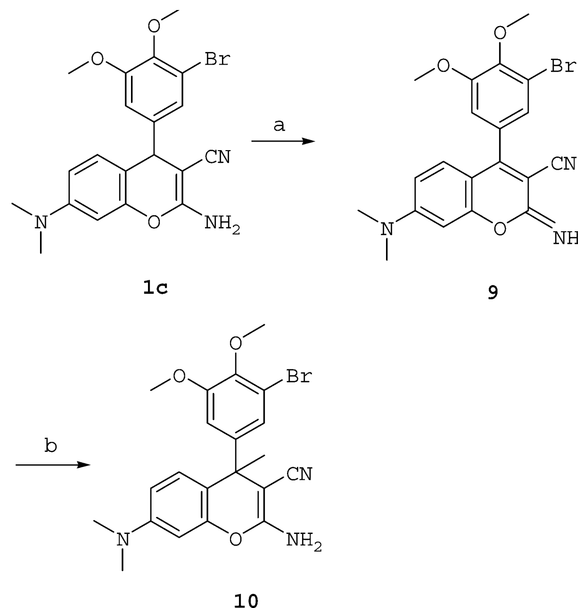
1b−**f**, **1h**−**j**, **2a**−**e**, **3a**−**n**, **4a**−**c**, **4f**, and **5a**−**c** were prepared in a manner similar to **1a** from the corresponding commercially available aldehydes by reaction with 3-(dimethylamino)phenol and malononitrile (Scheme 1). For compounds **4d** and **4e**, the corresponding aldehydes (**6** and **7**) were synthesized starting from methyl 5-methyl-3-pyridinecarboxylate (Scheme 2) and 5-bromonicotinic acid (Scheme 3). Compound **1g** was prepared in two steps by the initial formation of 2-(3-bromo-4-hydroxy-5-methoxybenzylidene)malononitrile (**8**) from reaction of 3-bromo-4-hydroxy-5-methoxybenzaldehyde and malononitrile (Scheme 4), followed by reaction of **8** with 3-(dimethylamino)phenol. Compound **10** was synthesized in two steps starting from **1c** (Scheme 5). First, **1c** was oxidized to 4-(3-bromo-4,5-dimethoxyphenyl)-3-cyano-7-(dimethylamino)-2-imino-2*H*-chromene (**9**) by reaction with 2,3-dichloro-5,6-dicyano-1,4-benzoquinone. Compound **9** was then methylated using copper(I) bromide−dimethyl sulfide and methyllithium to afford compound **10**.

Scheme 4[a]



[a] Conditions: (a) malononitrile, EtOH, piperdine, rt, 12 h; (b) 3-(dimethylamino)phenol, EtOH, piperdine, reflux, 12 h.

Scheme 5[a]



[a] Conditions: (a) molecular sieves, CH_2Cl_2, 2,3-dichloro-5,6-dicyano-1,4-benzoquinone, rt, 1 h; (b) (1) $Cu(I)Br-SMe_2$, THF, −78 °C, MeLi, 1 h; (2) **9**, THF, −78 °C, 30 min.

HTS Assays. 2-Amino-3-cyano-7-(dimethylamino)-4-(3-methoxy-4,5-methylenedioxyphenyl)-4*H*-chromene **1a** was identified as an inducer of apoptosis from a commercial compound library using our cell-based apoptosis induction HTS assay in HL60 B-cell promyelotic leukemia cancer cells as described.[8] Briefly, human HL60 cells, in a 96-well microtiter plate containing 10 μM of test compound, were incubated for 3 h for the induction of apoptosis. Caspase-3 fluorogenic substrate *N*-(Ac-DEVD)-*N′*-ethoxycarbonyl-R110[10] was then added to cells, and the samples were mixed by agitation and incubated at room temperature for 3 h. Using a fluorescent plate reader, employing excitation at 485 nm and emission at 525 nm, the fluorescence was measured and the amount of caspase activation was determined. Compounds that induce apoptosis and activate the caspases yield a fluorescent signal higher than the background (signal/background ratio). Compounds found to give a ratio of >3 are considered active and retested in triplicate for confirmation in both HL60 and T47D, a solid human breast cancer tumor cell line. Compounds confirmed to be active in both cell lines are then tested at several concentrations to provide a dose response for

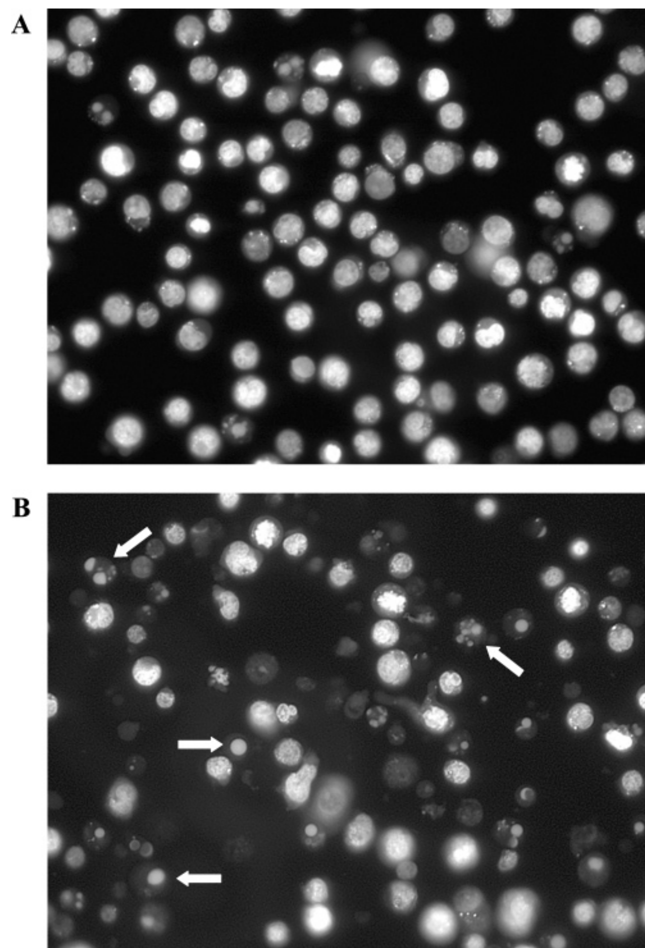


Figure 1. Fluorescent micrographs of Jurkat cells treated with drug and stained with a fluorescent DNA probe, Syto 16. Figure 1A depicts control cells. Figure 1B depicts cells treated with 0.1 μM of **1a** for 24 h, showing shrunken and fragmented nuclei.

the calculation of the caspase activation activity (EC_{50}). Compound **1a** was found to induce apoptosis and activate caspase in T47D cells with an EC_{50} value of 0.073 μM and a ratio of around 6 over untreated cells, similar to that of substituted *N*-phenylnicotinamides[11] and substituted indole-2-carboxylic acid benzylidene-hydrazides.[13]

Characterization of Compound 1a. The ability of compound **1a** to induce apoptosis was confirmed in a nuclear fragmentation assay, which is one of the hallmarks of caspase-mediated apoptosis. Jurkat cells were treated with 0.1 μM of compound **1a** for 24 h followed by staining with Syto 16 (Molecular Probes Inc, Eugene, OR), a DNA stain that allows the visualization of nuclear morphology. The compound-treated cells were characterized by shrunken and fragmented nuclei with condensed chromatin (Figure 1B). In contrast, the nuclei of Jurkat cells treated with vehicle control (DMSO) appeared to be normal with dispersed chromatin moderately stained with Syto 16 (Figure 1A).

The apoptosis-inducing activity of compound **1a** was also characterized by poly(ADP)ribose polymerase (PARP) cleavage in Jurkat cells. PARP, an enzyme important in genome surveillance, is one of the natural substrates that is cleaved by caspases during apoptosis. The ability of **1a** to induce PARP cleavage was tested by treating

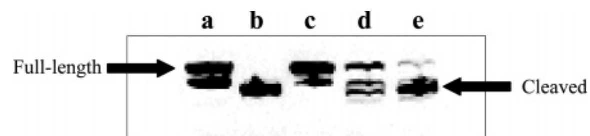


Figure 2. Western blots of the time-course of drug-induced poly(ADP)ribose polymerase (PARP) cleavage in Jurkat cells: (a) DMSO-treated control cells; (b) cells treated with 1 μM of staurosporine for 30 h; (c) cells treated with 2.5 μM of **1a** for 5 h; (d) cells treated with 2.5 μM of **1a** for 15 h; (e) cells treated with 2.5 μM of **1a** for 30 h.

Jurkat cells with the compound at a concentration of 2.5 μM for 5, 15, and 30 h. The cells were then lysed and the proteins transferred to PVDF membrane. The membranes were probed with a polyclonal antibody to PARP, followed by goat–anti-rabbit HRP second antibody. Figure 2c shows that PARP cleavage can be observed after cells were treated with **1a** for 5 h. At 30 h, almost all the PARP have been cleaved (Figure 2e), which is similar to what is observed with cells treated with 1 μM of staurosporine (Figure 2b), a potent apoptosis inducer for the same period of time.

The apoptosis-inducing activity of compound **1a** was also characterized by cell cycle analysis. T47D cells were treated with 0.1 μM of compound **1a** for 24 or 48 h, stained with propidium iodide, and analyzed by flow cytometry. An increase in the G_2/M DNA content in cells treated with compound **1a** was observed, as shown in Figure 3B,C. The subdiploid DNA content (apoptotic sub-G_1 area) of cells increased from 0.9% in the control cells (Figure 3A) to 12% upon compound treatment for 48 h (Figure 3C), indicating the presence of apoptotic cells that have undergone DNA degradation and nuclear fragmentation. A similar level of apoptotic cells (10%) is seen when T47D cells were treated with 0.1 μM of paclitaxel.[12] These results showed that treatment of T47D cells by compound **1a** for 48 h results in cell cycle arrest in the G_2/M phase, as well as induction of apoptosis.

Structure–Activity Relationship (SAR) Studies. The cell-based caspase HTS assay was used to test analogues of compound **1a** for SAR studies. A panel of three different human cancer cell lines was used for these experiments. Briefly, human breast cancer cell line T47D, human nonsmall cell lung cancer cell line H1299, and human colorectal cancer cell line DLD-1 were plated in 384-well microtiter plates containing various concentration of test compound and incubated at 37 °C for 24 h. After incubation, the samples were treated with the *N*-(Ac-DEVD)-*N*-ethoxycarbonyl-R110 fluorogenic substrate. The caspase activation activity (EC_{50}) of compound **1a** and its analogues in the three cancer cell lines is summarized in Table 1.

Table 1 shows that compound **1a** has an EC_{50} for caspase activation of 0.073, 0.093, and 0.083 μM in T47D, H1299, and DLD-1 cells, respectively. By maintaining the 2-amino, 3-cyano, and 7-dimethylamino groups of **1a**, the SAR at the 4-position was explored, beginning with trisubstituted analogues. Replacing the 4,5-methylenedioxy ring by 4,5-dimethoxy groups gave the trimethoxy-substituted analogue **1b**, which was slightly more potent than **1a** in the T47D cells, suggesting that the ring structure in the 4,5-position could be replaced by two substituents. Replacement of the

Entry	Sub	G1	S	G_2M
1A	0.9	67.8	13.6	17.3
1B	4.3	43.5	7.6	44.0
1C	12.0	11.0	18.8	57.0

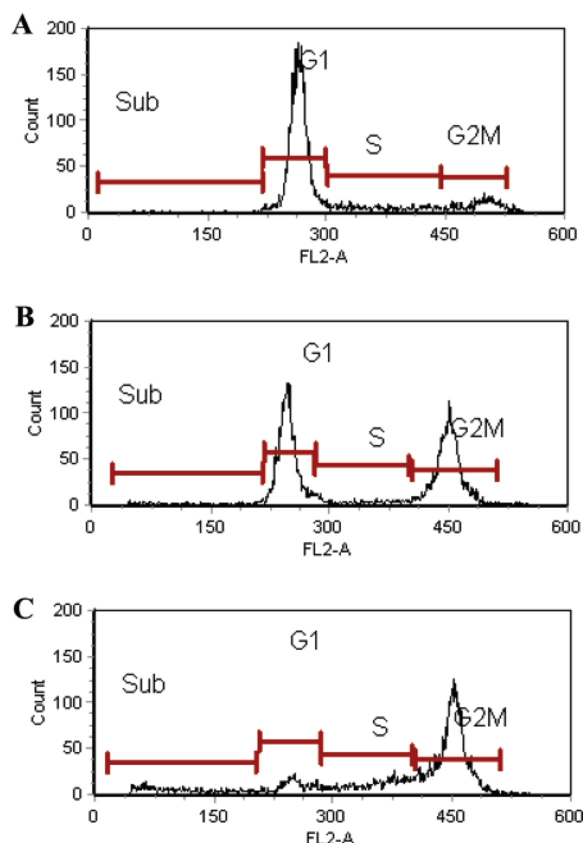


Figure 3. Drug-induced apoptosis in T47D cells as measured by flow cytometric analysis. The *x*-axis is the fluorescence intensity and the *y*-axis is the number of cells with that fluorescence intensity (A) Control cells showing most of the cells in G1 phase of the cell cycle. (B) Cells treated with 0.1 μM of compound **1a** for 24 h showing most of the cells arrested in G2/M phase. (C) Cells treated with 0.1 μM of compound **1a** for 48 h showing a progression from G2/M to cells with subdiploid DNA content, which are apoptotic cells with fragmented nuclei.

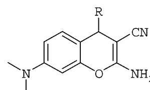
methoxy group at the 3-position of **1b** with a bromo group (**1c**) resulted in 2-fold increase in potency, suggesting that an electron-withdrawing group might be preferred in the 3-position of these 3,4,5-trisubstituted compounds. This was confirmed by the preparation of the corresponding chloro (**1d**) and iodo (**1e**) analogues, which are as active as the bromo analogue **1c**. The 4,5-methylenedioxy analogue **1f** was 6-fold less active than **1c**, suggesting that 4,5-dimethoxy groups are preferred over the 4,5-methylenedioxy ring structure with an electron withdrawing group in the 3-position. Replacing the 4-methoxy group in **1c** by a 4-hydroxy group (**1g**) resulted in a 2-fold reduction of potency, suggesting that the hydrophobic methoxy group is preferred over the hydrophilic hydroxy group in the 4-position. Compounds **1h** and **1i**, with a methoxy group at the 2-position, are >40-fold less active than the 3,4,5-trimethoxy analogue **1b**, suggesting that there might be a space-limited pocket around the 2-position, or due to steric effect, the 2-methoxy group forces the phenyl ring into an unfavorable position. Compound **1j** was >130-fold less active than **1c**, confirming that substitution in the 2-position is not preferred.

The 3,5-dimethoxy analogue **2a** is slightly more potent than the 3,4,5-trimethoxy analogue **1b**, indicating that the 4-methoxy group is not crucial for activity. In comparison, the 3-bromo-5-methoxy analogue **2b** is slightly less potent than **1c**, indicating that, when there is a bromo group in the 3-position, the 4-methoxy group does contribute to the potency. Compound **2b** also was slightly less potent than **2a**, suggesting that for 3,5-disubstituted analogues, an electron-donating group is preferred over an electron-withdrawing group. This was supported by the preparation of the 3,5-dichloro analogue **2c**, which was >3-fold less active than **2a**. The 3,4-dimethoxy analogue **2d** was >10-fold less potent than **2a**, further supporting the notion that substitution in the 3,5-position is important for activity and substitution in the 4-position contributes little to potency. Similar to what has been observed in the trisubstituted analogues, the 2,3-dimethoxy analogue **2e** was >44-fold less active than **2a**, further confirming that substitution in the 2-position is not preferred.

Monosubstituted analogues further highlight the importance of substitution in the 3- and/or 5-positions of the phenyl ring for activity. Monosubstitution at the 3-position of the phenyl ring with bromo (**3a**), methoxy (**3b**), and cyano (**3c**) groups resulted in potent compounds, which are only about 2–3-fold less potent than the 3,5-dimethoxy analogue **2a** and 3,4,5-trisubstituted analogue **1c**. The 3-fluoro (**3d**), 3-chloro (**3e**), and 3-methyl (**3f**) analogues also were found to be potent compounds with potencies around 100 nM. The 3-hydroxy analogue **3g** was >4-fold less potent than the 3-methoxy analogue **3b**, suggesting that a hydrophobic group is preferred in the 3-position. The 3-carboxylic acid analogue **3h** was >130-fold less potent than **3b**, indicating that a charged group is not tolerated in the 3-position. This dramatic reduction of activity of the carboxylic acid analogue could be due to a combination of low cell permeability and low affinity to its target. Replacing the methoxy group in the 3-position with a trifluoromethoxy group (**3i**) resulted in reduction of potency by >6-fold compared to **3b**, suggesting that there might be a size-limited pocket in the 3-position. The 3-benzyloxy (**3j**) and 3-dodecyloxy (**3k**) analogues are >40- and >130-fold less potent than the 3-methoxy analogue, further supporting the notion that there is a size-limited pocket in the 3-position. The nonsubstituted phenyl analogue (**3l**) was found to have a potency of 118 nM, which is only about 2-fold less active than the 3-methoxy analogue **3b**. The 4-methoxy (**3m**) and 2-methoxy (**3n**) analogues are >24- and > 4-fold less potent than the 3-methoxy analogue **3b**, and both are less active than the nonsubstituted phenyl analogue **3l**, confirming that substitution in the 3-position is preferred and substitution in the 2- and 4-positions does not contribute positively to the activity.

On the basis of the above SAR, we sought to replace the phenyl group by a pyridyl group, which might improve the aqueous solubility of the compound. The 3-pyridyl analogue **4a** was almost 2-fold more potent than the phenyl analogue **3l**, suggesting that the

Table 1. SAR of 4-Aryl-2-amino-3-cyano-7-(dimethylamino)-4*H*-chromene and Analogues in the Caspase Activation Assay



Cmpd No.	R	EC$_{50}$ (μM)[a] T47D	EC$_{50}$ (μM)[a] H1299	EC$_{50}$ (μM)[a] DLD-1
1a		0.073 ± 0.006	0.093 ± 0.012	0.083 ± 0.002
1b		0.045 ± 0.003	0.115 ± 0.018	0.102 ± 0.014
1c		0.019 ± 0.004	0.043 ± 0.001	0.066 ± 0.014
1d		0.018 ± 0.004	0.036 ± 0.006	0.065 ± 0.016
1e		0.018 ± 0.002	0.034 ± 0.005	0.064 ± 0.010
1f		0.245 ± 0.045	0.754 ± 0.015	0.293 ± 0.034
1g		0.041 ± 0.005	0.144 ± 0.028	0.064 ± 0.009
1h		3.16 ± 0.16	3.74 ± 0.28	5.09 ± 0.076
1i		1.77 ± 0.034	2.29 ± 0.076	3.24 ± 0.29
1j		2.64 ± 0.22	3.37 ± 0.24	4.12 ± 0.09
2a		0.023 ± 0.001	0.073 ± 0.013	0.077 ± 0.041
2b		0.031 ± 0.008	0.063 ± 0.007	0.125 ± 0.011
2c		0.076 ± 0.016	0.233 ± 0.045	0.242 ± 0.064
2d		0.253 ± 0.013	0.510 ± 0.062	0.347 ± 0.015
2e		1.02 ± 0.01	2.13 ± 0.23	1.27 ± 0.03
3a		0.064 ± 0.011	0.337 ± 0.044	0.087 ± 0.003
3b		0.065 ± 0.007	0.200 ± 0.026	0.081 ± 0.002
3c		0.063 ± 0.004	0.215 ± 0.030	0.082 ± 0.001
3d		0.081 ± 0.011	0.304 ± 0.024	0.129 ± 0.014
3e		0.112 ± 0.017	0.514 ± 0.044	0.158 ± 0.004
3f		0.134 ± 0.010	0.420 ± 0.066	0.168 ± 0.002
3g		0.272 ± 0.017	0.959 ± 0.066	0.532 ± 0.024
3h		>10	>10	>10
3i		0.450 ± 0.073	1.14 ± 0.089	0.457 ± 0.030
3j		2.52 ± 0.12	3.56 ± 0.12	4.79 ± 0.07
3k		>10	>10	>10
3l		0.118 ± 0.005	0.277 ± 0.038	0.169 ± 0.004
3m		1.6 ± 0.1	2.0 ± 0.1	2.6 ± 0.2
3n		0.28 ± 0.01	0.56 ± 0.07	0.33 ± 0.01
4a		0.068 ± 0.003	0.155 ± 0.009	0.123 ± 0.007
4b		0.465 ± 0.017	1.430 ± 0.152	0.959 ± 0.058
4c		0.381 ± 0.020	0.855 ± 0.157	0.78 ± 0.054
4d		0.033 ± 0.006	0.046 ± 0.003	0.026 ± 0.003
4e		0.011 ± 0.001	0.027 ± 0.003	0.018 ± 0.001
4f		0.244 ± 0.014	0.622 ± 0.035	0.438 ± 0.020
5a		0.290 ± 0.016	0.583 ± 0.038	0.343 ± 0.008
5b		0.43 ± 0.07	1.08 ± 0.08	0.59 ± 0.04
5c		1.82 ± 0.15	2.23 ± 0.13	2.74 ± 0.13
5d		0.20 ± 0.05	0.30 ± 0.06	0.27 ± 0.04
5e		0.15 ± 0.03	0.24 ± 0.07	0.16 ± 0.04
10		7.3± 0.5	7.8± 0.6	10.4± 0.3
Colchicine (D)	NA	0.014 ± 0.001	0.034 ± 0.016	0.19 ± 0.01
Vinblastin	NA	0.025 ± 0.008	0.073 ± 0.049	0.129 ± 0.068
Paclitaxel	NA	0.030 ± 0.004	0.163 ± 0.011	0.075 ± 0.016

[a] Data are the mean of three or more experiments and are reported as mean ± standard error of the mean (SEM). NA = not applicable.

nitrogen in the 3-pyridyl group contributes to activity. In contract, the 2-pyridyl (**4b**) and 4-pyridyl (**4c**) analogues were >4- and >3-fold less active than **3l**, indicating that the nitrogen in either the 2- or the 4-position contributes negatively to activity. Monosubstitution of the 3-pyridyl analogue at the 5-position with a bromo (**4d**) and a methyl (**4e**) group increased potency by >2- and >6-fold, respectively, confirming the importance of substitution in the 3-(5-) positions in both the phenyl and the pyridyl ring system. The corresponding 3-methyl-substituted 2-pyridyl analogue **4f** was >20-fold less potent than **4e**, confirming the importance of the position of the nitrogen in the pyridyl group. Therefore, in the pyridyl series it is important to have the nitrogen in the 3-position with a substitution at the 5-position. This SAR corresponds to the 3,5-disubstitutions for the phenyl series and suggests that the 3-pyridyl group is a good replacement for the phenyl group.

The nonaromatic cyclohexyl analogue (**5a**) was >2.5-fold less active than **3l**, suggesting that a planar structure of either a phenyl or pyridyl group is preferred in the 4-position of the chromene structure. Extending the nonsubstituted phenyl ring from the 4-position via an ethyl linker (**5b**) resulted in >3.5-fold reduction in potency relative to **3l**, suggesting that the binding pocket in the 4-position of chromene is size limited. This is in agreement with the substituted phenyl series in which a large group substituted in the 3-postion of the phenyl group is not tolerated. Introduction of a bicyclic quinolinyl group (**5c**) in the 4-position of chromene also

Table 2. Comparison of Caspase Activation Activity and Inhibition of Cell Proliferation Activity of 4-Aryl-2-amino-3-cyano-7-(dimethylamino)-4*H*-chromenes

	T47D			DLD-1		
	EC_{50}[a] (μM)	GI_{50}[b] (μM)	GI_{50}/EC_{50}	EC_{50}[a] (μM)	GI_{50}[b] (μM)	GI_{50}/EC_{50}
1c	0.019 ± 0.004	0.092 ± 0.013	4.8	0.066 ± 0.014	0.070 ± 0.013	1.1
2a	0.023 ± 0.001	0.085 ± 0.010	3.7	0.077 ± 0.041	0.078 ± 0.013	1.0
1b	0.045 ± 0.003	0.11 ± 0.024	2.4	0.102 ± 0.014	0.29 ± 0.053	2.8
3l	0.118 ± 0.005	0.455 ± 0.027	3.9	0.169 ± 0.004	0.746 ± 0.074	4.4
1f	0.245 ± 0.045	0.605 ± 0.035	2.5	0.293 ± 0.034	0.735 ± 0.074	2.5
5a	0.290 ± 0.016	0.462 ± 0.092	1.6	0.343 ± 0.008	0.722 ± 0.077	2.1
3j	2.52 ± 0.12	8.31± 0.72	3.3	4.79 ± 0.07	>10	>2.1
1j	2.64 ± 0.22	4.68 ± 0.72	1.8	4.12 ± 0.09	9.89 ± 0.56	2.4

[a] From Table 1. [b] Data are the mean of three experiments and are reported as mean ± standard error of the mean (SEM).

resulted in large drop of potency, confirming that there is a size-limited pocket in the 4-position of chromene. The 2-thiophenyl analogue **5d** is about 2-fold less active than the phenyl analogue **3l**, and the 4-bromo-2-thiophenyl analogue **5e** is slightly more than 2-fold less active than the 5-bromophenyl analogue **3a**, suggesting that a five-member ring heterocycle could partially replace the six-member ring phenyl group.

The introduction of an additional methyl group in the 4-position of chromene of **1c** resulted in the 4,4-disubstituted analogue **10**, which is 178-fold less active than its hydrogen-substituted analogue **1c**. Structural modeling, comparing compounds **1c** and **10** using ChemBats3D (CambridgeSoft, Cambridge, MA), did not indicate that the extra methyl group in the 4-position of **10** altered the 3D orientation of the phenyl group relative to the chromene ring. An alternate possibility is that the loss of potency is simply an issue of pocket size and that the methyl group is not tolerated within the binding site because of unfavorable steric interaction, which is consistent with the SAR found for the 2-position of the phenol ring.

The activity of compounds **1c** and **4e**, two of the most potent compounds in this series, is comparable to the well-known antimitotic agent colchicine (Chart 1, compound D). Compound **1c** is about as potent as colchicine, while compound **4e** is slightly more potent than colchicine in the caspase activation assay. Compounds **1c** and **4e** also are slightly more potent than vinblastin and paclitaxel, two widely used anticancer drugs, in the caspase activation assay. Interestingly, some structural similarity between compound **1c** and colchicine was observed, including the preference for methoxy groups in the phenyl ring,[14] suggesting that compound **1c** and colchicine might interact with the same target.

The activities of these compounds toward the human nonsmall cell lung cancer cell line H1299 and colorectal cancer cell line DLD-1 was roughly parallel to their activity toward T47D cells. H1299 cells were slightly less sensitive (about 2-fold less sensitive as measured by the EC_{50} value) to the compounds than T47D cells in this assay. DLD-1 cells also were slightly less sensitive (about 2−3-fold less as measured by the EC_{50} value) to the compounds than T47D cells.

Selected compounds were also tested by the traditional inhibition of cell proliferation (GI_{50}) assay to confirm that the active compounds can inhibit tumor cell growth, as well as to determine whether there is a correlation between the activity from the caspase activation assay and the cell proliferation assay. The growth inhibition assays in T47D and DLD-1 cells were run in a 96-well microtiter plate as described previously.[11] The GI_{50}, along with the EC_{50} data and the ratio of GI_{50}/EC_{50}, are summarized in Table 2.

Table 2 shows that compounds **1c**, **2a**, and **1b** all are potent inhibitors of tumor cells growth. Compound **1c** has a GI_{50} value of 0.092 μM and 0.070 μM in T47D and DLD-1 cells, respectively. In general, the compound that is more active in the apoptosis induction assay, as measured by caspase activation, also is more potent in the growth inhibition assay. For example, in T47 D cells, compounds **1c** is 132- and 90-fold more potent than **3j** in the caspase assay and growth inhibition assay, respectively. In DLD-1 cells, compound **1c** is 72- and >140-fold more potent than **3j** in the caspase assay and growth inhibition assay, respectively. These data suggest that generally there is a correlation between the caspase activation activity and inhibition of cell proliferation activity for these compounds. Similar to what has been observed for the substituted *N*-phenylnicotinamides,[11] these data indicate that the cell-based caspase activation HTS assay not only is useful for the identification of apoptosis inducers, but also can be used for subsequent optimization and SAR studies of screening hits.

One of the serious problems of the currently used cancer chemotherapeutic agents is multidrug resistance. Many tumor cells overexpress the *p*-glycoprotein, a plasma membrane transporter with a broad substrate specificity that includes most of the conventional chemotherapeutic drugs.[15] This transporter efficiently removes the drugs from the interior of the cell, resulting in protection of the cancer cells from the toxic effects of the drugs. Significantly, when compound **1c** was tested against the human uterus sarcoma MED-SA cells and the multidrug resistant MED-SA/DX5 cells,[11] it was found that **1c** is highly active with GI_{50} values of 2 nM in both cells. In comparison, paclitaxel and vinblastin are effective against MED-SA cells but at least 160- and >20-fold less effective against MED-SA/DX5 cells, respectively.[11,16] This underscores a major potential advantage of this series of compounds—they appear to be effective against cells that overexpress the *p*-glycoprotein—and may therefore be useful in treating patients with cancers that are resistant to traditional antitumor agents, including the taxanes.[17]

Since tubulin inhibitors are known to arrest cells in the G_2/M phase and some structural similarity between compound **1c** and colchicine was observed, we suspected that this series of 4-arylchromenes might be inhibitors of tubulin polymerization. The effect of compound **1c** on tubulin polymerization was measured using a cell-

free assay in which the amount of polymerized tubulin in the presence and absence of drug is determined by measuring the increase in absorbance at 340 nm.[11] Compound **1a** inhibits tubulin polymerization with an IC_{50} value of 400 nM, a level of potency similar to vinblastin (IC_{50} value of 300 nM), a well-known tubulin inhibitor.[11,16]

To locate the binding site of **1c**, the ability of **1c** to block the interaction of tubulin with [^{3}H]colchicine was measured. Compound **1c** displaced [^{3}H]colchicine with an IC_{50} of 8 μM, suggesting that **1c** binds at or close to the colchicine site on β-tubulin.[16] These data suggests that compound **1c**, and the 4-aryl-4*H*-chromene series of compounds, most probably induce apoptosis through the inhibition of tubulin polymerization.

Conclusion

In conclusion, we have discovered a series of 4-aryl-4*H*-chromenes as potent inducers of apoptosis using our cell- and caspase-based HTS assay. The ability of compound **1a**, the screening hit, to induce apoptosis was confirmed by nuclear fragmentation and PARP cleavage assays in Jurkat cells, and flow cytometry assays in T47D cells. SAR studies of the 4-position of the 4*H*-chromenes showed that substituted phenyl and pyridyl are the preferred group. These groups include 3,4,5-trisubstituted phenyl, such as 3-bromo-4,5-dimethoxyphenyl (**1c**, MX58151),[16] 3-chloro-4,5-dimethoxyphenyl (**1d**), and 3-iodo-4,5-dimethoxyphenyl (**1e**), as well as 5-substituted-3-pyridyl, such as 5-bromo-3-pyridyl (**4d**) and 5-methyl-3-pyridyl (**4e**). Compounds **1c** and **4e** are about as potent as or slightly more potent than colchicine, vinblastin, and paclitaxel in the caspase activation assay. Interestingly, introduction of an additional Me group in the 4-position resulted in compound **10**, which is 178-fold less active than the corresponding 4-H analogue **1c**.

The 4-aryl-4*H*-chromenes were found to inhibit tubulin polymerization, which might be the main mechanism of action of this series of apoptosis inducers. Compound **1c** retains activity in cells resistant toward current antimitotic agents, taxanes and vinca alkaloids. Therefore, they might have advantage for the treatment of drug resistance cancers. Moreover, the 4-aryl-4*H*-chromenes were found to disrupt preformed endothelial cell capillary tubules, suggesting that they are likely to work as tumor vasculature targeting agents,[16] similar to combretastatin A-4 phosphate (CA-4P) and ZD-6126.[18] Additional SAR studies and in vivo studies of the 4-aryl-4*H*-chromenes series of apoptosis inducers are in progress and will be reported in future publications.[19]

Experimental Sections

General Methods and Materials. Commercial-grade reagents and solvents were obtained from Acros, Aldrich, Lancaster, Salor, or TCI and were used without further purification except as indicated. All reactions were stirred magnetically; moisture-sensitive reactions were performed under argon in flame-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}H NMR spectra were recorded at 300 MHz. All samples were prepared as dilute solutions in either deuteriochloroform ($CDCl_3$) with v/v 0.05% tetramethylsilane (TMS) or dimethyl-d_6 sulfoxide (CD_3SOCD_3) with v/v 0.05% TMS. Chemical shifts are reported in parts per million (ppm) downfield from TMS (0.00 ppm) and *J* coupling constants are reported in hertz. Elemental analyses were performed by Numega Resonance Labs, Inc. (San Diego, CA). Melting points were determined on a glass capillary melting point apparatus. Human leukemia cancer cells HL-60 and Jurkat, human breast cancer cells T47D, human colon cancer cells DLD-1, human nonsmall cell lung cancers H1299, human uterus sarcoma MES-SA cells, and human uterus sarcoma doxorubicin-resistant MES-SA/DX5 cells were obtained from American Type Culture Collection (Manassas, VA). Colchicine, vinblastin, and paclitaxel were obtained from Sigma, and [^{3}H]colchicine was from American Radiolabeled Chemicals (St. Louis, MO). Tubulin was obtained from Cytoskeleton (Boulder, CO). Compounds **1a, 5d**, and **5e** were obtained from Chembridge (San Diego, CA) and their structures were confirmed by ^{1}H NMR.

5-Methyl-3-pyridinemethanol (6b). To a solution of methyl 5-methyl-3-pyridinecarboxylate (**6a**, 5.02 g, 33.1 mmol) in THF (10 mL) at −78 °C was added $LiAlH_4$ (0.701 g, 17.5 mmol) in small portions over 5 min while the internal temperature was maintained below −70 °C. The mixture was stirred at −78°C for 1 h, and then the cold bath was removed. The reaction mixture was stirred at room temperature for 6.5 h. TLC of the reaction mixture indicated the presence of starting material, additional $LiAlH_4$ (0.071 g, 1.7 mmol) was added, and the mixture was stirred overnight. The reaction mixture was quenched with water (5 mL), stirred at room temperature for 0.5 h, and dried over $MgSO_4$. The mixture was filtered through a layer of Celite and the filter cake was washed with ethyl acetate (100 mL). Evaporation of the solvent gave compound **6b** as a yellow oil (4.05 g, 99%): ^{1}H NMR ($CDCl_3$) 8.40 (s, 1H), 8.37 (s, 1H), 7.31 (s, 1H), 4.71 (s, 2H), 2.35 (s, 3H).

5-Methyl-3-pyridinecarboxaldehyde (6c). A black mixture of **6b** (4.05 g, 32.9 mmol) and activated MnO_2 (13.4 g, 131 mmol) in anhydrous dichloromethane (50 mL) was refluxed for 18 h. The resulting black mixture was filtered through a layer of Celite and the filter cake was washed with dichloromethane (150 mL). The filtrate was concentrated in vacuo to yield compound **6c** as a yellow oil (3.23 g, 81%): ^{1}H NMR ($CDCl_3$) 10.10 (s, 1H), 8.89 (s, 1H), 8.68 (s, 1H), 7.98 (s, 1H), 2.45 (s, 3H).

5-Bromo-3-pyridinecarboxaldehyde (7b). To a yellow solution of oxalyl chloride (15.0 mL, 30.0 mmol, 2.0 M in dichloromethane) and anhydrous dichloromethane (10 mL) at 0 °C was added anhydrous DMF (1.5 mL, 19 mmol). The resulting white suspension was equilibrated to room temperature and filtered and the white solid was collected. The white solid (2.13 g, 16.6 mmol) was then added to a three-neck round-bottom flask fitted with an internal thermometer and dissolved in anhydrous CH_3CN (25 mL) and anhydrous THF (55 mL). The clear solution was cooled to −74 °C, and then 5-bromonicotinic acid (**7a**, 0.672 g, 3.33 mmol) and anhydrous pyridine (0.11 mL, 1.4 mmol) were added while the internal temperature was maintained below −70 °C. The reaction mixture was warmed to −37 °C over 1 h and then cooled to −77 °C. The reducing agent Li(^{t}BuO)$_3$AlH (6.65 mL, 6.65 mmol) was added slowly *to maintain the internal temperature below −71 °C* and was followed by the addition of copper iodide (0.050 g, 0.27 mmol). The mixture was stirred at −81 °C for 20 min and then 1 M HCl (20 mL) was added in small portions to maintain the internal temperature below −67 °C. After the addition of 1 M HCl, the mixture was equilibrated to room temperature, diluted with ethyl acetate (150 mL), dried over Na_2SO_4, filtered through Celite, and washed with additional ethyl acetate. The organic filtrate was washed with water (2 × 20 mL), dried over Na_2SO_4, filtered, and concentrated to yield compound **7b** as a brown solid (0.050 g, 8%): ^{1}H NMR ($CDCl_3$) 10.09 (s, 1H), 9.03 (s, 1H), 8.92 (s, 1H), 8.33 (s, 1H).

2-Amino-3-cyano-7-(dimethylamino)-4-(3-methoxy-4,5-methylenedioxyphenyl)-4*H*-chromene (1a). To a mixture of 3-(dimethylamino)phenol (0.76 g, 5.5 mmol), 5-methoxypiperonal (1.0 g, 5.5 mmol), and malononitrile (0.37 g, 5.5

mmol) in ethanol (20 mL) was added piperidine (1.2 mL, 11 mmol). The mixture was stirred at room temperature overnight and then the solvent was evaporated. The resulting solid was collected by filtration and washed with diethyl ether. Recrystallization of the black solid (1:1, DMSO:water) yielded (1.5 g, 74%) of compound **1a** as a light brown solid: mp 168−171 °C; ^{1}H NMR (DMSO-d_6) 6.85 (s, 1H), 6.82 (s, 2H), 6.52 (d, J = 1.5, 1H), 6.49−6.45 (m, 1H), 6.29 (d, J = 1.5, 1H), 6.21 (d, J = 2.7, 1H), 5.94 (d, J = 6.3, 2H), 4.54 (s, 1H), 3.81 (s, 3H), 2.86 (s, 6H).

The following compounds were prepared from the corresponding substituted benzaldehyde, 3-(dimethylamino)phenol, and malononitrile by a procedure similar to that described for the preparation of compound **1a**.

2-Amino-4-(3-bromo-4,5-dimethoxyphenyl)-3-cyano-7-(dimethylamino)-4*H*-chromene (1c): white solid (24%); recrystallized from 5:1, hexanes:EtOAc; mp 200−202 °C; ^{1}H NMR (CDCl$_3$) 6.89 (d, J = 1.8, 1H), 6.79 (d, J = 8.7, 1H), 6.72 (d, J = 1.8, 1H), 6.46−6.43 (m, 1H), 6.28 (d, J = 2.7, 1H), 4.58 (s, 1H), 4.57 (s, 2H), 3.84 (s, 3H), 3.83 (s, 3H), 2.94 (s, 6H). Anal. ($C_{20}H_{20}BrN_3O_3$) C, H, N.

2-Amino-4-(3-chloro-4,5-dimethoxyphenyl)-3-cyano-7-(dimethylamino)-4*H*-chromene (1d): tan solid (57%); recrystallized from 5:1, hexanes:EtOAc; mp 190−192 °C; ^{1}H NMR (CDCl$_3$) 6.82−6.69 (m, 3H), 6.48−6.44 (m, 1H), 6.30 (d, J = 2.7, 1H), 4.59 (s, 3H), 3.86 (s, 3H), 3.85 (s, 3H), 2.85 (s, 6H). Anal. ($C_{20}H_{20}ClN_3O_3$) C, H, N.

2-Amino-4-(3-bromo-5-methoxyphenyl)-3-cyano-7-(dimethylamino)-4*H*-chromene (2b): white solid (50%); recrystallized from 5:1, hexanes:EtOAc; mp 210−212 °C; ^{1}H NMR (DMSO-d_6) 6.99 (s, 1H), 6.91 (s, 2H), 6.85 (s, 1H), 6.84 (d, J = 8.7, 1H), 6.77 (s, 1H), 6.49−6.46 (m, 1H), 6.24 (d, J = 3.0, 1H), 4.61 (s, 1H), 3.75 (s, 3H), 2.87 (s, 6H). Anal. ($C_{19}H_{18}BrN_3O_2$) C, H, N.

2-Amino-3-cyano-4-(3,5-dichlorophenyl)-7-(dimethylamino)-4*H*-chromene (2c): yellow solid (24%); recrystallized from 1:1, hexanes:dichloromethane; mp 212−214 °C; ^{1}H NMR (DMSO-d_6) 7.47 (t, J = 1.8, 1H), 7.20 (d, J = 2.1, 2H), 6.98 (s, 2H), 6.83 (d, J = 8.7, 1H), 6.49 (dd, J = 8.9 and 2.6, 1H), 6.24 (d, J = 2.4, 1H), 4.73 (s, 1H), 2.88 (s, 6H). Anal. ($C_{18}H_{15}Cl_2N_3O$) C, H, N.

2-Amino-3-cyano-4-(3,4-dimethoxyphenyl)-7-(dimethylamino)-4*H*-chromene (2d): white solid (23%); recrystallized from 5:1, hexanes:EtOAc; mp 158−160 °C; ^{1}H NMR (CDCl$_3$) 6.81 (s, 1H), 6.79 (s, 1H), 6.75 (d, J = 1.8, 1H), 6.69 (d, J = 1.8, 1H), 6.45−6.41 (m, 1H), 6.29 (d, J = 3.0, 1H), 4.60 (s, 1H), 4.54 (s, 2H), 3.85 (s, 3H), 3.83 (s, 3H), 2.93 (s, 6H). Anal. ($C_{20}H_{21}N_3O_3 * 0.6H_2O$) C, H, N.

2-Amino-4-(3-carboxyphenyl)-3-cyano-7-(dimethylamino)-4*H*-chromene (3h): yellow solid (80%); recrystallized from 1:1:0.1, hexanes:EtOAc:MeOH; mp 204−206 °C; ^{1}H NMR (DMSO-d_6) 7.72−7.68 (m, 1H), 7.64 (s, 1H), 7.29−7.25 (m, 2H), 6.84 (s, 2H), 6.76 (d, J = 9.0, 1H), 6.45 (dd, J = 8.9 and 2.6, 1H), 6.23 (d, J = 2.7, 1H), 4.62 (s, 1H), 2.86 (s, 6H). Anal. ($C_{19}H_{17}N_3O_3$) C, H, N.

2-Amino-3-cyano-7-(dimethylamino)-4-(3-pyridyl)-4*H*-chromene (4a): brown solid (93%); recrystallized from 1:1:0.1, hexanes:EtOAc:MeOH; mp 210−214 °C (dec); ^{1}H NMR (CDCl$_3$) 8.49−8.47 (m, 2H), 7.52−7.49 (m, 1H), 7.26−7.22 (m, 1H), 6.75 (d, J = 8.7, 1H), 6.45−6.41 (m, 1H), 6.30 (d, J = 2.4, 1H), 4.69 (s, 1H), 4.62 (s, 2H), 2.93 (s, 6H). Anal. ($C_{17}H_{16}N_4O * 0.3H_2O$) C, H, N.

2-Amino-3-cyano-7-(dimethylamino)-4-(2-pyridyl)-4*H*-chromene (4b): brown solid (35%); recrystallized from 1:1, hexanes:dichloromethane; mp 206−208 °C; ^{1}H NMR (DMSO-d_6) 8.46 (dd, J = 5.7 and 1.8, 1H), 7.73 (td, J = 7.7 and 1.8, 1H), 7.23−7.20 (m, 2H), 6.88 (s, 2H), 6.85 (d, J = 9.0, 1H), 6.44 (dd, J = 8.9 and 2.6, 1H), 6.23 (d, J = 3.0, 1H), 4.70 (s, 1H), 2.85 (s, 6H). Anal. ($C_{17}H_{16}N_4O$) C, H, N.

2-Amino-3-cyano-7-(dimethylamino)-4-(4-pyridinyl)-4*H*-chromene (4c): brown solid (47%); recrystallized from 1:1, hexanes:dichloromethane; mp 202−204 °C; ^{1}H NMR (CDCl$_3$) 8.54−8.52 (m, 2H), 7.13−7.11 (m, 2H), 6.75 (d, J = 8.7, 1H), 6.45−6.42 (m, 1H), 6.30 (d, J = 2.7, 1H), 4.64 (s, 1H), 4.63 (s, 2H), 2.94 (s, 6H). Anal. ($C_{17}H_{16}N_4O$) C, H, N.

2-Amino-3-cyano-7-(dimethylamino)-4-phenyl-4*H*-chromene (3l). To a mixture of 3-dimethylaminophenol (0.129 g, 0.940 mmol), malononitrile (0.062 g, 0.94 mmol), and benzaldehyde (96 μL, 0.94 mmol) in ethanol (5 mL) was added piperidine (0.20 mL, 2.0 mmol). The white suspension was stirred at room temperature for 2 h and the resulting precipitate was collected by filtration, washed with methanol, and then dried in vacuo to yield compound **3l** as a white solid (0.202 g, 74%): mp 206−207 °C; ^{1}H NMR (DMSO-d_6) 7.32−7.27 (m, 2H), 7.22−7.14 (m, 3H), 6.82 (s, 2H), 6.78 (d, J = 8.7, 1H), 6.45 (dd, J = 8.7 and 2.4, 1H), 6.23 (d, J = 2.4, 1H), 4.59 (s, 1H), 2.86 (s, 6H). Anal. ($C_{18}H_{17}N_3O$) C, H, N.

The following compounds were prepared from the corresponding substituted benzaldehyde, 3-(dimethylamino)phenol, and malononitrile by a procedure similar to that described for the preparation of compound **3l.**

2-Amino-3-cyano-7-(dimethylamino)-4-(3,4,5-trimethoxyphenyl)-4*H*-chromene (1b): white solid (78%); mp 179−181 °C; ^{1}H NMR (DMSO-d_6) 6.90−6.84 (m, 3H), 6.48−6.46 (m, 3H), 6.23 (s, 1H), 4.57 (s, 1H), 3.72 (s, 6H), 3.62 (s, 3H), 2.87 (s, 6H). Anal. ($C_{21}H_{23}N_3O_4$) C, H. N.

2-Amino-3-cyano-7-(dimethylamino)-4-(3-iodo-4,5-dimethoxyphenyl)-4*H*-chromene (1e): white solid (18%); mp 176−177 °C; ^{1}H NMR (DMSO-d_6) 7.02 (d, J = 1.8, 1H), 6.96 (s, 1H), 6.88 (s, 2H), 6.86 (d, J = 8.7, 1H), 6.49−6.46 (m, 1H), 6.22 (d, J = 2.4, 1H), 4.59 (s, 1H), 3.78 (s, 3H), 3.66 (s, 3H), 2.87 (s, 6H). Anal. ($C_{20}H_{20}IN_3O_3$) C, H, N.

2-Amino-4-(3-bromo-4,5-methylenedioxyphenyl)-3-cyano-7-(dimethylamino)-4*H*-chromene (1f): brown solid (56%); mp 232−234 °C (dec); ^{1}H NMR (DMSO-d_6) 6.87 (s, 2H), 6.84−6.80 (m, 2H), 6.67 (d, J = 1.2, 1H), 6.50−6.46 (m, 1H), 6.22 (d, J = 2.4, 1H), 6.08 (d, J = 6.9, 2H), 4.58 (s, 1H), 2.87 (s, 6H). Anal. ($C_{19}H_{16}BrN_3O_3$) C, H, N.

2-Amino-3-cyano-4-(3,5-dimethoxyphenyl)-7-(dimethylamino)-4*H*-chromene (2a): white solid (38%); mp 169−170 °C; ^{1}H NMR (DMSO-d_6) 6.85 (d, J = 8.7, 1H), 6.81 (s, 2H), 6.46 (dd, J = 8.7 and 2.7, 1H), 6.36−6.34 (m, 1H), 6.31 (d, J = 2.1, 2H), 6.22 (d, J = 2.4, 1H), 4.52 (s, 1H), 3.70 (s, 6H), 2.86 (s, 6H). Anal. ($C_{20}H_{21}N_3O_3$) C, H, N.

2-Amino-3-cyano-7-(dimethylamino)-4-(3-methylphenyl)-4*H*-chromene (3f): white solid (24%); mp 174−175 °C; ^{1}H NMR (DMSO-d_6) 7.18 (t, J = 7.5, 1H), 7.00 (d, J = 7.2, 1H), 6.95 (d, J = 2.1, 2H), 6.80 (s, 2H), 6.78 (d, J = 8.7, 1H), 6.46−6.43 (m, 1H), 6.22 (d, J = 2.4, 1H), 4.54 (s, 1H), 2.86 (s, 6H), 2.26 (s, 3H). Anal. ($C_{19}H_{19}N_3O$) C, H, N.

2-Amino-4-(3-benzyloxyphenyl)-3-cyano-7-(dimethylamino)-4*H*-chromene (3j): white solid (76%); mp 197−198 °C; ^{1}H NMR (DMSO-d_6) 7.46−7.33 (m, 5H), 7.22 (t, J = 7.8, 1H), 6.87−6.79 (m, 5H), 6.75 (d, J = 7.2, 1H), 6.45 (dd, J = 8.9 and 2.6, 1H), 6.22 (d, J = 2.1, 1H), 5.04 (s, 2H), 4.56 (s, 1H), 2.86 (s, 6H). Anal. ($C_{25}H_{23}N_3O_2$) C, H, N.

2-Amino-3-cyano-7-(dimethylamino)-4-(3-dodecyloxyphenyl)-4*H*-chromene (3k): white solid (54%); mp 94 °C; ^{1}H NMR (DMSO-d_6) 7.19 (t, J = 7.8, 1H), 6.83−6.69 (m, 6H), 6.45 (dd, J = 8.6 and 2.6, 1H), 6.22 (d, J = 2.7, 1H), 4.55 (s, 1H), 3.90 (t, J = 6.3, 2H), 2.86 (s, 6H), 1.70−1.65 (m, 2H), 1.38−1.24 (m, 18H), 0.85 (t, J = 6.6, 3H). Anal. ($C_{30}H_{41}N_3O_2$) C, H, N.

2-Amino-3-cyano-7-(dimethylamino)-4-(4-methoxyphenyl)-4*H*-chromene (3m): yellow solid (80%); mp 174−176 °C; ^{1}H NMR (CDCl$_3$) 7.12−7.10 (m, 2H), 6.85−6.76 (m, 3H), 6.42 (dd, J = 8.4 and 2.4, 1H), 6.28 (d, J = 2.4, 1H), 4.60 (s, 1H), 4.51 (br s, 2H), 3.77 (s, 3H), 2.92 (s, 6H). Anal. ($C_{19}H_{19}N_3O_2$) C, H, N.

2-Amino-3-cyano-7-(dimethylamino)-4-(2-methoxyphenyl)-4*H*-chromene (3n): yellow solid (30%); mp 152−153 °C (dec); ^{1}H NMR (CDCl$_3$) 7.20−7.14 (m, 1H), 7.00 (dd, J = 7.5 and 1.8, 1H), 6.91−6.84 (m, 3H), 6.41 (dd, J = 8.7 and 2.7, 1H), 6.28 (d, J = 2.4, 1 H), 5.17 (s, 1H), 4.50 (br s, 2H), 3.85 (s, 3H), 2.91(s, 6H). Anal. ($C_{19}H_{19}N_3O_2$) C, H, N.

2-Amino-4-(5-bromo-3-pyridyl)-3-cyano-7-(dimethylamino)-4*H*-chromene (4d): brown solid (8%); mp 230−232 °C

(dec); ^{1}H NMR (DMSO-d_6) 8.58 (t, J = 2.3, 1H), 8.44 (d, J = 1.9, 1H), 7.75 (d, J = 2.2, 1H), 7.01 (br s, 2H), 6.82 (d, J = 8.8, 1H), 6.49 (d, J = 8.8, 1H), 6.25 (s, 1H), 4.77 (s, 1H), 2.88 (s, 6H). Anal. ($C_{17}H_{15}BrN_4O$) C, H, N.

2-Amino-3-cyano-7-(dimethylamino)-4-(5-methyl-3-pyridyl)-4*H*-chromene (4e): yellow solid (92%); mp 232−234 °C; ^{1}H NMR (DMSO-d_6) 8.26 (d, J = 0.8, 2H), 7.29 (s, 1H), 6.90 (br s, 2H), 6.77 (d, J = 8.5, 1H), 6.48−6.45 (m, 1H), 6.24 (d, J = 2.2, 1H), 4.66 (s, 1H), 2.87 (s, 6H), 2.25 (s, 3H). Anal. ($C_{18}H_{18}N_4O$) C, H, N.

2-Amino-3-cyano-7-(dimethylamino)-4-(6-methyl-2-pyridyl)-4*H*-chromene (4f): tan solid (66%); mp 212−214 °C; ^{1}H NMR ($CDCl_3$) 7.49 (t, J = 7.5, 1H), 7.00−6.93 (m, 3H), 6.43−6.39 (m, 1H), 6.28 (s, 1H), 4.84 (s, 1H), 4.66 (s, 2H), 2.91 (s, 6H), 2.56 (s, 3H). Anal. ($C_{18}H_{18}N_4O$) C, H, N.

2-Amino-3-cyano-7-(dimethylamino)-4-(3-quinolyl)-4*H*-chromene (5c): yellow solid (69%); mp 224−226 °C; ^{1}H NMR (DMSO-d_6) 8.71 (d, J = 2.1, 1H), 8.12 (d, J = 2.1, 1H), 7.99 (dd, J = 7.7 and 4.0, 2H), 7.73 (td, J = 7.7 and 1.5, 1H), 7.61 (t, J = 7.5, 1H), 6.99 (s, 2H), 6.81 (d, J = 8.7, 1H), 6.46 (dd, J = 8.9 and 2.6, 1H), 6.28 (d, J = 2.4, 1H), 4.91 (s, 1H), 2.87 (s, 6H). Anal. ($C_{21}H_{18}N_4O$) C, H, N.

2-Amino-4-(2-bromo-4,5-dimethoxyphenyl)-3-cyano-7-(dimethylamino)-4*H*-chromene (1j). To a purple suspension of 3-(dimethylamino)phenol (0.250 g, 1.02 mmol), malononitrile (0.067 g, 1.0 mmol), and 6-bromoveratraldehyde (0.140 g, 1.02 mmol) in ethanol (3.4 mL) was added piperidine (50 μL, 0.51 mmol). The brown suspension was stirred at room temperature for 28 h, and the resulting precipitate was collected by filtration, washed with ethanol, and purified by flash column chromatography (elution with EtOAc:hexanes, 1:2) to give compound **1j** as a yellow solid (0.13 g, 29%): mp 94−98 °C; ^{1}H NMR ($CDCl_3$) 6.99 (s, 1H), 6.83 (d, J = 8.5, 1H), 6.58 (s, 1H), 6.41 (dd, J = 8.8 and 2.5, 1H), 6.27 (d, J = 2.8, 1H), 5.23 (s, 1H), 4.57 (s, 2H), 3.86 (s, 3H), 3.75 (s, 3H), 2.93 (s, 6H). Anal. ($C_{20}H_{20}BrN_3O_3{\cdot}0.3H_2O$) C, H, N.

The following compounds were prepared from the corresponding substituted benzaldehyde, 3-(dimethylamino)phenol, and malononitrile by a procedure similar to that described for the preparation of compound **1j**.

2-Amino-3-cyano-4-(2,3-dimethoxyphenyl)-7-(dimethylamino)-4*H*-chromene (2e): white solid (58%); mp 72−74 °C; ^{1}H NMR (DMSO-d_6) 6.98 (t, J = 7.2, 1H), 6.88 (d, J = 7.5, 1H), 6.77 (s, 2H), 6.72 (d, J = 8.4, 1H), 6.61 (d, J = 7.2, 1H), 6.44−6.40 (m, 1H), 6.22 (d, J = 1.8, 1H), 4.86 (s, 1H), 3.79 (s, 3H), 3.65 (s, 3H), 2.85 (s, 6H). Anal. ($C_{20}H_{21}N_3O_3$) C, N. H calcd, 6.02; found, 6.74.

2-Amino-4-(3-bromophenyl)-3-cyano-7-(dimethylamino)-4*H*-chromene (3a): yellow solid (62%); mp 180−182 °C; ^{1}H NMR (DMSO-d_6) 7.43−7.39 (m, 1H), 7.33 (t, J = 1.8, 1H), 7.29 (t, J = 10.2, 1H), 7.20−7.17 (m, 1H), 6.92 (s, 2H), 6.81 (d, J = 8.4, 1H), 6.49−6.45 (m, 1H), 6.24 (d, J = 2.7, 1H), 4.66 (s, 1H), 2.87 (s, 6H). Anal. ($C_{18}H_{16}BrN_3O_2{\cdot}0.2H_2O$) C, H, N.

2-Amino-3-cyano-7-(dimethylamino)-4-(3-fluorophenyl)-4*H*-chromene (3d): white solid (38%); mp 157−158 °C; ^{1}H NMR (DMSO-d_6) 7.39−7.31 (m, 1H), 7.06−6.95 (m, 3H), 6.89 (s, 2H), 6.82 (d, J = 8.4, 1H), 6.47 (dd, J = 8.7 and 2.4, 1H), 6.23 (d, J = 2.1, 1H), 4.66 (s, 1H), 2.86 (s, 6H). Anal. ($C_{18}H_{16}FN_3O$) C, H, N.

2-Amino-3-cyano-7-(dimethylamino)-4-(3-hydroxyphenyl)-4*H*-chromene (3g): white solid (25%); mp 176−178 °C; ^{1}H NMR (DMSO-d_6) 9.31 (s, 1H), 7.07 (t, J = 7.8, 1H), 6.81−6.79 (m, 3H), 6.64−6.55 (m, 3H), 6.48−6.44 (m, 1H), 6.22 (d, J = 2.4, 1H), 4.48 (s, 1H), 2.86 (s, 6H). Anal. ($C_{18}H_{17}N_3O_2$) C, H, N.

2-Amino-3-cyano-7-(dimethylamino)-4-(3-trifluoromethoxyphenyl)-4*H*-chromene (3i): yellow solid (47%); mp 166−168 °C; ^{1}H NMR (DMSO-d_6) 7.45 (t, J = 7.5, 1H), 7.21−7.15 (m, 3H), 6.92 (s, 2H), 6.82 (d, J = 8.7, 1H), 6.49−6.46 (m, 1H), 6.24 (d, J = 2.7, 1H), 4.73 (s, 1H), 2.87 (s, 6H). Anal. ($C_{19}H_{16}F_3N_3O_2$) C, H, N.

2-Amino-3-cyano-7-(dimethylamino)-4-(2,3,4-trimethoxyphenyl)-4*H*-chromene (1h). To a purple solution of 3-(dimethylamino)phenol (0.14 g, 1.0 mmol), malononitrile (0.067 g, 1.0 mmol), and 2,3,4-trimethoxybenzaldehyde (0.20 g, 1.0 mmol) in ethanol (3.4 mL) was added piperidine (50 μL, 0.51 mmol). The purple solution was stirred at room temperature for 24 h and then the reaction solution was concentrated in vacuo. The crude product was purified by flash column chromatography (elution with EtOAc:hexanes, 2:1) to give compound **1h** as a yellow solid (0.20 g, 52%): mp 74−78 °C; ^{1}H NMR (DMSO-d_6) 6.73−6.71 (m, 5H), 6.45−6.41 (m, 1H), 6.22 (d, J = 2.4, 1H), 4.74 (s, 1H), 3.76 (s, 3H), 3.74 (s, 3H), 3.66 (s, 3H), 2.85 (s, 6H). Anal. ($C_{21}H_{23}N_3O_4$) C, H, N.

The following compounds were prepared from the corresponding substituted benzaldehyde, 3-(dimethylamino)phenol, and malononitrile by a procedure similar to that described for the preparation of compound **1h.**

2-Amino-3-cyano-7-(dimethylamino)-4-(3-methoxyphenyl)-4*H*-chromene (3b): yellow solid (19%); mp 161−162 °C; ^{1}H NMR ($CDCl_3$) 7.19 (t, J = 0.6, 1H), 6.82−6.72 (m, 4H), 6.44−6.40 (m, 1H), 6.28 (d, J = 2.7, 1H), 4.61 (s, 1H), 4.54 (s, 2H), 3.77 (s, 3H), 2.92 (s, 6H). Anal. ($C_{19}H_{19}N_3O_2$) C, H, N.

2-Amino-3-cyano-4-(3-cyanophenyl)-7-(dimethylamino)-4*H*-chromene (3c): yellow solid (25%); mp 188−190 °C; ^{1}H NMR (DMSO-d_6) 7.70 (d, J = 7.5, 1H), 7.65 (s, 1H), 7.56−7.50 (m, 2H), 6.96 (s, 2H), 6.79 (d, J = 8.4, 1H), 6.49−6.45 (m, 1H), 6.24 (d, J = 2.4, 1H), 4.75 (s, 1H), 2.87 (s, 6H). Anal. ($C_{19}H_{16}N_4O$) C, H, N.

2-Amino-4-(3-chlorophenyl)-3-cyano-7-(dimethylamino)-4*H*-chromene (3e): yellow solid (33%); mp 172−173 °C; ^{1}H NMR (DMSO-d_6) 7.35 (t, J = 7.5, 1H), 7.29−7.25 (m, 1H), 7.19 (t, J = 1.8, 1H), 7.16−7.13 (m, 1H), 6.91 (s, 2H), 6.81 (d, J = 8.4, 1H), 6.49−6.45 (m, 1H), 6.23 (d, J = 2.7, 1H), 4.66 (s, 1H), 2.87 (s, 6H). Anal. ($C_{18}H_{16}ClN_3O$) C, H, N.

2-Amino-3-cyano-4-cyclohexyl-7-(dimethylamino)-4*H*-chromene (5a): yellow solid (31%); mp 148−152 °C; ^{1}H NMR (DMSO-d_6) 6.96 (d, J = 8.4, 1H), 6.72 (s, 2H), 6.51 (dd, J = 8.7 and 2.4, 1H), 6.20 (d, J = 2.4, 1H), 3.19 (d, J = 3.3, 1H), 2.87 (s, 6H), 2.82 (s, 1H), 1.65−0.86 (m, 10H). Anal. ($C_{18}H_{23}N_3O$) C, H, N.

2-Amino-3-cyano-7-(dimethylamino)-4-(2-phenylethyl)-4*H*-chromene (5b): yellow solid (17%); mp 140−142 °C; ^{1}H NMR (DMSO-d_6) 7.36−7.08 (m, 6H), 6.75 (s, 2H), 6.56 (dd, J = 8.4 and 2.4, 1H), 6.21 (d, J = 2.4, 1H), 3.58 (t, J = 6.9, 1H), 2.87 (s, 6H), 2.34−2.24 (m, 2H), 1.97−1.79 (m, 2H). Anal. ($C_{20}H_{21}N_3O$) C, H, N.

2-Amino-3-cyano-7-(dimethylamino)-4-(2,4,5-trimethoxyphenyl)-4*H*-chromene (1i). To a purple solution of 3-(dimethylamino)phenol (0.14 g, 1.0 mmol), malononitrile (0.067 g, 1.0 mmol), and 2,4,5-trimethoxybenzaldehyde (0.20 g, 1.0 mmol) in ethanol (3.4 mL) was added piperidine (50 μL, 0.51 mmol). The purple solution was heated at 50 °C for 10 h and the resulting precipitate was collected by filtration, washed with ethanol, and dried in vacuo to give compound **1i** as a yellow solid (0.17 g, 44%): mp 200−202 °C; ^{1}H NMR (DMSO-d_6) 6.80 (d, J = 8.7, 1H), 6.71 (s, 2H), 6.68 (s, 1H), 6.55 (s, 1H), 6.44−6.40 (m, 1H), 6.20 (d, J = 2.4, 1H), 4.88 (s, 1H), 3.76 (d, J = 3.9, 6H), 3.59 (s, 3H), 2.84 (s, 6H). Anal. ($C_{21}H_{23}N_3O_4$) C, H, N.

2-(3-Bromo-4-hydroxy-5-methoxybenzylidene)malononitrile (8). To a mixture of 3-bromo-4-hydroxy-5-methoxybenzaldehyde (2.31 g, 10.0 mmol) and malononitrile (0.660 g, 10.0 mmol) in ethanol (20 mL) was added piperidine (0.50 mL, 0.50 mmol). The solution was stirred at room temperature overnight and the resulting precipitate was collected by filtration and dried to yield compound **8** (2.14 g, 77%) as a red solid, which was used for the next reaction.

2-Amino-4-(3-bromo-4-hydroxy-5-methoxyphenyl)-3-cyano-7-(dimethylamino)-4*H*-chromene (1g). To a mixture of **8** (0.279 g, 1.00 mmol) and 3-(dimethylamino)phenol (0.137 g, 1.00 mmol) in ethanol (10 mL) was added piperidine (0.05 mL, 0.05 mmol) and the solution was refluxed overnight. The solvent was removed in vacuo and the crude material was purified by column chromatography (elution with EtOAc: hexanes, 1:2) to give compound **1g** (0.035 g, 8.4%) as a white solid: mp 218−221 °C; ^{1}H NMR ($CDCl_3$) 6.88 (d, J = 2.1, 1H), 6.78 (d, J = 8.7, 1H), 6.67 (d, J = 2.1, 1H), 6.44 (dd, J = 2.4,

8.7, 1H), 6.28 (d, $J = 2.4$, 1H), 5.81 (s, 1H), 4.55 (br s, 3H), 3.87 (s, 3H), 2.94 (s, 6H). Anal. ($C_{19}H_{18}BrN_3O_3$) C, H, N.

4-(3-Bromo-4,5-dimethoxyphenyl)-3-cyano-7-(dimethylamino)-2-imino-2*H*-chromene (9). To a solution of compound **1c** (2.00 g, 4.65 mmol) in dichloromethane (23 mL) was added 2,3-dichloro-5,6-dicyano-1,4-benzoquinone (1.05 g, 4.65 mmol). The resulting green mixture was stirred at room temperature for 5 h. The reaction mixture was then diluted with ethyl acetate (250 mL), washed with sodium bicarbonate saturated solution (2 × 50 mL) and brine (50 mL), dried over sodium sulfate, and concentrated to yield 0.90 g (45%) of compound **9** as an orange solid: ^{1}H NMR (DMSO-d_6) 8.33 (s, 1H), 7.28 (d, $J = 1.9$, 1H), 7.21 (d, $J = 1.9$, 1H), 6.87 (d, $J = 9.1$, 1H), 6.56 (dd, $J = 9.1$ and 2.5, 1H), 6.40 (d, $J = 2.5$, 1H), 3.84 (s, 3H), 3.82 (s, 3H), 3.03 (s, 6H).

2-Amino-4-(3-bromo-4,5-dimethoxyphenyl)-3-cyano-7-(dimethylamino)-4-methyl-4*H*-chromene (10). To a mixture of copper bromide dimethyl sulfide (1.20 g, 5.84 mmol) in anhydrous THF (16.7 mL) at −78 °C was slowly added methyllithium (1.6 M in ether, 7.30 mL, 11.7 mmol). The internal temperature of the reaction mixture was kept below −72 °C throughout the addition process. To the mixture was then added an orange solution of the compound **9** (0.50 g, 1.8 mmol) in anhydrous THF (20 mL) dropwise over 45 min while the internal temperature was maintained below −70 °C. The resulting orange mixture was stirred for 30 min at −78 °C and then was warmed to 0 °C. It was then quenched with ammonium chloride saturated solution (20 mL), extracted with ethyl acetate (3 × 100 mL), washed with brine (25 mL), dried over sodium sulfate, and concentrated. The residue was purified by flash column chromatography (elution with EtOAc:hexanes, 1:2) to give compound **10** as a yellow solid (0.11 g, 21%): mp 72−74 °C; ^{1}H NMR ($CDCl_3$) 6.86−6.76 (m, 3H), 6.45 (dd, $J = 8.7$ and 2.6, 1H), 6.28 (d, $J = 2.6$, 1H), 4.46 (br s, 2H), 3.84 (s, 3H), 3.80 (s, 3H), 2.93 (s, 6H), 1.87 (s, 3H). Anal. ($C_{21}H_{22}BrN_3O_3{\cdot}0.5H_2O$) C, H, N.

Caspase Activation Assay (EC_{50}). T47D, H1299, and DLD-1 cells were grown according to media component mixtures designated by American Type Culture Collection in RPMI-1640 +10% FCS in a 5% CO_2−95% humidity incubator at 37 °C. Cells were harvested using trypsin, washed at 600*g*, and resuspended at 0.65 × 10^6 cells/mL into RPMI media + 10% FCS. An aliquot of 22.5 μL of cells was added to a well of a 384-well microtiter plate containing 2.5 μL of 0.05−100 μM of test compound in RPMI-1640 containing 25 mM HEPES media solution with 10% DMSO (0.005 to 10 μM final). An aliquot of 22.5 μL of cells was added to a well of a 384-well microtiter plate containing 2.5 μL of RPMI-1640 media solution with 10% DMSO and without test compound as the control sample. The samples were then incubated at 37 °C for 24 h in a 5% CO_2−95% humidity incubator. After incubation, the samples were removed from the incubator and 25 μL of a solution containing 14 μM of *N*-(Ac-DEVD)-*N'*-ethoxycarbonyl-R110 fluorogenic substrate,[10] 20% sucrose, 20 mM DTT, 200 mM NaCl, 40 mM Na PIPES buffer pH 7.2, and 250 μg/mL lysolecithin was added. The samples were incubated at room temperature for 3 h. Using a fluorescent plate reader (Model Spectrafluor Plus Tecan), an initial reading ($T = 0$) was made approximately 1−2 min after addition of the substrate solution employing excitation at 485 nm and emission at 525 nm, to determine the background fluorescence of the control sample. After the 3 h incubation, the samples were read for fluorescence as above ($T = 3$ h).

Calculation. The relative fluorescence unit values (RFU) were used to calculate the sample readings as follows: The activity of caspase activation was determined by the ratio of the net RFU value for the test compound to that of control samples. Compound **1a** was found to have a ratio (maximum response) of 6.0. In comparison, colchicine and paclitaxel were found to have a ratio of 7.5 and 6.4, respectively. The EC_{50} (μM) was determined by a sigmoidal dose−response calculation (XLFit3, IDBS), as the concentration of compound that produces the 50% maximum response. The caspase activation activity (EC_{50}) in three cancer cell lines, T47D, H1299, and DLD-1, are summarized in Table 1.

Morphological Assessment of Nuclear Fragmentation of Apoptotic Cells. Jurkat cells, grown and harvested as above, were treated with 0.1 μM of compound **1a** for 24 h followed by staining of the nucleus with Syto 16, a fluorescent DNA dye. The cells were then observed under a fluorescent microscope for chromosomal condensation and nuclear fragmentation (490 nm). The nuclei of Jurkat cells treated with vehicle control (DMSO) are seen to be round with dispersed chromatin that is moderately stained with Syto16 (Figure 1A). In contrast, Jurkat cells treated with 0.1 μM of compound **1a** for 24 h have shrunken and fragmented nuclei (Figure 1B), which is a hallmark of caspase-mediated apoptosis. These results support the caspase activation assays, showing that compound **1a** can induce a key cellular marker of apoptosis.

Induction of PARP Cleavage in Jurkat Cells. The ability of compound **1a** to induce PARP cleavage was tested by treating Jurkat cells with the compound at a concentration of 2.5 μM for 5, 15, and 30 h. Control cultures were treated with DMSO vehicle or staurosporine, a known apoptosis inducer. At the end of the incubation period, the cells were harvested by centrifugation, washed with PBS, and lysed in a buffer containing 50 mM Tris-Cl, pH 7.4, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, and a cocktail of protease inhibitors (Complete Protease Inhibitor Tablets, Roche Molecular Systems). The amount of protein in each sample was measured by the method of Bradford using a BioRad protein assay kit. Samples of lysate containing equivalent amounts of protein were separated by SDS−PAGE and transferred to PVDF membrane. The membranes were probed with a polyclonal antibody to PARP (Enzyme Systems Products), followed by goat−anti-rabbit HRP second antibody. Antibody binding was visualized by chemiluminescence (Pierce SuperSignal). As illustrated in Figure 2, compound **1a** induced PARP cleavage starting at 15 h, and by 30 h virtually all of PARP was converted into its cleaved form. These results provide additional evidence that **1a** induces caspase-mediated apoptosis in cells.

Cell Cycle Analysis and Measurement of Apoptosis. T47D cells were maintained and harvested as described above. Cells (1 × 10^6) were treated with 0.1 μM of compound **1a** for 24 or 48 h at 37 °C. As a control, cells were also incubated with an equivalent amount of solvent (DMSO). Cells were harvested at 1200 rpm and washed twice with 5 mM EDTA/PBS. Cells were then resuspended in 300 μL of EDTA/PBS and 700 μL of 100% ethanol, vortexed, and incubated at room temperature for 1 h. Samples were centrifuged at 1200 rpm for 5 min, and the supernatant was removed. A solution containing 100 μg/mL of propidium iodide and 1 mg/mL of RNAse A (fresh) was added to the samples and incubated for 1 h at room temperature. Samples were then transferred to 12 × 75 mm polystyrene tubes and analyzed on a flow cytometer. All flow cytometry analyses were performed on a FACScalibur (Becton Dickinson) using Cell Quest analysis software. On the *x*-axis is plotted the fluorescence intensity and on the *y*-axis is plotted the number of cells with that fluorescence intensity. The T47D control cell population profile is shown in Figure 3A with most of the cells in the G_1 phase. An increase in the G2/M DNA content of cells from 17% to 44% was observed when cells were treated with compound **1a** at 0.1 μM for 24 h (Figure 3B). After the cells were treated with 0.1 μM of compound **1a** for 48 h (Figure 3C), G2/M DNA content of cells increased to 57%. Concurrently, the subdiploid DNA content of cells (Figure 3C) increased from 0.9% to 12%. The subdiploid amount of DNA (M1) is indicative of apoptotic cells that have undergone DNA degradation and nuclear fragmentation.

Cell Growth Inhibition Assays (GI_{50}). Cells were grown and harvested as described above. An aliquot of 45 μL of cells (4.4 × 10^4 cells/mL) was added to a well of a 96-well microtiter plate, then 5 μL of 0.01−100 μM of test compound (0.001 to 10 μM final concentration) in RPMI-1640 media solution with 10% DMSO was added. An aliquot of 45 μL of cells was added

to a well of a 96-well microtiter plate containing 5 μL of RPMI-1640 media solution with 10% DMSO and without test compound as the control sample for maximal cell proliferation (L_{max}). The samples were then incubated at 37 °C for 48 h in a 5% CO_2−95% humidity incubator. After incubation, the samples were removed from the incubator and 25 μL of CellTiter-Glo reagent (Promega) was added. The samples were mixed by agitation and incubated at room temperature for 10−15 min. Plates were then read using a luminescent plate reader (Model Spectrafluor Plus Tecan Instrument) to the give L_{test} value.

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

Calculation. GI_{50} (dose for 50% inhibition of cell proliferation) is the concentration where $[(L_{test} - L_{start})/(L_{max} - L_{start})] \times 0.5$. The GI_{50} (μM) values of compound **1b**, **1e**, **1f**, **1g**, **2b**, **3h**, **5a**, and **5b** in T47D and DLD-1 cells are summarized in Table 2 in comparison with the caspase activation activity (EC_{50}).

Tubulin Polymerization Assay. Lyophilized tubulin (Cytoskeleton #ML113, 1 mg, MAP-rich) was assayed for the effect of the test compound on tubulin polymerization according to the recommended procedure of the manufacturer. To 1 μL of each experimental compound (from a 100 x stock) in a 96-well was added 99 μL of supplemented tubulin supernatant. Incubation was done in a Molecular Devices plate reader at 37 °C, and absorbance readings at 340 nm were recorded every minute for 1 h. The IC_{50} for tubulin inhibition was the concentration found to decrease the initial rate of tubulin polymerization by 50% as calculated with Prism 3.0.

Colchicine Binding Assay. A radioactive 96-well plate tubulin-binding assay was used to measure the ability of drugs to compete with [^{3}H]colchicine on tubulin. Briefly, 5.5 μg/well of biotin-labeled tubulin (Cytoskeleton, Denver, CO), diluted in 80 mM PIPES pH 6.8 containing 1 mM EGTA, 1 mM $MgCl_2$, and 1 mM GTP, was coated into a FlashPlate streptavidin-coated 96-well plate (Perkin-Elmer, Woodbridge, Ontario, Canada) and incubated for 30 min at 37 °C. The wells were then washed in a buffer of 80 PIPES pH 6.8, 1 mM EGTA, and 1 mM $MgCl_2$. A serial dilution of the compound to be tested was made (10^{-4}–10^{-9} M) in 80 mM PIPES, 1 mM EGTA, 1 mM $MgCl_2$, and 1 mM GTP, and 0.36 μM of [^{3}H]colchicine was added to a final volume of 100 μL. The reaction mixture was incubated at 37°C for 30 min, and the wells were then washed in 80 mM PIPES pH 6.8, 1 mM EGTA, and 1 mM $MgCl_2$. The wells were allowed to dry and then 50 μL of Scintisafe Econo 1 (Fisher Scientific, St-Laurent, Quebec, Canada) was added to each well and each was counted for radioactivity in a 1450 MicroBeta Trilux (Wallac, Turku, Finland). Each concentration of the drugs was performed in triplicate and IC_{50}'s were generated using the PrismPad computer program (Graph-Pad Software, Inc., San Diego, CA).

Supporting Information Available: Table of elemental analysis data for new and targeted compounds **1b**−**j**, **2a**−**e**, **3a**−**n**, **4a**−**f**, **5a**−**c**, and **10**. This material is available free of charge via the Internet at http://pubs.acs.org.

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