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Structure−Activity Relationship and Molecular Mechanisms of Ethyl 2-Amino-4-(2-ethoxy-2-oxoethyl)-6-phenyl-4*H*-chromene-3-carboxylate (sHA 14-1) and Its Analogues

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Rapid development of multiple drug resistance against current therapies is a major barrier in the treatment of cancer. Therefore, anticancer agents that can overcome acquired drug resistance in cancer cells are of great importance. Previously, we have demonstrated that ethyl 2-amino-4-(2-ethoxy-2-oxoethyl)-6-phenyl-4*H*-chromene-3-carboxylate (**5a**, sHA 14-1), a stable analogue of ethyl 2-amino-6-bromo-4-(1-cyano-2-ethoxy-2-oxoethyl)-4*H*-chromene-3-carboxylate (**6**, HA 14-1), mitigates drug resistance and synergizes with a variety of cancer therapies in leukemia cells. Structure−activity relationship (SAR) studies of **5a** guided the development of ethyl 2-amino-6-(3′,5′-dimethoxyphenyl)-4-(2-ethoxy-2-oxoethyl)-4*H*-chromene-3-carboxylate (**5q**, CXL017), a compound with low micromolar cytotoxicity against a wide-range of hematologic and solid tumor cells. More excitingly, our studies of **5q** in camptothecin (CCRF-CEM/C2) and mitoxantrone (HL-60/MX2) resistant cancer cells highlight its ability to selectively kill drug-resistant cells over parent cancer cells. **5q** inhibits tumor cell growth through the induction of apoptosis, with detailed mechanism of its selectivity toward drug-resistant cancer cells under investigation. These results suggest that **5q** is a promising candidate for treatment of cancers with multiple drug resistance.

Introduction

Cancer is a disease in which mutated and damaged cells continue to survive and proliferate beyond their normal life span. Extensive research has led to the development of a plethora of chemotherapeutic agents; however, none of these agents are capable of completely eliminating cancer.[1−4] Drug resistance, especially acquired drug resistance, is a prime cause contributing to the current inadequacy of chemotherapy and poses a major challenge in the treatment of cancer. Moreover, acquired drug resistance among cancer patients often leads to cross-resistance to a broad spectrum of anticancer agents, possibly by altering multiple pathways in cancer cells.[5,6] Because most anticancer agents in use have a low therapeutic index, increasing dosage to overcome drug resistance is not feasible. Consequently, there is a vital need to develop new therapeutic entities to surmount drug resistance to chemotherapy.

Most, if not all, classical chemotherapeutic agents eliminate cancerous cells via induction of apoptosis.[3,4] Therefore, alterations leading to evasion of apoptosis is a major mechanism by which cancer cells develop drug resistance. Apoptosis is a programmed cell death process that removes cells in an orderly way without triggering inflammation. Two pathways can activate this process: the intrinsic pathway and the extrinsic pathway. Both pathways lead to the activation of executioner caspases, such as caspase-3 and -7, that induce apoptosis via cleavage of proteins essential for cell survival.[7,8] Apoptosis is regulated by a fine balance between pro-apoptotic and antiapoptotic signals, and a disruption of this balance results in various diseases.[9,10] Overexpression of antiapoptotic factors assist cells to evade apoptosis, leading to cancer development and drug resistance. Hence, drugs that directly induce apoptosis by inhibiting antiapoptotic factors provide a promising strategy for overcoming drug resistance in cancer therapy.

B-cell lymphoma-2 (Bcl-2[a]) proteins are a key family of proteins that modulate apoptosis. In mammals, there are at least 24 Bcl-2 family proteins which can be classified into pro-apoptotic and antiapoptotic proteins. Overexpression of anti-apoptotic Bcl-2 family proteins is a major cause for drug resistance in cancers.[11,12] Therefore, antagonizing these proteins is a potential approach for overcoming drug resistance. Structurally, the antiapoptotic proteins consist of four conserved Bcl-2 homology (BH) domains BH1−BH4 and a transmembrane domain.[13] The transmembrane domain of these proteins helps them anchor to membranes in an organelle-specific manner.[14,15] The BH1, BH2, and BH3 domains of the antiapoptotic Bcl-2 family proteins form a long hydrophobic cleft that is proposed to physically interact with and functionally antagonize the pro-apoptotic proteins.[16] Agents that disrupt this interaction can possibly lead to the release of

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[a] Abbreviations: SAR, structure−activity relationship; Bcl-2, B-cell lymphoma-2; BH, Bcl-2 homology; ROS, reactive oxygen species; PARP, poly(ADP-ribose) polymerase; CT, camptothecin; MX, mitoxantrone; AML, acute myeloid leukemia; ALL, acute lymphoblastic leukemia; FP, fluorescent polarization; TPP, triphenylphosphine; PBS, phosphate-buffered saline; PVDF, polyvinylidine fluoride; CI, combination index.

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pro-apoptotic Bcl-2 family proteins, providing a plausible approach to overcome drug resistance induced by overexpression of antiapoptotic Bcl-2 family proteins.[16] Multiple antagonists of antiapoptotic Bcl-2 family proteins based on this approach have been developed and some are currently in clinical trials as single agents or in combination therapy.[17]

Ethyl 2-amino-6-bromo-4-(1-cyano-2-ethoxy-2-oxoethyl)-4*H*-chromene-3-carboxylate (**6**, HA 14-1, Figure 1) is an early Bcl-2 antagonist that was discovered through a fluorescent polarization (FP) based high-throughput screening[2] and has been shown to synergize with various anticancer therapies of diverse mechanisms of action.[18−20] In spite of its advantages, studies on **1** have revealed that it is unstable under standard tissue culture conditions with a half-life of 15 min.[21] Moreover, decomposition of **6** leads to reactive oxygen species (ROS) generation, which might be responsible for its observed activity instead of its ability to antagonize Bcl-2 proteins.

Efforts to identify a stable analogue of **6** led to the development of **5a** (Figure 1) that is stable in cell culture medium for more than 24 h and does not generate ROS.[22] In addition, **5a** retains the benefits of **6**, such as overcoming drug resistance and synergism with chemotherapeutic agents of varied mechanisms of action.[22] In this study, we will discuss the SAR of **5a** that led to the development of **5q** and provide preliminary biological evaluations.

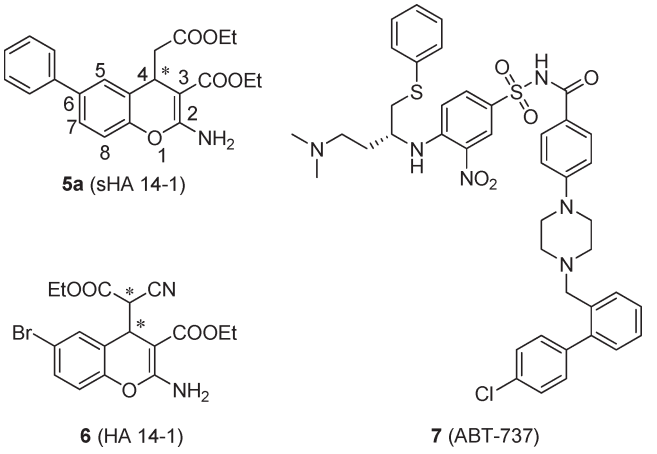


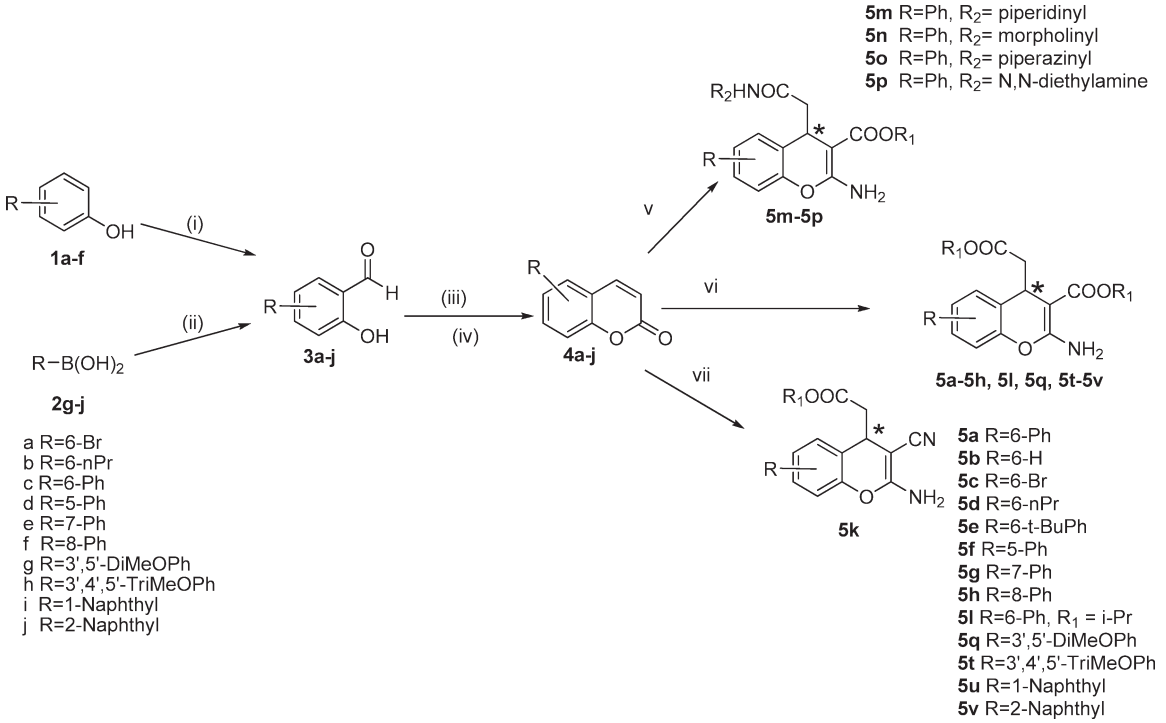
Figure 1. Structures of **5a**, **6**, and **7**.

Results

Design. 5a was designed based on the hypothesis that removal of the cyano group from **6** would lead to a decreased rate of decomposition and improved stability by reducing the stability of the carbanion formed during the decomposition of **6**.[21] Previous studies have demonstrated that **5a** is indeed a more stable analogue of **6**, with enhanced antagonism against antiapoptotic Bcl-2 proteins.[22] A systematic SAR study of **5a** was carried out with an aim to develop more potent analogues.

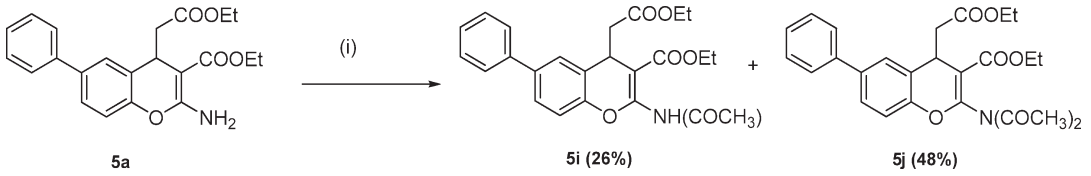
Structure−Activity Relationship of 5a. The design of analogues of **5a** began with substitutions at the 6-position of the chromene core with small to bulky groups of varying lipophilicity. Specifically, hydrogen, bromo, *n*-propyl, and *t*-butyl phenyl substituents were explored at the 6-position, affording analogues **5b**, **5c**, **5d**, and **5e**, respectively (Scheme1, Table 1). A preliminary in vitro cytotoxicity evaluation indicated that the cytotoxicity of these analogues generally increased with lipophilicity. However, the highly lipophilic and steric *t*-butyl phenyl analogue (**5e**) was slightly less active compared to **5a** (Table 1). A possible reason for this observation could be due to decreased solubility or unfavorable

Scheme 1[a]



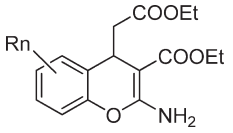
[a] Reagents and conditions: (i) $MgCl_2$, Et_3N, paraformaldehyde, anhydrous CH_3CN, reflux; (ii) 5-bromosalicylaldehyde, $Pd(OAc)_2$, K_2CO_3, H_2O, room temp; (iii) DMA, $POCl_3$ 60 °C, 4−8 h; (iv) $NaHCO_3$, 60 °C, 0.5 h; (v) ethyl cyanoacetate, benzene (C_6H_6)/dry ethanol, R_2-H (base), room temp; (vi) ethyl cyanoacetate, R_1OH, $NaOR_1$, room temp; (vii) malononitrile, R_1OH, $NaOR_1$, room temp.

Scheme 2[a]



[a] Reagents and condition: (i) Acetic anhydride, reflux, 8 h.

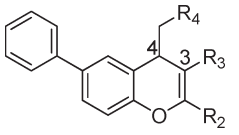
Table 1. IC_{50} Values (μM) for Analogues of **5a** with Substitution at positions 5, 6, 7, and 8 in JURKAT Cells

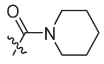
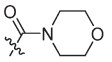
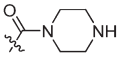


compd	R_5	R_6	R_7	R_8	$IC_{50} \pm SEM$[a]	CLogP[b]
5a	H	Ph	H	H	27.2 ± 3.4	5.8
5b	H	H	H	H	91.43 ± 6.16	2.8
5c	H	Br	H	H	57.29 ± 2.53	4.9
5d	H	*n*-Pr	H	H	57.27 ± 1.58	5.5
5e	H	*t*-BuPh	H	H	35.51 ± 2.99	7.6
5f	Ph	H	H	H	44.3 ± 3.2	5.8
5g	H	H	Ph	H	51.6 ± 2.8	5.8
5h	H	H	H	Ph	49.8 ± 3.0	5.8

[a] Results are given as the mean of three independent experiments with triplicate in each experiment. [b] The ClogP values mentioned in this table were calculated using the ChemBiodraw Ultra software.

Table 2. IC_{50} Values (μM) for Analogues of **5a** with Modification at Position 2, 3, and 4 in JURKAT Cells



Compound	R_2	R_3	R_4	$IC_{50} \pm SEM$[a]
5i	$NH(COCH_3)$	H	H	67.7 ± 1.5
5j	$N(COCH_3)_2$	H	H	70.6 ± 5.2
5k	NH_2	CN	OEt	52.5 ± 0.9
5l	NH_2	COOiPr	COOiPr	38.7 ± 4.1
5m	NH_2	COOEt		58.5 ± 2.6
5n	NH_2	COOEt		>100
5o	NH_2	COOEt		53.7 ± 1.1
5p	NH_2	COOEt		49.6 ± 0.3

[a] Results are given as the mean of three independent experiments with triplicate in each experiment.

steric interactions with the binding site of its biological target.

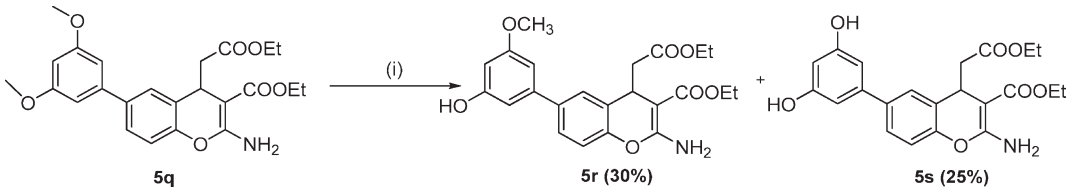
As the modifications at the 6-position did not improve the activity of **5a**, the effect of shifting the phenyl ring to various positions on the chromene core (5, 7, and 8) was next explored, providing analogues **5f**, **5g**, and **5h**, respectively (Scheme 1, Table 1). In vitro cytotoxicity evaluations showed that the phenyl substituent at position 5, 7, or 8 resulted in decreased cytotoxicity compared to **5a**. Hence, the phenyl substituent at the 6-position was retained for further studies.

The effect of altering functional groups at other positions (namely 2, 3, and 4) on the chromene nucleus was next examined. The role of the 2-amino group was probed by synthesis and evaluation of the monoacetylated (**5i**) and diacetylated (**5j**) analogues (Scheme 2, Table 2). Replacement of one or both hydrogen atoms on the amino group led to decreased cytotoxicity compared to the parent compound **5a**. Next, the effect of other functional groups at the 3-position was evaluated. This was achieved by replacing the ethyl ester with a cyano functional group, leading to analogue **5k** (Scheme 1, Table 2). Biological evaluation indicated that **5k** was less active than the parent compound, **5a**. Additionally, replacing the ethyl ester with an isopropyl ester at the 3- and 4-position also resulted in a slight decrease in cytotoxicity demonstrated by analogue **5l** (Scheme 1, Table 2). Further exploration of the influence of the ethyl ester functionality at the 4-position led to the synthesis of various amides such as piperidinyl, morpholinyl, piperazinyl, and diethylamino analogues **5m**−**5p** (Scheme 1, Table 2). Unfortunately, all of the amide analogues exhibit decreased cytotoxicity compared to the parent **5a**.

Having studied the various modifications on the chromene core structure with no success in improving activity of **5a**, we sought to explore the SAR on the 6-phenyl ring. We started by substituting the 6-phenyl ring with a 3′,5′-dimethoxy substituent. This modification led to the development of **5q**, which is ~30 times more cytotoxic than **5a** (Table 3). Demethylation of **5q** led to the synthesis of compounds **5r** and **5s**, as depicted in Scheme 3. Conversion of a single methoxy group to a hydroxyl group led to a 4-fold decrease in cytotoxicity and replacing both methoxy groups with hydroxyl groups led to a 16-fold decrease in cytotoxicity (Table 3). Subsequently, a 3′,4′,5′-trimethoxy analogue (**5t**) was synthesized. **5t** showed a 14-fold decrease in activity compared to **5q** (Table 3), suggesting that the 4′-position of the 6-phenyl ring does not tolerate substitution. To further confirm the steric effect at the 4′-position of 6-phenyl ring, we synthesized the 1-naphthyl and 2-naphthyl analogues **5u** and **5v**, respectively. Biological evaluation of these compounds showed that **5u** is 3-fold more cytotoxic than **5v**, supporting the notion that substitution at the 4′-position of the 6-phenyl ring would result in decreased cytotoxicity. This is possibly due to a steric effect, consistent with 4′-*t*-butylphenyl substitution in **5e** (Table 1).

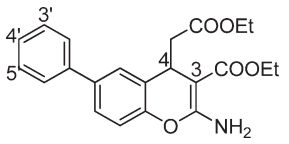
In Vitro Cytotoxicity of 5q in Various Cancer Cell Lines. As **5q** was ~30-fold more cytotoxic than **5a** in JURKAT cells, we further explored its activity in other cancer cell lines. In vitro cytotoxicity studies of **5q**, **5a**, and **7** (ABT-737)[23] (Figure 1), a well-known Bcl-2 antagonist, in 11 different malignant cell lines demonstrated that **5q** was universally

Scheme 3[a]



[a] Reagents and condition: (i) BBr_3 5 equiv, dry CH_2Cl_2, 3 Å molecular sieves, −78 to 0 °C, 4 h.

Table 3. IC_{50} Values for Analogues of **5a** with Modification on the 6-Phenyl Ring in JURKAT Cells



Compound	$R_{2'}$	$R_{3'}$	$R_{4'}$	$R_{5'}$	$IC_{50} \pm SEM$[a]
5q	H	OCH_3	H	OCH_3	1.2 ± 0.1
5r	H	OH	H	OCH_3	4.2 ± 0.7
5s	H	OH	H	OH	18.3 ± 2.7
5t	H	OCH_3	OCH_3	OCH_3	13.6 ± 0.1
5u			H	H	5.83 ± 0.02
5v	H			H	18.7 ± 3.2

[a] Results are given as the mean of three independent experiments with triplicate in each experiment.

more cytotoxic than **5a** and was of comparable cytotoxicity to **7** (Table 4).

Induction of PARP Cleavage and Caspase-3/7 Activation by 5q in NALM-6 Cells. Poly(ADP-ribose) polymerase (PARP) is a protein involved in a number of cellular processes including DNA repair and programmed cell death. PARP cleavage is carried out by caspases during apoptosis and is a biomarker for apoptosis. NALM-6 cells treated with varying concentrations of **5q** for 24 h show a dose-dependent increase in PARP cleavage with the distinct cleavage band observable from 3.1 μM. Complete cleavage was observed at 25 μM. Slight cleavage was observed with **5a** at a concentration of 50 μM (Figure 2A). Capsase-3/7 activation, another marker for apoptosis, was also examined. Treatment of NALM-6 cells with varying concentrations of **5q** and **5a** for 24 h induces caspase-3/7 activation in a dose-dependent manner. Consistent with its increased cytotoxicity, **5q** induced higher caspase-3/7 activation at a much lower concentration than **5a** (Figure 2B).

Characterization of Camptothecin and Mitoxantrone Resistant Leukemia Cell Lines. CCRF-CEM/C2 and HL-60/MX2 cells obtained from ATCC are camptothecin (CT) and mitoxantrone (MX) drug-resistant forms of CCRF-CEM and HL-60 parent cells, respectively.[24−27] To establish the resistant nature of CCRF-CEM/C2 and HL-60/MX2 cells, the cytotoxicity of CT and MX in CCRF-CEM and HL-60 parent and resistant cells were evaluated, respectively. CCRF-CEM/C2 cells demonstrated a 1879-fold loss of sensitivity to CT compared to the parent CCRF-CEM cells

Table 4. IC_{50} Values (μM) for **5q** in Comparison with **5a** in 11 Different Cancer Cell Lines

cell line	**5q**	**5a**	**7**	type of cancer
JURKAT	1.2	27.2	1.38	T-cell leukemia
NALM-6	4.74	44.2	N/A[a]	B-cell leukemia
Reh	4.16	20.18	N/A	B-cell leukemia
DU-145	11.49	14.73	27.6	prostate cancer
HCT-116	8.83	18.49	4.06	colon cancer
Hepa1c1c7	10.87	22.78	8.68	hepatocarcinoma
H460	5.14	15.53	8.03	lung cancer
MCF7	15.48	18.87	21.26	breast cancer
SU86.86	7.03	18.69	4.24	ductal carcinoma

[a] N/A: not available.

(Figure 3A). HL-60/MX2 cells demonstrated a 23-fold loss of sensitivity to MX compared to the parent HL-60 cells (Figure 3B). Because overexpressing antiapoptotic Bcl-2 family proteins is one of the major mechanisms by which cancer cells acquire drug resistance, the expression levels of several antiapoptotic Bcl-2 family proteins, specifically Bcl-2, Bcl-X_L, and Mcl-1, in CCRF-CEM, CEM/C2, HL-60, and HL-60/MX2 cells were evaluated by Western Blot analysis. As shown in Figure 3C, CCRF-CEM/C2 cells overexpress Bcl-X_L and Mcl-1, while HL-60/MX2 cells overexpress Bcl-2 and Mcl-1 compared to their corresponding parent cells.

We next evaluated the sensitivity of these drug-resistant cells to the major first line therapies for acute myeloid leukemia (AML) and acute lymphoblastic leukemia (ALL) including cytarabine, 6-thioguanine, doxorubicin, and vincristine.[28−31] Studies showed that these drug-resistant cell lines demonstrate cross-resistance to doxorubicin and cytarabine while displaying similar sensitivity to others such as vincristine and 6-thioguanine (Figures 4 and 5).

Cytotoxicity of 5q in CCRF-CEM and HL-60 Parent and Resistant Cells. We next evaluated the ability of **5q** to target MX and CT resistant cells, using **7** as a control. Interestingly, both HL-60/MX2 and CCRF-CEM/C2 cells demonstrated collateral sensitivity to **5q** − more sensitive to **5q** than their corresponding parent cells (Figure 6). Conversely, HL-60/MX2 cells were resistant to **7**, while CCRF-CEM/C2 cells were collaterally sensitive to **7** (Figure 6).

Combination of 5q with Mitoxantrone and Camptothecin in HL-60 and CCRF-CEM Resistant Cells. Subsequently combination studies were carried out to evaluate the potential of **5q** to function as a chemosensitizer to help overcome drug resistance. Specifically, studies of **5q** in combination with MX and CT in HL-60/MX2 and CCRF-CEM/C2 cells, respectively, were carried out to determine if **5q** could sensitize the resistant cells. The combination index of these drugs were analyzed using the Chou and Talalay method (Table 5). Moderate synergistic effect was observed when **5q** was administered in combination with MX in HL-60/MX2

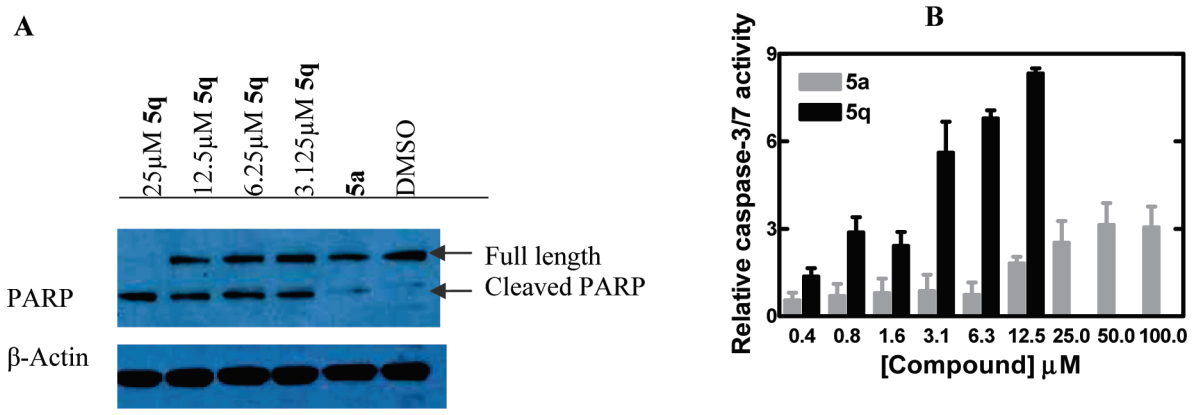
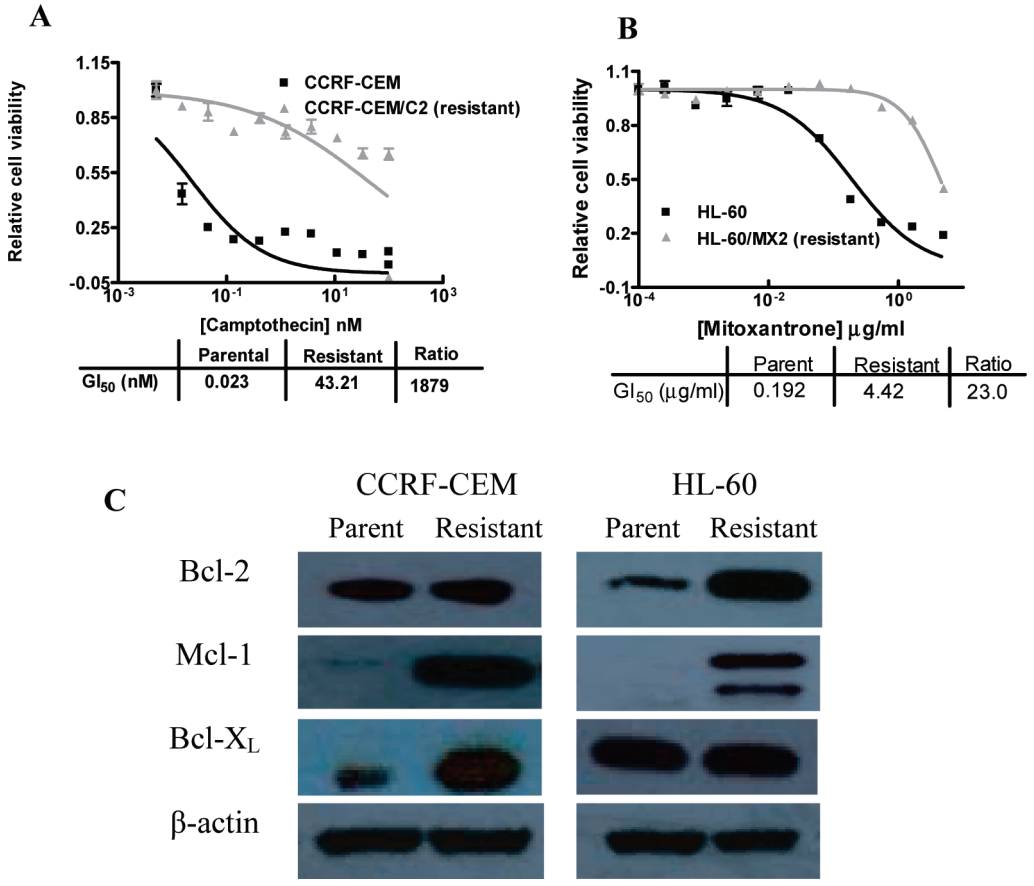


Figure 2. (A) Dose-dependent PARP cleavage in NALM-6 cells treated with **5q** and **5a** (50 μM) for 24 h. (B) Dose−response caspase-3/7 activation in NALM-6 cells induced after treatment with **5q** and **5a** for 24 h.



	Parental	Resistant	Ratio
GI_{50} (nM)	0.023	43.21	1879

	Parent	Resistant	Ratio
GI_{50} (μg/ml)	0.192	4.42	23.0

Figure 3. In vitro cytotoxicity of camptothecin in (A) CCRF-CEM and mitoxantrone in (B) HL-60 parent and resistant cells. (C) Western Blot analysis of overexpression of antiapoptotic Bcl-2 family proteins.

cells, while a combination of **5q** with CT in CCRF-CEM/C2 cells showed antagonism.

Discussion and Conclusion

Compound **5a**, an analogue of **6**, was stable in cell culture medium for more than 24 h and did not generate ROS. In addition, **5a** retained the benefits of **6**, such as overcoming drug resistance and synergism with chemotherapeutic agents of various mechanisms of action.[22] SAR studies of **5a** starting with substitution at the 6-position of the chromene nucleus established that a phenyl ring substitution at the 6-position was optimal for cytotoxicity. Modifications at positions 2, 3, 4, 5, 7, and 8 on the chromene nucleus all led to a decrease in activity, suggesting that these functional groups play an important role in the cytotoxicity of **5a**. Substituting the 6-phenyl ring with methoxy groups at the 3′,5′-position led to the discovery of **5q** with a 30-fold increase in potency compared to **5a**. Subsequent studies on the effect of the methoxy groups resulted in the synthesis of **5r** and **5s**. **5r** (3′-OH, 5′-OCH_3) demonstrated a 4-fold decrease in activity compared to **5q**, while **5s** (3′,5′-diOH) exhibited a 16-fold decrease in activity, suggesting that the methoxy substituents were critical for the activity of **5q**. These results prompted us to substitute the

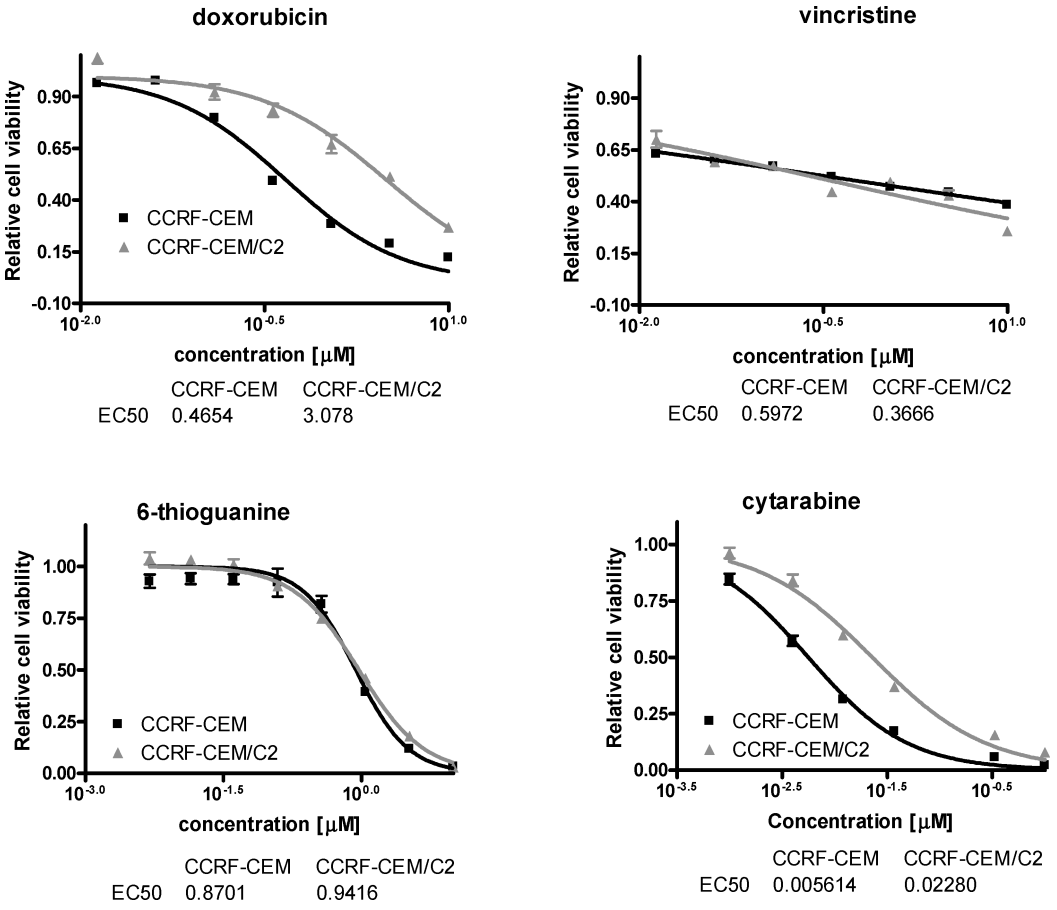


Figure 4. Cross-resistance studies in CCRF-CEM/C2 and CCRF-CEM cell lines.

phenyl ring with methoxy groups at 3′, 4′, and 5′-position, leading to **5t**. However, **5t** was 13-fold less potent in activity when compared to **5q**, indicating that the 4′-position on the phenyl ring is sensitive to substitutions. These results led us to explore the steric effect on the 4′-position of the 6-phenyl ring. Replacing the phenyl ring with 1-napthyl and 2-napthyl groups led to compounds **5u** and **5v**. Compound **5v** was 3-fold less potent than **5u** in activity. Because 1-napthyl and the 2-napthyl group only differ in their spatial orientation, these results suggest that substitution at the 4′-position of 6-phenyl ring leading to a decrease in activity is likely due to a steric effect.

Compound **5q**, the most potent analogue of this series, is 30-fold more potent than **5a** in JURKAT cells. It also demonstrates improved potency over **5a** in a variety of solid and hematological malignancies. An insight into the mechanism of action of **5q** demonstrated that it induces cell death via apoptosis as characterized by activation of caspase-3/7 and induction of PARP cleavage in a dose-dependent manner.

Overcoming drug resistance is an important feature of **5a**, and hence further studies were carried out on **5q** to determine its ability to overcome drug resistance. Upregulation of antiapoptotic Bcl-2 family proteins has been extensively associated with chemoresistance and poor outcomes in hematopoetic malignancies.[10,32] Because **5a** and its analogues are putative Bcl-2 antagonists, we sought to determine their ability to overcome drug resistance in hematological malignancies overexpressing Bcl-2 proteins. For this purpose, we acquired CCRF-CEM and HL-60 cells resistant to CT (CCRF-CEM/C2) and MX (HL-60/MX2), respectively[24−27] and characterized their expression levels for antiapoptotic Bcl-2 proteins, including Bcl-2, Bcl-X_L, and Mcl-1. Western Blot analysis revealed the overexpression of Mcl-1 protein in both drug-resistant cell lines. Bcl-2 protein was overexpressed in HL-60/MX2 cells, while Bcl-X_L was overexpressed in CCRF-CEM/C2-resistant cells. Cytotoxic evaluation of these cells with current major chemotherapeutic agents for the treatment of ALL and AML, cytarabine, doxorubicin, vincristine, and 6-thioguanine, demonstrated that CCRF-CEM/C2 cells were cross-resistant to doxorubicin and cytarabine while HL-60/MX2 cells were cross-resistant to doxorubicin. Both cells demonstrated similar sensitivity to the rest of the drugs compared to their parent cells. These results suggest that CCRF-CEM/C2 and HL-60/MX2 demonstrate certain cross-resistance to standard therapies.

In vitro cytotoxicity studies of **5q** with HL-60/MX2 and CCRF-CEM/C2 cell lines revealed that both cell lines were collaterally sensitive to **5q**. While compound **7**, a well-known Bcl-2 antagonist, demonstrated collateral sensitivity with CCRF-CEM/C2, it was insensitive to HL-60/MX2 cells. Although both resistant cell lines overexpress antiapoptotic Bcl-2 proteins, the inability of **7** to overcome resistance in HL-60/MX2 cells suggests that there might be multiple pathways apart from overexpressing antiapoptotic Bcl-2 family proteins that are responsible for drug resistance in these cell lines, especially in HL-60/MX2. **5q**, with selective cytotoxicity against both resistant cell lines, may be a good lead candidate as a single agent for drug-resistant cancer treatment and is currently under in vivo evaluation.

We next explored if **5q** could synergize with CT and MX when treated against drug-resistant CCRF-CEM/C2 and

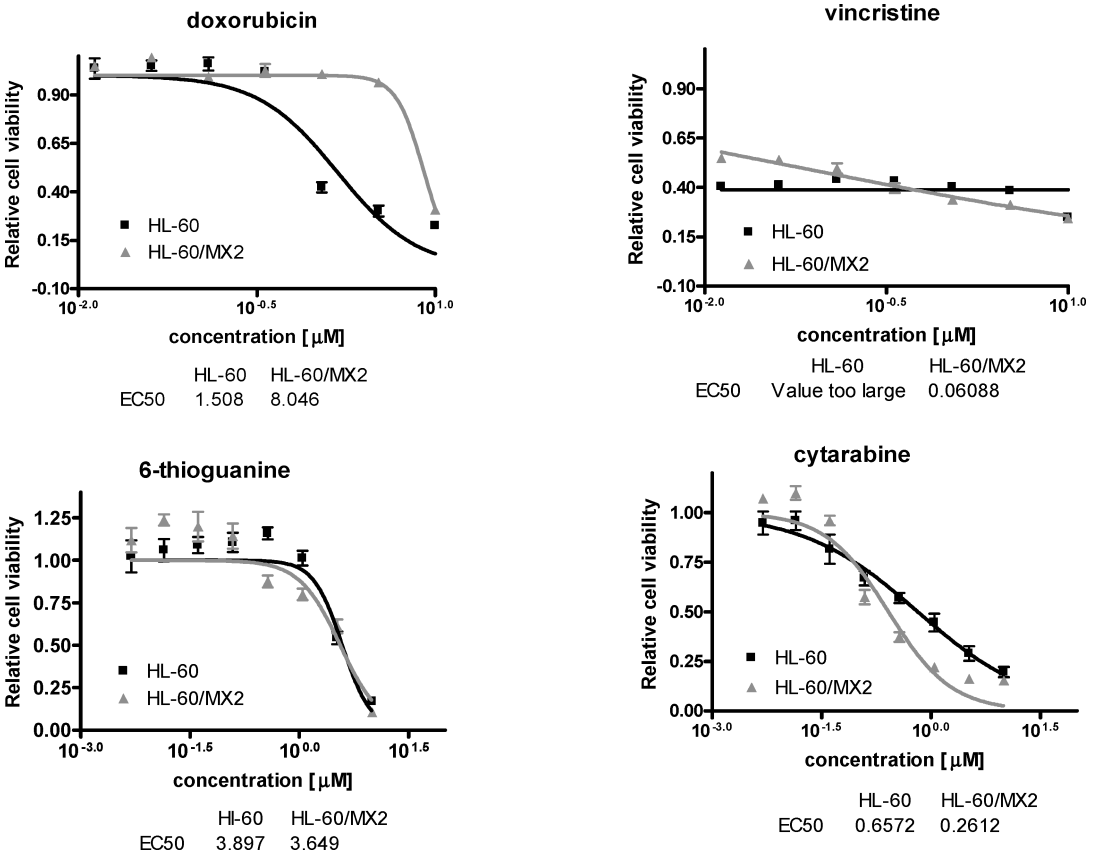


Figure 5. Cross-resistance studies in HL-60/MX2 and HL-60 cell lines.

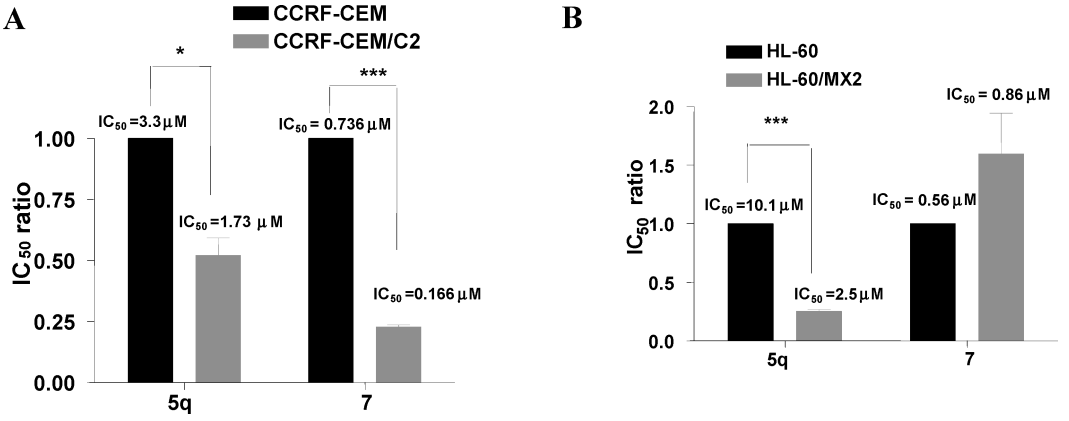


Figure 6. Sensitivity of (A) camptothecin-resistant CCRF-CEM cells and (B) mitoxantrone-resistant HL-60 cells to **5q** and **7**. (* $P < 0.01$, ** $P < 0.001$, *** $P < 0.0001$ for the comparison between CCRF-CEM and HL-60 parent and resistant cells treated with **5q** or **7** at all concentrations tested).

HL-60/MX2. Intriguingly, results from these studies demonstrate that **5q** moderately synergizes with MX in HL-60/MX2-resistant cells but it antagonizes with CT in CCRF-CEM/C2-resistant cells. These results further suggest that the mechanisms of drug resistance in these two cell lines are probably different. Although overexpression of antiapoptotic Bcl-2 family proteins likely plays a role in development of drug resistance in these cell lines, there are other cellular changes that may contribute to drug resistance.[5] Therefore, it may be necessary to target multiple cellular changes to effectively overcome drug resistance.

Because **5q** was more potent than **5a** in cytotoxicity and **5a** is a putative antagonist against antiapoptotic Bcl-2 family proteins, we performed a FP assay to determine its affinity toward various antiapoptotic Bcl-2 proteins following established procedures.[33] Our results revealed that **5q** failed to disrupt the interaction between Flu-Bak peptide and the antiapoptotic Bcl-2 family proteins, including Bcl-2, Bcl-X_L, and Mcl-1. As overexpressing antiapoptotic Bcl-2 family proteins in HL-60/MX2 and CCRF-CEM/C2 drug-resistant cells fail to induce resistance to **5a** and **5q**, it is possible that **5a** and **5q** activate apoptotic machineries that are downstream of antiapoptotic Bcl-2 family proteins and that are intact in the two drug-resistant cancer cells, supported by the successful induction of apoptosis by **5q** in NALM-6 cells. On the other hand, the antiapoptotic Bcl-2 proteins used in the competition-based

Table 5. Dose−Effect Relationships of Single Drugs **5q**, Mitoxantrone (MX), Camptothecin (CT), and Their Combinations in HL-60/MX2 and CCRF-CEM/C2 Resistant Cells[a]

single drugs and combinations	parameters *r*	CI value			
		ED_{50}	ED_{75}	ED_{90}	ED_{95}
5q	0.99				
MX	0.99				
5q + MX (2:1)	0.95	1.19 ± 0.42	0.86 ± 0.17	0.74 ± 0.12	0.74 ± 0.21
5q	0.95				
CT	0.94				
5q + CT(1:4)	0.95	1.04 ± 0.27	1.18 ± 0.41	1.59 ± 0.74	2.14 ± 1.2

[a] D_m: Median-effect dose (concentration which inhibits cell growth by 50%, **5q**, MX, CT, μM); *r*: linear correlation coefficient of the median-effect plot (indicates conformity of data). CI (combination index) was calculated by the combination index equation of Chou and Talalay. CI < 1, CI = 1, and CI > 1 indicate synergism, additive effect, and antagonism, respectively at varied effective dose (ED_{50}, ED_{75}, ED_{90}, and ED_{95}) levels. All values shown above are data obtained from three different experiments.

assay may not fully mimic the naturally membrane-bound proteins that the results based on this assay can not completely rule out the possibility of **5a** and its analogues as antagonists against antiapoptotic Bcl-2 family proteins. Further investigations are undergoing to elucidate the detailed mechanism for the collateral sensitivity of **5q** toward drug-resistant cancer cells, especially toward identifying its cellular target(s).

Material and Methods

Chemistry. All commercial reagents and anhydrous solvents were purchased from vendors and were used without further purification or distillation unless otherwise stated. Analytical thin layer chromatography was performed on Whatman silica gel 60 Å with fluorescent indicator (partisil K6F). Compounds were visualized by UV light and/or stained with potassium permanganate solution followed by heating. Flash column chromatography was performed on Whatman silica gel 60 Å (230−400 mesh). NMR (^{1}H, ^{13}C) spectra were recorded on a Varian 300/400 MHz or a Bruker 400 MHz spectrometer and calibrated using an internal reference. ESI mode mass spectra were recorded on a Bruker BiotofII mass spectrometer. All compounds synthesized are racemic mixtures and are more than 95% pure, analyzed using elemental analyses or HPLC. M−H−W Laboratories, Phoenix, AZ, performed the elemental analyses. **7** was synthesized by following reported procedures.[23]

General Procedure for the Synthesis of Salicylaldehyde. 5-Bromosalicylaldehyde (1 g, 4.97 mmol), K_2CO_3 (2.061 g, 14.91 mmol), boronic acid (0.9954 g, 5.47 mmol), triphenylphosphine (TPP) (1 mol %), and $Pd(OAc)_2$ (1 mol %) were taken in DME:water (1:1) (12 mL). The mixture was stirred at room temperature under an atmosphere of nitrogen for 24 h. The reaction mixture was acidified using HCl (1N) on an ice bath, followed by extraction with ethyl acetate. The extracts were combined, dried ($MgSO_4$), and the solvent was removed under vacuum. The crude solid was purified by flash chromatography to isolate the desired salicylaldehyde.

2-Hydroxy-5-(3′,5′-dimethoxyphenyl)benzaldehyde (3g). Yield: 45%. ^{1}H NMR ($CDCl_3$): δ 11.01 (1H, s, OH), 9.98 (1H, s, CHO), 7.77−7.74 (2H, m, Ar Hs), 7.08 (1H, d, J = 9 Hz, Ar H), 6.67 (2H, d, J = 3 Hz, 2′, 6′-H), 6.48−6.46 (1H, m, 4′-H), 3.86 (6H, s, 3′,5′-OCH_3). ^{13}C NMR (100 MHz, $CDCl_3$): δ 196.63, 161.27 161.15, 135.74, 133.23, 131.91, 120.62, 118.05, 105.01, 99.14, 55.45. ESI-MS (positive): m/z 281.27 $(M + Na)^+$

2-Hydroxy-5-(3′,4′,5′-trimethoxyphenyl)benzaldehyde (3h). Yield: 38%. ^{1}H NMR ($CDCl_3$): δ 11.01 (1H, s, OH), 9.98 (1H, s, CHO), 7.74−7.70 (2H, m, Ar Hs), 7.08 (1H, d, J = 8.4 Hz, Ar H), 6.72 (2H, s, 2′,6′-H), 3.94 (6H, s, 3′,5′-OCH_3), 3.89 (3H, s, 4′-OCH_3). ^{13}C NMR (75 MHz, $CDCl_3$): δ 196.58, 160.95, 153.65, 137.77, 135.70, 135.36, 133.49, 131.73, 120.62, 118.11, 104.64, 104.05, 60.98, 56.27. ESI-MS (positive): m/z 311.3 $(M + Na)^+$

2-Hydroxy-5-(napthalen-1-yl)benzaldehyde (3i). Yield: 40%. ^{1}H NMR ($CDCl_3$): δ 11.01 (1H, s, OH), 9.87 (1H, s, CHO), 8.2 (1H, d, J = 1.6 Hz, 6H), 8.1−8.02 (5H, m, Ar), 7.86 (1H, dd, J = 1.6, 8.4 Hz, 4H), 7.6 (2H, m, Ar), 7.14 (1H, d, J = 8.4 Hz, 3-H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 196.63, 160.95, 138.69, 136.7, 135.2, 134.73, 133.86, 132.59, 128.48, 128.07, 127.02, 126.37, 125.99, 125.99, 125.40, 120.50, 117.65. ESI-MS (positive) m/z 271.28 $(M + Na)^+$

2-Hydroxy-5-(napthalen-2-yl)benzaldehyde (3j). Yield: 52%. ^{1}H NMR ($CDCl_3$): δ 11.05 (1H, s, OH), 10.03 (1H, s, CHO), 8.0 (1H, d, J = 1.6 Hz, 6-H), 7.94−7.86 (5H, m, Ar), 7.71 (1H, dd, J = 1.6, 8.4 Hz, 4-H), 7.55−7.48 (2H, m, Ar), 7.13 (1H, d, J = 8.4 Hz, 3-H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 196.73, 160.82, 136.61, 136, 133.64, 133.24, 132.54, 132.14, 128.76, 128.07, 127.69, 126.57, 126.13, 125.23, 124.88, 120.77, 118.27. ESI-MS (positive) m/z 271.28 $(M + Na)^+$

General Procedure for the Synthesis of Coumarin. To *N*,*N*-dimethylacetamide (1.98 mmol) stirred at 0 °C, phosphorus oxychloride (1.98 mmol) was added slowly. The reaction mixture was allowed to stir at 0 °C for 30 min followed by addition of the corresponding salicylaldehyde (0.99 mmol). The reaction mass was then heated at 68−70 °C for 3 h. Following this, the reaction mass was cooled to room temperature and saturated $NaHCO_3$ solution (10 mL) was added to it. The reaction mass was heated at 68−70 °C for another 30 min, cooled, and acidified (1 N HCl), followed by extraction with methylene chloride. The extracts were combined, dried (anhydrous $MgSO_4$), and concentrated under reduced pressure to afford a residue, which upon column chromatography afforded the desired coumarin.

6-(3′,5′-Dimethoxyphenyl)-2*H*-chromen-2-one (4g). Yield: 53%. ^{1}H NMR ($CDCl_3$): δ 7.77−7.65 (3H, m, Ar), 7.41 (1H, d, J = 9 Hz, 8-H), 6.69 (2H, m, Ar), 6.49 (2H, m), 3.85 (6H, s, 3′,5′-OCH_3). ^{13}C NMR (75 MHz, $CDCl_3$): δ 161.26, 160.65, 155.49, 153.57, 143.42, 141.56, 137.77, 130.76, 126.11, 118.96, 117.21, 117.07, 105.47, 99.48, 55.47. ESI-MS (positive): m/z 305.29 $(M + Na)^+$

6-(3′,4′,5′-Trimethoxyphenyl)-2*H*-chromen-2-one (4h). Yield: 50%. ^{1}H NMR ($CDCl_3$): δ 7.77 (2H, d, J = 9.6 Hz, 2′,6′-H), 7.73 (1H, dd, J = 2.4, 8.4 Hz, 7-H), 7.63 (1H, d, J = 2.2 Hz, 5-H), 7.41 (1H, d, J = 8.7 Hz, 8-H), 6.75 (1H, m), 6.49 (1H, d, J = 9.6 Hz, CHCO), 3.94 (6H, s, 3′,5′-OCH_3), 3.90 (3H, s, 4′-OCH_3). ^{13}C NMR (75 MHz, $CDCl_3$): δ 160.05, 153.8, 153.61, 143.62, 138.22, 135.61, 130.95, 126.18, 119.22, 117.48, 117.39, 109.99, 61.24, 56.51. ESI-MS (positive): m/z 335.32 $(M + Na)^+$

6-(Naphthalene-1-yl)-2*H*-chromene-2-one (4i). Yield: 55%. ^{1}H NMR ($CDCl_3$): δ 7.94−7.89 (2H, m, Ar),7.82 (1H, J = 8.4 Hz, Ar), 7.75 (1H, J = 9.6 Hz, 4-H), 7.66 (1H, J = 8.4 Hz, Ar), 7.59−7.41 (6H, m, Ar), 6.50 (1H, J = 9.6 Hz, CHCO). ^{13}C NMR (100 MHz, $CDCl_3$): δ 160.76, 153.38, 143.43, 138.19, 137.23, 133.81, 133.63, 131.45, 128.95, 128.48, 128.29, 127.17, 126.45, 126.16, 126.05, 125.39, 118.77, 117.07, 116.79. ESI-MS (positive): m/z 295.3 $(M + Na)^+$

6-(Naphthalene-2-yl)-2*H*-chromene-2-one (4j). Yield: 63.8%. ^{1}H NMR ($CDCl_3$): δ 8.04 (1H, d, J = 1.6 Hz, 5-H), 7.97−7.94 (1H, d, J = 8.8 Hz, 4H), 7.93−7.88 (3H, m, Ar), 7.83−7.80 (2H, m, Ar), 7.72 (1H, dd, J = 1.6, 8.4 Hz, 7-H), 7.55−7.51 (2H, m, Ar), 7.45 (1H, d, J = 8.4 Hz, 8-H), 6.49 (1H, d, J = 9.6 Hz, CHCO). ^{13}C NMR (100 MHz, $CDCl_3$): δ 206.89, 160.65, 153.48, 143.51, 136.67, 133.58, 132.72, 131.00, 128.82, 128.16, 127.71, 126.64, 126.35, 126.31, 125.92, 125.12. 119.13, 117.40, 117.11. ESI-MS (positive): m/z 295.3 $(M + Na)^+$

General Procedure for the Synthesis of Substituted Ethyl-4*H*-chromene-3-carboxylate Compounds (5a−5h). Freshly cut sodium (0.096 mmol) was added to anhydrous ethanol (2 mL), followed by the addition of ethyl cyanoacetate (0.192 mmol). The reaction mixture was stirred at room temperature under an inert atmosphere for 30 min, followed by the addition of a solution of the corresponding coumarin (0.08 mmol) in anhydrous ethanol (1 mL). The resulting reaction mixture was stirred at room temperature. Upon consumption of the coumarin, the reaction mass was concentrated, diluted with water (30 mL), and extracted using methylene chloride (3 × 20 mL). The organics were combined, dried ($MgSO_4$), and the solvent removed under vacuum to afford an oil. This crude oil was subjected to column chromatography to afford the pure product.

Ethyl 2-Amino-4-(2-ethoxy-2-oxoethyl)-6-phenyl-4*H*-chromene-3-carboxylate (5a). Yield: 53%. ^{1}H NMR ($CDCl_3$): δ 7.44 (7H, m, Ar), 7.03 (1H, d, J = 8.4 Hz, 8-H), 6.32 (2H, br s, NH_2), 4.36 (1H, dd, J = 4.5, 6.9 Hz, 4-H), 4.24 (2H, q, J = 7.2 Hz, $COOCH_2CH_3$), 4.02 (2H, q, J = 7.2 Hz, $COOCH_2CH_3$), 2.70 (1H, dd, J = 4.8, 15.0 Hz, HCHCO), 2.62 (1H, dd, J = 6.9, 15.0 Hz, HCHCO), 1.34 (3H, t, J = 6.9 Hz, $COOCH_2CH_3$), 1.12 (3H, t, J = 7.2 Hz, $COOCH_2CH_3$). ^{13}C NMR (75 MHz, $CDCl_3$): δ 171.88, 169.21, 161.61, 149.58, 140.43, 137.61, 128.92, 127.31, 127.19, 126.99, 126.52, 126.03, 116.276, 60.36, 59.69, 43.79, 31.48, 14.74, 14.21. HRMS-ESI (positive) calcd for $(C_{22}H_{23}NO_5 + Na)^+$ 404.1468; found 404.08 $(M + Na)^+$. Anal. ($C_{22}H_{23}NO_5$) C, H, N. Calcd C, 69.28; H, 6.08; N, 3.67. Found C, 69.86; H, 6.24; N, 3.44.

Ethyl 2-Amino-4-(2-ethoxy-2-oxoethyl)-4*H*-chromene-3-carboxylate (5b). Yield: 54%. ^{1}H NMR ($CDCl_3$): δ 7.21 (2H, m, Ar), 7.07 (1H, dt, J = 1.5, 7.5, 8.7 Hz, Ar H), 6.96 (1H, dd, J = 1.2, 8.1 Hz) 6.34 (2H, br s, NH_2), 4.29 (1H, dd, J = 4.8, 7.5 Hz, 4-H), 4.22 (2H, q, J = 6.9 Hz, $COOCH_2CH_3$), 4.03 (2H, q, J = 7.2 Hz, $COOCH_2CH_3$), 2.64 (1H, dd, J = 4.8, 15.0 Hz, HCHCO), 2.57 (1H, dd, J = 7.2, 14.7 Hz, HCHCO), 1.32 (3H, t, J = 7.2 Hz, $COOCH_2CH_3$), 1.15 (3H, t, J = 7.2 Hz, $COOCH_2CH_3$). ^{13}C NMR (75 MHz, $CDCl_3$): δ 171.69, 169.03, 161.38, 149.29, 131.38, 130.84, 128.03, 117.78, 116.87, 76.51, 60.59, 59.87, 43.56, 31.31, 14.79, 14.35. HRMS-ESI (positive) calcd for $(C_{16}H_{19}NO_5 + Na)^+$: 328.1155; found 328.1128 $(M + Na)^+$. Anal. ($C_{16}H_{19}NO_5$) C, H, N. Calcd C, 62.94; H, 6.27; N, 4.59. Found C, 62.94; H 6.19; N, 4.52.

Ethyl 2-Amino-6-bromo-4-(2-ethoxy-2-oxoethyl)-4*H*-chromene-3-carboxylate (5c). Yield: 52%. ^{1}H NMR ($CDCl_3$): δ 7.38 (1H, d, J = 2.4 Hz, 5-H), 7.29 (1H, dd, J = 2.4, 8.4 Hz, 7-H), 6.84 (1H, d, J = 9.0 Hz, 8-H), 6.30 (2H, br s, NH_2), 4.22 (3H, m, 4-H and $COOCH_2CH_3$), 4.05 (2H, q, J = 7.2 Hz, $COOCH_2CH_3$), 2.64 (1H, dd, J = 4.8, 15 Hz, HCHCO), 2.57 (1H, dd, J = 6.9, 15 Hz, HCHCO), 1.31 (3H, t, J = 7.2 Hz, $COOCH_2CH_3$), 1.18 (3H, t, J = 7.2 Hz, $COOCH_2CH_3$). ^{13}C NMR ($CDCl_3$): δ 171.69, 169.03, 161.38, 149.29, 131.38, 130.84, 128.03, 117.78, 116.87, 76.51, 60.59, 59.87, 43.56, 31.31, 14.79, 14.35. HRMS-ESI (positive) calcd for $(C_{16}H_{18}BrNO_5 + Na^+)$: 406.0261; found, 406.0266 $(M + Na)^+$. Anal. ($C_{16}H_{18}BrNO_5$) C, H, N. Calcd C, 50.02; H, 4.72; N, 3.65. Found C, 50.54; H, 5.06; N, 3.64.

Ethyl 2-Amino-4-(2-ethoxy-2-oxoethyl)-6-propyl-4*H*-chromene-3-carboxylate (5d). Yield: 53%. ^{1}H NMR ($CDCl_3$): δ 7.03 (1H, d, J = 1.8 Hz, 5-H), 6.98 (1H, dd, J = 2.1 Hz, 8.4 Hz, 7-H), 6.86 (1H, d, J = 8.4 Hz, 8-H), 6.28 (2H, br s, NH_2), 4.23 (3H, m, 4-H, $COOCH_2CH_3$), 4.02 (2H, q, J = 6.9 Hz, $COOCH_2CH_3$), 2.63 (1H, J = 14.7 Hz, 7.5 Hz, CH_2), 2.54 (1H, J = 14.7 Hz, 7.5 Hz, CH_2), 2.51 (2H, t, J = 7.5 Hz, $CH_2CH_2CH_3$), 1.586 (2H, m, $CH_2CH_2CH_3$), 1.32 (3H, t, J = 6.9 Hz, $COOCH_2CH_3$), 1.156 (3H, t, J = 6.9 Hz, $COOCH_2CH_3$), 0.904 (3H, t, J = 7.2 Hz, $CH_2CH_2CH_3$). ^{13}C NMR (75 MHz, $CDCl_3$): δ 171.96, 169.25, 161.83, 148.13, 138.84, 128.25, 127.75, 125.35, 115.58, 76.94. 60.26, 59.57, 43.90, 37.45, 31.44, 24.73, 14.72, 14.22, 13.81. HRMS-ESI (positive) calcd for $(C_{19}H_{25}NO_5+Na)^+$ 370.1632; found 370.1652 $(M+Na)^+$. Anal. ($C_{19}H_{25}NO_5$) C, H, N. Calcd C, 65.69; H, 7.25; N, 4.03. Found C, 65.23; H, 7.11; N, 3.90.

Ethyl 2-Amino-6-(4-*tert*-butylphenyl)-4-(2-ethoxy-2-oxoethyl)-4*H*-chromene-3-carboxylate (5e). Yield: 56%. ^{1}H NMR ($CDCl_3$): δ 7.45 (6H, m, Ar), 7.01 (1H, d, J = 8.7 Hz, 8-H), 6.34 (2H, br s, NH_2), 4.35 (1H, dd, J = 4.5, 6.6 Hz, 4-H), 4.24 (2H, q, J = 7.2 Hz, $COOCH_2CH_3$), 4.02 (2H, q, J = 7.2 Hz, $COOCH_2CH_3$), 2.69 (1H, dd, J = 5.1, 15.0 Hz, HCHCO), 2.62 (1H, dd, J = 6.6, 15.0 Hz, HCHCO), 1.33 (12H, m, $C(CH_3)_3$ and $COOCH_2CH_3$), 1.13 (3H, t, J = 7.2 Hz, $COOCH_2CH_3$). ^{13}C NMR (75 MHz, $CDCl_3$): δ 171.88, 169.23, 161.63, 150.32, 149.38, 137.50, 137.43, 126.98, 126.60, 126.35, 125.94, 125.87, 116.19, 76.82, 60.33, 59.66, 43.77, 35.08, 34.66, 31.49, 14.74, 14.21. HRMS-ESI (positive) calcd for $(C_{26}H_{31}NO_5 + Na)^+$ 460.2120; found 460.2106 $(M + Na)^+$. Anal. ($C_{26}H_{31}NO_5$) C, H, N. Calcd C, 71.37; H, 7.14; N, 3.2. Found C, 69.72; H, 7.16; N, 3.12.

Ethyl 2-Amino-4-(2-ethoxy-2-oxoethyl)-5-phenyl-4*H*-chromene-3-carboxylate (5f). Yield: 51%. ^{1}H NMR ($CDCl_3$): δ 7.44 (7H, m, Ar), 7.03 (1H, d, J = 8.4 Hz, 8-H), 6.32 (2H, br s, NH_2), 4.36 (1H, dd, J = 4.5, 6.9 Hz, 4-H), 4.23 (2H, q, J = 7.2 Hz, $COOCH_2CH_3$), 4.02 (2H, q, J = 7.2 Hz, $COOCH_2CH_3$), 2.69 (1H, dd, J = 4.5, 14.7 Hz, HCHCO), 2.62 (1H, dd, J = 6.9, 14.7 Hz, HCHCO), 1.33 (3H, t, J = 6.9 Hz, $COOCH_2CH_3$), 1.12 (3H, t, J = 7.2 Hz, $COOCH_2CH_3$). ^{13}C NMR (75 MHz, $CDCl_3$): δ 171.98, 169.29, 161.71, 149.68, 140.53, 137.71, 129.01, 127.40, 127.28, 127.08, 126.60, 126.14, 116.36, 76.95, 60.44, 59.77, 43.90, 31.59, 14.82, 14.29. HRMS-ESI (positive) calcd for $(C_{22}H_{23}NO_5 + Na)^+$ 404.1468; found 404.1464 $(M + Na)^+$. Anal. ($C_{22}H_{23}NO_5$) C, H, N. Calcd C, 69.28; H, 6.08; N, 3.67. Found C, 68.88; H, 6.05; N, 3.41.

Ethyl 2-Amino-4-(2-ethoxy-2-oxoethyl)-7-phenyl-4*H*-chromene-3-carboxylate (5g). Yield: 56%. ^{1}H NMR ($CDCl_3$): δ 7.41 (6H, m, Ar), 7.21 (1H, s, Ar), 7.15 (1H, s, Ar), 6.28 (2H, br s, NH_2), 4.28 (1H, dd, J = 4.5, 7.2 Hz, 4-H), 4.19 (2H, q, J = 6.9 Hz, $COOCH_2CH_3$), 4.00 (2H, q, J = 6.9 Hz, $COOCH_2CH_3$), 2.64 (1H, dd, J = 4.5, 15 Hz, HCHCO), 2.56 (1H, dd, J = 6.9, 15 Hz, HCHCO), 1.28 (3H, t, J = 7.2 Hz, $COOCH_2CH_3$), 1.12 (3H, t, J = 7.2 Hz, $COOCH_2CH_3$). ^{13}C NMR (75 MHz, $CDCl_3$): δ 172.03, 169.29, 161.74, 150.46, 141.93, 140.25, 129.05, 128.97, 127.82, 127.19, 124.77, 123.32, 114.52, 76.99, 60.45, 59.77, 43.84, 31.20, 14.83, 14.33. HRMS-ESI (positive) calcd for $(C_{22}H_{23}NO_5 + Na)^+$ 404.1468; found 404.1452 $(M + Na)^+$. Anal. ($C_{22}H_{23}NO_5$) C, H, N. Calcd C, 69.28; H, 6.08; N, 3.67. Found C, 70.00; H, 6.25; N, 3.62.

Ethyl 2-Amino-4-(2-ethoxy-2-oxoethyl)-8-phenyl-4*H*-chromene-3-carboxylate (5h). Yield: 54%. ^{1}H NMR ($CDCl_3$): δ 7.44 (5H, m, Ar), 7.22 (2H, m, Ar), 7.15 (1H, t, J = 7.2 Hz, Ar), 6.22 (2H, br s, NH_2), 4.36 (1H, dd, J = 4.8, 7.5 Hz, 4-H), 4.23 (2H, q, J = 7.5 Hz, $COOCH_2CH_3$), 4.06 (2H, q, J = 7.5 Hz, $COOCH_2CH_3$), 2.67 (1H, dd, J = 4.2, 14.7 Hz, HCHCO), 2.58 (1H, dd, J = 7.5, 14.7 Hz, HCHCO), 1.33 (3H, t, J = 7.2 Hz, $COOCH_2CH_3$), 1.17 (3H, t, J = 7.2 Hz, $COOCH_2CH_3$). ^{13}C NMR (75 MHz, $CDCl_3$): δ 171.98, 169.16, 161.72, 146.93, 137.42, 129.74, 129.41, 128.38, 128.01, 127.56, 126.53, 124.51, 77.21, 60.44, 59.74, 44.07, 31.87, 14.82, 14.35. HRMS-ESI (positive) calcd for $(C_{22}H_{23}NO_5 + Na)^+$ 404.1468; found 404.1471 $(M+Na)^+$. Anal. ($C_{22}H_{23}NO_5$) C, H, N. Calcd C, 69.28; H, 6.08; N, 3.67. Found C, 68.80; H, 6.64; N, 3.56.

Ethyl 2-Acetamido-4-(2-ethoxy-2-oxoethyl)-6-phenyl-4*H*-chromene-3-carboxylate (5i). Compound **5a** (0.030 g, 0.078 mmol) was taken in acetic anhydride (2 mL). The resultant reaction mixture was heated under reflux for 8 h. Following this, the

acetic anhydride was removed under reduced pressure. The crude residue was diluted with water and extracted with methylene chloride. The organics were combined, dried (anhydrous $MgSO_4$), and solvent removed under vacuum to afford an oily liquid. This was subjected to column chromatography to afford **5i** as a colorless oil. Yield: 26%. Also obtained was **5j** as a colorless oil. Yield: 48%. ^{1}H NMR ($CDCl_3$): δ 10.98 (1H, s, N−H), 7.45 (7H, m, Ar), 7.20 (1H, d, J = 8.1 Hz, 8-H), 4.44 (1H, dd, J = 5.1, 7.2 Hz, 4-H), 4.28 (2H, q, J = 7.2 Hz, $COOCH_2CH_3$), 4.03 (2H, q, J = 6.9 Hz, $COOCH_2CH_3$), 2.79 (1H, dd, J = 5.4, 15.3 Hz, HCHCO), 2.71 (1H, dd, J = 7.2, 15.3 Hz, HCHCO), 2.30 (3H, s, $NHCOCH_3$) 1.35 (3H, t, J = 7.2 Hz, $COOCH_2CH_3$), 1.12 (3H, t, J = 7.2 Hz, $COOCH_2CH_3$). ^{13}C NMR (75 MHz, $CDCl_3$): δ 171.45, 168.60, 168.24, 155.84, 149.33, 140.26, 138.71, 129.08, 127.62, 127.12, 127.09, 124.93, 117.14, 85.69, 61.10, 60.75, 43.71, 31.61, 25.89, 14.57, 14.29. HRMS-ESI (positive) calcd for $(C_{24}H_{25}NO_6 + Na)^+$ 446.1574; found 446.1597. Anal. ($C_{24}H_{25}NO_6 \cdot 0.5C_6H_{14}$) C, H, N. Calcd C, 69.62; H, 6.41; N, 2.73. Found C, 69.62; H, 6.51; N, 2.87.

Ethyl 2-(*N*-Acetylacetamido)-4-(2-ethoxy-2-oxoethyl)-6-phenyl-4*H*-chromene-3-carboxylate (5j). Yield: 48%. ^{1}H NMR ($CDCl_3$): δ 7.45 (7H, m, Ar), 7.10 (1H, d, J = 2.7 Hz, 8-H), 4.58 (1H, dd, J = 5.4, 6.6 Hz, 4-H), 4.22 (2H, q, J = 7.2 Hz, $COOCH_2CH_3$), 4.05 (2H, q, J = 7.5 Hz, $COOCH_2CH_3$), 2.79 (1H, dd, J = 5.4, 15.3 Hz, HCHCO), 2.71 (1H, dd, J = 6.9, 15.0 Hz, HCHCO), 2.49 (3H, s, $N(COCH_3)$, 2.39 (3H, s, $N(COCH_3)$, 1.29 (3H, t, J = 7.2 Hz, $COOCH_2CH_3$), 1.14 (3H, t, J = 7.2 Hz, $COOCH_2CH_3$). ^{13}C NMR (75 MHz, $CDCl_3$): δ 171.56, 171.42, 171.13, 164.48, 151.44, 150.31, 140.23, 139.09, 129.12, 127.70, 127.37, 127.15, 124.00, 116.87, 107.17, 77.28, 61.65, 60.93, 42.52, 33.92, 26.07, 25.75, 14.38, 14.29. HRMS-ESI (positive) calcd for $(C_{26}H_{27}NO_7 + Na)^+$ 488.168; found 488.1713. Anal. ($C_{26}H_{27}NO_7 \cdot 0.5C_6H_{14}$) C, H, N. Calcd C, 68.50; H, 6.74; N, 2.75. Found C, 68.50; H, 6.17; N, 2.61.

Ethyl 2-(2-Amino-3-cyano-6-phenyl-4*H*-chromen-4-yl)acetate (5k). Freshly cut sodium (0.096 mmol) was taken in anhydrous ethanol (2 mL), followed by the addition of malononitrile (0.192 mmol) in anhydrous ethanol (0.5 mL). The reaction mixture was stirred at room temperature under an inert atmosphere for 30 min, followed by the addition of a solution of 6-phenylcoumarin (0.08 mmol) in ethanol (1 mL). The resulting reaction mixture was stirred at room temperature. Upon consumption of the 6-phenylcoumarin, the reaction mass was concentrated, diluted with water (30 mL), and extracted using methylene chloride (3 × 20 mL). The organics were combined, dried (anhydrous $MgSO_4$), and the solvent removed under vacuum to afford an oily liquid. This crude oil was subjected to column chromatography to afford the pure product as a cream solid. Yield: 52%. ^{1}H NMR ($CDCl_3$): δ 7.43 (7H, m, Ar), 7.04 (1H, d, J = 8.4 Hz, 8-H), 4.72 (2H, br s, NH_2), 4.12 (3H, m, 4-H and $COOCH_2CH_3$), 2.78 (1H, dd, J = 5.7, 15.3 Hz, HCHCO), 2.71 (1H, dd, J = 6, 15.6 Hz, HCHCO), 1.20 (3H, t, J = 7.2 Hz, $COOCH_2CH_3$). ^{13}C NMR (75 MHz, $CDCl_3$): δ 170.89, 160.95, 148.85, 140.01, 138.42, 129.08, 127.69, 127.28, 127.06, 126.83, 122.97, 119.92, 116.95, 61.11, 58.24, 43.75, 32.39, 14.48. HRMS-ESI (positive) calcd for $(C_{20}H_{18}N_2O_3 + Na)^+$ 357.1210; found 357.1204. Anal. ($C_{20}H_{18}N_2O_3$) C, H, N. Calcd C, 71.84; H, 5.43; N, 8.38. Found C, 71.14; H, 5.79; N, 7.95.

Isopropyl 2-Amino-4-(2-isopropoxy-2-oxoethyl)-6-phenyl-4*H*-chromene-3-carboxylate (5l). Yield: 48%. ^{1}H NMR ($CDCl_3$): δ 7.42 (7H, m, Ar), 7.02 (1H, d, J = 8.4 Hz, 8-H), 6.30 (2H, br s, NH_2), 5.11 (1H, m, $COOCH(CH_3)_2$), 4.92 (1H, m, $COOCH(CH_3)_2$), 4.34 (1H, dd, J = 4.5, 7.2 Hz, 4-H), 2.67 (1H, dd, J = 4.2, 15.0 Hz, HCHCO), 2.61 (1H, dd, J = 7.2, 14.7 Hz, HCHCO), 1.33 (3H, d, J = 6.6 Hz, $COOCH(CH_3)_2$), 1.31 (3H, d, J = 6.6 Hz, $COOCH(CH_3)_2$), 1.12 (3H, d, J = 6.6 Hz, $COOCH(CH_3)_2$), 1.04 (3H, d, J = 6.6 Hz, $COOCH(CH_3)_2$). ^{13}C NMR (75 MHz, $CDCl_3$): δ 171.26, 161.65, 161.52, 149.65, 140.55, 137.63, 128.99, 127.38, 127.08, 126.57, 126.09, 116.35, 67.75, 67.71, 66.82, 59.76, 44.15, 31.61, 22.56, 22.34, 21.92, 14.83. ESI-MS (positive): m/z 432.08 $(M + Na)^+$. Anal. ($C_{24}H_{27}NO_5$) C, H, N. Calcd C, 70.40; H, 6.65; N, 3.42. Found C, 70.27; H, 5.85; N, 3.85.

Ethyl 2-Amino-4-(2-oxo-2-(piperidin-1-yl)ethyl)-6-phenyl-4*H*-chromene-3-carboxylate (5m). Piperidine (0.096 mmol) was taken in anhydrous ethanol (2 mL), followed by the addition of ethyl cyanoacetate (0.192 mmol). The resultant solution was stirred at room temperature under an inert atmosphere for 30 min, followed by the addition of a solution of 6-phenylcoumarin (0.08 mmol) in ethanol (1 mL). The resulting reaction mixture was stirred at room temperature, and upon consumption of the 6-phenylcoumarin, it was concentrated to half its volume. The reaction mass was then diluted with water (30 mL) and extracted using methylene chloride (3 × 20 mL). The organics were combined, dried (anhydrous $MgSO_4$), and the solvent removed under reduced pressure to afford a solid residue. This residue was subjected to column chromatography to afford the pure product as a cream solid. Yield: 28%. ^{1}H NMR ($CDCl_3$): δ 7.51 (3H, m, Ar), 7.38 (4H, m, Ar), 7.03 (1H, d, J = 8.4 Hz, 8-H), 6.32 (2H, br s, NH_2), 4.40 (1H, dd, J = 4.2, 9 Hz, 4-H), 4.24 (2H, q, J = 6.9 Hz, $COOCH_2CH_3$), 3.59−3.11 (4H, m, $N(CH_2)_2$), 2.70 (1H, dd, J = 4.5, 13.8 Hz, HCHCO), 2.56 (1H, dd, J = 9.3, 13.8 Hz, HCHCO), 1.6−1.3 (6H, m, $(CH_2)_3$), 1.34 (3H, t, J = 6.9 Hz, $COOCH_2CH_3$). ^{13}C NMR (75 MHz, $CDCl_3$): δ 169.47, 169.41, 161.81, 149.66, 140.56, 137.55, 128.98, 127.87, 127.35, 127.11, 126.45, 116.29, 78.04, 59.82, 47.13, 42.79, 42.56, 32.26, 26.47, 25.63, 24.67, 14.84. HRMS-ESI (positive) calcd for $(C_{25}H_{28}N_2O_4 + Na)^+$ 443.1937; found 443.1954. Anal. ($C_{25}H_{28}N_2O_4$) C, H, N. Calcd C, 71.41; H, 6.71; N, 6.66. Found C, 71.25; H 6.92; N, 7.02.

Ethyl 2-Amino-4-(2-morpholino-2-oxoethyl)-6-phenyl-4*H*-chromene-3-carboxylate (5n). Synthesized from 6-phenylcoumarin, morpholine, and ethyl cyanoacetate following similar procedure as for **5m** to afford a solid. Yield: 30%. ^{1}H NMR ($CDCl_3$): δ 7.43 (7H, m, Ar), 7.04 (1H, d, J = 8.4 Hz, 8-H), 6.34 (2H, br s, NH_2), 4.42 (1H, dd, J = 5.1, 9.3 Hz, 4-H), 4.24 (2H, q, J = 7.2 Hz, $COOCH_2CH_3$), 3.49 (4H, m, $(OCH_2)_2$), 3.25 (4H, m, $(CON(CH_2)_2)$, 2.68 (1H, dd, J = 4.8, 13.8 Hz, HCHCO), 2.58 (1H, dd, J = 9, 13.8 Hz, HCHCO), 1.33 (3H, t, J = 6.9 Hz, $COOCH_2CH_3$). ^{13}C NMR (75 MHz, $CDCl_3$): δ 169.87, 169.25, 161.83, 149.74, 140.32, 137.69, 129.07, 127.67, 127.50, 127.05, 126.61, 126.26, 116.39, 77.85, 66.91, 66.56, 59.88, 46.54, 42.01, 41.89, 32.30, 14.87. HRMS-ESI (positive) calcd for $(C_{24}H_{26}N_2O_5 + Na)^+$ 445.1734; found 445.1754. Anal. ($C_{24}H_{26}N_2O_5 \cdot 0.22CH_2Cl_2$) C, H, N. Calcd C, 65.89; H, 6.03; N, 6.34. Found C, 65.89; H, 6.45; N, 6.34.

Ethyl 2-Amino-4-(2-oxo-2-(piperazin-1-yl)ethyl)-6-phenyl-4*H*-chromene-3-carboxylate (5o). Synthesized from 6-phenylcoumarin, piperazine, and ethyl cyanoacetate following similar procedure as for **5m** and using benzene as a solvent to afford an oil. Yield: 58%. ^{1}H NMR ($CDCl_3$): δ 7.43 (7H, m, Ar), 7.04 (1H, d, J = 8.1 Hz, 8-H), 6.32 (2H, br s, NH_2), 4.41 (1H, dd, J = 4.2, 8.7 Hz, 4-H), 4.24 (2H, q, J = 6.9 Hz, $COOCH_2CH_3$), 3.52−3.18 (4H, m, 2 × $N(CH_2)$), 2.69 (6H, m, CH_2CO and 2 × CH_2), 1.33 (3H, t, J = 6.9 Hz, $COOCH_2CH_3$). ^{13}C NMR (75 MHz, $CDCl_3$): δ 169.60, 169.18, 161.73, 149.58, 140.27, 137.451, 128.91, 127.61, 127.30, 126.93, 126.39, 116.21, 59.73, 47.11, 46.12, 45.759, 42.58, 42.20, 32.14, 14.74. HRMS-ESI (positive) calcd for $(C_{24}H_{27}N_3O_4 + Na)^+$ 444.2066 found 444.2056. Anal. ($C_{24}H_{27}N_3O_4$) C, H, N. Calcd C, 68.55; H, 6.23; N, 9.99. Found C, 61.89; H, 5.94; N, 8.74.

Ethyl 2-Amino-4-(2-(diethylamino)-2-oxoethyl)-6-phenyl-4*H*-chromene-3-carboxylate (5p). Synthesized from 6-phenylcoumarin, diethylamine, and ethyl cyanoacetate following the general procedure for **5m** to afford a solid. Yield: 53%. ^{1}H NMR ($CDCl_3$): δ 7.53 (3H, m, Ar), 7.40 (3H, m, Ar), 7.31 (1H, m, Ar), 7.02 (1H, d, J = 8.1 Hz, 8-H), 6.31 (2H, br s, NH_2), 4.49 (1H, dd, J = 4.2, 9 Hz, 4-H), 4.25 (2H, q, J = 6.9 Hz, $COOCH_2CH_3$), 3.27 (2H, m, $CONCH_2CH_3$), 2.99 (2H, m, $CONCH_2CH_3$), 2.61 (1H, dd, J = 4.5, 14.1 Hz, HCHCO), 2.53 (1H, dd, J = 9, 14.1 Hz, HCHCO) 1.34 (3H, t, J = 7.2 Hz, $COOCH_2CH_3$), 0.89 (6H, m,

2 × $CONCH_2CH_3$). ^{13}C NMR (75 MHz, $CDCl_3$): δ 170.30, 169.37, 161.63, 149.48, 140.37, 137.41, 128.84, 127.76, 127.22, 126.96, 126.24, 116.11, 78.19, 59.71, 42.35, 42.06, 40.36, 32.02, 14.78, 14.30, 13.13. HRMS-ESI (positive) calcd for $(C_{24}H_{28}N_2O_4+Na)^+$ 437.1940 found 437.1925. Anal. ($C_{24}H_{28}N_2O_4$) C, H, N. Calcd C, 70.57; H, 6.91; N, 6.86. Found C, 70.31; H, 6.71; N, 6.54.

Ethyl 2-Amino-6-(3′,5′-dimethoxyphenyl)-4-(2-ethoxy-2-oxoethyl)-4*H*-chromene-3-carboxylate (5q). Yield: 52%. ^1H NMR ($CDCl_3$): δ 7.45 (1H, d, J = 2.1 Hz, 5-H), 7.39 (1H, dd, J = 2.4, 8.4 Hz, 7-H), 7.01 (1H, d, J = 8.4 Hz, 8-H), 6.60 (2H, d, J = 2.4 Hz, 2′,6′-H), 6.45 (1H, t, J = 2.1 Hz, 4′-H), 4.35 (1H, dd, J = 5.1, 6.9 Hz, 4-H), 4.23 (2H, q, J = 6.9 Hz, $COOCH_2CH_3$), 4.02 (2H, q, J = 6.9 Hz, $COOCH_2CH_3$), 3.84 (6H, s, 3′, 5′-OCH_3), 2.69 (1H, dd, J = 4.8, 14.7 Hz, HCHCO), 2.62 (1H, dd, J = 7.2, 15.0 Hz, HCHCO), 1.33 (3H, t, J = 7.2 Hz, $COOCH_2CH_3$), 1.13 (3H, t, J = 7.2 Hz, $COOCH_2CH_3$). ^{13}C NMR (75 MHz, $CDCl_3$): δ 171.94, 169.28, 161.69, 161. 29, 149.84, 142.76, 137.65, 127.31, 126.69, 126.08, 116.30, 105.34, 99.44, 60.44, 59.79, 55.66, 43.86, 31.59, 14.83, 14.33. ESI-MS (positive): m/z 464.47 $(M + Na)^+$. Anal. ($C_{24}H_{27}NO_7$) C, H, N. Calcd C, 65.29; H, 6.16; N, 3.17. Found C, 63.78; H, 6.92; N, 3.26.

Ethyl 2-Amino-4-(2-ethoxy-2-oxoethyl)-6-(3′-hydroxy-5′-methoxyphenyl)-4*H*-chromene-3-carboxylate (5r). Compound **5q** (0.050 g, 0.12 mmol) was dissolved using anhydrous methylene chloride followed by addition of molecular sieves (3 Å). The resultant solution was stirred for an hour at −78 °C to remove any residual moisture. Boron tribromide (BBr_3) (0.61 mmol) was slowly added to this solution. The resultant mixture was stirred at −78 °C for 1 h. The reaction mixture was then gradually warmed to 0 °C, and stirring was continued at this temp for 1 h. Finally, after 3 h, the reaction was quenched using saturated aq sodium bicarbonate solution, followed by extraction using ethyl acetate. The organic layer was dried using $MgSO_4$, and the solvent was removed under pressure to get the crude product. The crude mixture was subjected to column chromatography using (2:1) (hexane:ethyl acetate) to get **5r**. Yield: 25%. Also obtained was **5s** in 30% yield. ^1H NMR ($CDCl_3$): δ 7.43 (1H, d, J = 2.1 Hz, 5-H), 7.37 (1H, dd, J = 2.1,8.4 Hz, 7-H), 6.99 (1H, d, J = 8.4 Hz, 8-H), 6.65 (1H, dd, J = 1.5, 2.1 Hz, 2′-H), 6.60 (1H, dd, J = 1.5, 2.1 Hz, 6′-H), 6.39 (1H, t, J = 2.1 Hz, 4′-H), 5.15 (1H, br s, OH), 4.34 (1H, dd, J = 4.8, 6.6 Hz, 4-H), 4.23 (2H, q, J = 6.9 Hz, $COOCH_2CH_3$), 4.02 (2H, q, J = 6.9 Hz, $COOCH_2CH_3$), 3.83 (3H, s, OCH_3), 2.65 (1H, dd, J = 4.5, 15 Hz, HCHCO), 2.62 (1H, dd, J = 6.9, 14.7 Hz, HCHCO), 1.33 (3H, t, J = 6.9 Hz, $COOCH_2CH_3$), 1.13 (3H, t, J = 6.9 Hz, $COOCH_2CH_3$). ^{13}C NMR (100 MHz, $CDCl_3$): δ 171.94, 169.28, 161.69, 161.29, 149.84, 142.76, 137.65, 127.31, 126.69, 126.08, 116.30, 105.34, 86.2, 60.44, 55.66, 43.86, 31.59, 14.83, 14.33. ESI-MS (positive): m/z 450.16 $(M + Na)^+$.

Ethyl 2-Amino-6-(3′,5′-dihydroxyphenyl)-4-(2-ethoxy-2-oxoethyl)-4*H*-chromene-3-carboxylate (5s). Yield: 30%. ^1H NMR ($CDCl_3$): δ 7.36 (1H, d, J = 2.1 Hz, 5-H), 7.31 (1H, dd, J = 2.1, 8.1 Hz, 7-H), 6.95 (1H, d, J = 8.1 Hz, 8-H), 6.58 (2H, d, J = 2.1 Hz, 2′, 6′-H), 6.37 (1H, t, J = 2.1 Hz, 4′-H), 5.78 (2H, bs, 2 × OH), 4.32 (1H, dd, J = 4.8, 6.6 Hz, 4-H), 4.23 (2H, q, J = 7.2 Hz, $COOCH_2CH_3$), 4.02 (2H, q, J = 7.2 Hz, $COOCH_2CH_3$), 2.67 (1H, dd, J = 4.8, 14.4 Hz, HCHCO), 2.62 (1H, dd, J = 6.6, 14.4 Hz, HCHCO), 1.33 (3H, t, J = 7.2 Hz, $COOCH_2CH_3$), 1.12 (3H, t, J = 7.2 Hz, $COOCH_2CH_3$). ^{13}C NMR (100 MHz, $CDCl_3$): δ 170.81, 166.75, 163.95, 157.32, 157.25, 150.22, 142.5, 137.65, 127.85, 126.82, 122.85, 117.39, 106.63, 106.57, 102.16, 62.67, 61.48, 51.47, 38.45, 35.80, 14.49, 14.02. ESI-MS (positive): m/z 436.15 $(M + Na)^+$.

Ethyl 2-Amino-4-(2-ethoxy-2-oxoethyl)-6-(3′,4′,5′-trimethoxyphenyl)-4*H*-chromene-3-carboxylate (5t). Yield: 60%. ^1H NMR ($CDCl_3$): δ 7.43 (1H, d, J = 2.1 Hz, 5-H), 7.39 (1H, dd, J = 2.1, 8.4 Hz, 7-H), 7.03 (1H, d, J = 8.4 Hz, 8-H), 6.7 (2H, s, 2′, 6′-H), 6.34 (1H, br s, NH_2), 4.37 (1H, dd, J = 4.8, 7.2 Hz, 4-H), 4.26 (2H, q, J = 6.9 Hz, $COOCH_2CH_3$), 4.06 (2H, q, J = 6.9 Hz, $COOCH_2CH_3$), 3.92 (6H, s, 3′,5′- OCH_3), 3.88 (3H, s, 4′-OCH_3), 2.72 (1H, dd, J = 4.8, 14.4 Hz, HCHCO), 2.65 (1H, dd, J = 7.2, 14.4 Hz, HCHCO), 1.35 (3H, t, J = 7.5 Hz, $COOCH_2CH_3$), 1.16 (3H, t, J = 7.2 Hz, $COOCH_2CH_3$). ^{13}C NMR (100 MHz, $CDCl_3$): δ 171.71, 169, 161.45, 153.45, 149.41, 137.58, 136.31, 126.95, 126.31, 116.07, 104.18, 60.95, 60.17, 59.54, 56.18, 43.69, 31.34, 14.57, 14.10. ESI-MS (positive): m/z 494.5 $(M + Na)^+$.

Ethyl 2-Amino-4-(2-ethoxy-2-oxoethyl)-6-(naphthalen-1-yl)-4*H*-chromene-3-carboxylate (5u). Yield: 55%. ^1H NMR ($CDCl_3$): δ 7.91 (1H, m, Ar), 7.86 (2H, m, Ar), 7.52−7.36(5H, m, Ar), 7.33−7.30 (1H, m, Ar), 7.09 (1H, d, J = 6.3 Hz, 8-H), 6.19 (2H, br s, NH_2), 4.37 (1H, dd, J = 5.1, 6.9 Hz, 4-H), 4.26 (2H, q, J = 6.9 Hz, $COOCH_2CH_3$), 4.03 (2H, q, J = 6.9 Hz, $COOCH_2CH_3$), 2.68 (1H, dd, J = 4.8, 14.4 Hz, HCHCO), 2.62 (1H, dd, J = 6.6, 14.4 Hz, HCHCO), 1.33 (3H, t, J = 7.2 Hz, $COOCH_2CH_3$), 1.12 (3H, t, J = 7.2 Hz, $COOCH_2CH_3$). ^{13}C NMR (100 MHz, $CDCl_3$); 170.37, 167.76, 160.39, 148.35, 138.52, 136.19, 133.08, 130.94, 129.28, 128.72, 127.66, 127.08, 126.29, 125.49, 125.277, 125.2, 124.75, 115.27, 63.45, 60.65, 60.02, 44.45, 32.35, 25.78, 15.79, 15.28. ESI-MS (positive): m/z 454.48. $(M + Na)^+$.

Ethyl 2-Amino-4-(2-ethoxy-2-oxoethyl)-6-(naphthalen-2-yl)-4*H*-chromene-3-carboxylate (5v). Yield: 54%. ^1H NMR ($CDCl_3$): δ 7.98 (1H, s), 7.91−7.85 (3H, m), 7.70−7.67 (1H, m), 7.61−7.60 (1H, d, J = 2.1 Hz), 7.55−7.46 (3H, m), 7.09(1H, d, J= 8.4 Hz, 8-H), 6.34 (2H, br s, NH_2), 4.40 (1H, dd, J = 4.8, 7.2 Hz, 4-H), 4.25 (2H, q, J = 7.2 Hz, $COOCH_2CH_3$), 4.02 (2H, q, J = 7.2 Hz, $COOCH_2CH_3$), 2.67 (1H, dd, J = 4.4, 14.8 Hz, HCHCO), 2.62 (1H, dd, J = 7.6, 14.8 Hz, HCHCO), 1.34 (3H, t, J = 8 Hz, $COOCH_2CH_3$), 1.13 (3H, t, J = 7.2 Hz, $COOCH_2CH_3$). ^{13}C NMR (100 MHz, $CDCl_3$); 171.74, 167.76, 161.46, 149.54, 137.36, 133.08, 132.52, 130.94, 128.45, 128.12, 127.63, 127.29, 126.63, 126.34, 126.05, 125.92, 125.42, 125.28, 116.24, 60.23, 59.546, 43.70, 31.39, 25.78, 14.57, 14.07. ESI-MS (positive): m/z 454.48. $(M + Na)^+$.

Cell Cultures. CCl-240 (HL-60), CRL-2257 (HL-60/MX2), CCL-119 (CCRF-CEM), CRL-2264 (CCRF-CEM/C2), CRL-8286 (Reh), HTB-81 (DU-145), HTB-22 (MCF7), CCL-247 (HCT-116), CRL-2026 (Hepa1c1c7), HTB-177 (H460), and CRL-1837 (SU86.86) cells were purchased from ATCC, and the NALM-6 cell line was originally characterized at the University of Minnesota and kindly provided by Dr. Tucker Lebien.[34] HL-60 cell line was grown in IMDM glutamax media supplemented with 20% FBS. HL-60/MX2, CCRF-CEM, and CCRF-CEM/C2 cells were grown in RPMI 1640 purchased from ATCC supplemented with 10% FBS. NALM-6, Reh, DU-145, MCF7, HCT-116, Hepa1c1c7, H460, and SU86.86 cell lines were cultured in RPMI 1640 (Invitrogen) medium supplemented with 10 mM HEPES, 1 mM sodium pyruvate, 4.5 g/L glucose, 1.5 g/L sodium bicarbonate, 10% FBS, and 1% penicillin−streptomycin (antibiotic). All cell lines were incubated at 37 °C with 5% CO_2 in air atmosphere.

Cell Viability Measurement. The in vitro cytotoxicity of these small molecules was assayed by determining their ability to inhibit growth of tumor cells. In brief, tumor cells were plated in a 96-well plate (at a density of 1×10^4 cells/well) and were treated with a series of dilutions of test compounds of varied concentrations with 1% DMSO in the final cell medium (cells treated with medium containing 1% DMSO served as a control). After a 48 h treatment, the relative cell viability in each well was determined by using Cell Titer-Blue Cell Viability Assay kit according to manufacturer's protocol. The IC_{50} of each candidate was determined by fitting the relative viability of the cells to the drug concentration by using a dose−response model in Prism program from GraphPad Software, Inc. (San Diego, CA).

Western Blot Analysis. Cultured cells were plated at a density of 4×10^6 cells/well in six-well plates. Cells were treated with the test compounds with various concentrations and incubated at 37 °C for 24 h. After 24 h, cells were harvested and washed with ice-cold phosphate-buffered saline (PBS). Cell pellets were suspended in RIPA (25 mM Tris·HCl pH 7.6, 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS) buffer supplemented with 1% protease inhibitor from Sigma. The cell suspension was incubated on ice for 30 min and centrifuged at 12000g for 10 min. The supernatant was collected, and the

protein concentration in the supernatant was quantified by Bradford method with BSA as the standard. Samples were separated at 150 V through electrophoresis for 1 h on 12% SDS-PAGE. The proteins were blotted to polyvinylidine fluoride (PVDF) membrane from Millipore (Boston, MA) and probed using the antibodies described as follows: the anti-Bcl-2 (B3170) antibody (Sigma Aldrich, Inc.) was used at a final dilution of 1:1000, PARP (9532) antibody (Cell Signaling Technology) was used at a final dilution of (1:300), and Bcl-X_L(sc-8392) and Mcl-1(sc-819) antibody (Santa-Cruz Biotechnology, Inc.) were used at a final dilution of (1:200). Bound antibodies were detected using appropriate peroxidase-coupled secondary antibodies followed by detection using the supersignal chemiluminescence system from Pierce (Rockford, IL).

Caspase-3/7 Activation Assay. Apo-ONE Homogeneous Caspase-3/7 Assay kit was used to measure the caspase-3/7 activity according to the manufacturer's instructions. Briefly, Apo-ONE Caspase-3/7 reagent (50 μL) was added to each well containing 50 μL of treated cell suspension (4×10^4 cells) in a 96-well plate. The suspension in the wells were mixed gently and incubated at 37 °C for 30−60 min. The fluorescence intensity of the sample in each well was measured with excitation at 485 nm and emission at 530 nm. Caspase-3/7 activity in each well was normalized to the vehicle-treated control.

Synergism Assay. Synergistic interactions between MX, CT, and **5q** was examined by using median dose−effect analysis as described by Chou and Talalay.[35] Briefly, tumor cells were treated with serial dilutions of each agent individually and in combination simultaneously at a fixed dose ratio for 48 h. The cytotoxic effects of the treatment were measured by evaluating the cell viability using the cell viability assay and the long-term survival assay. Fractional effect was calculated as fraction of cells killed by the individual agent or the combination in treated versus untreated cells. Median dose effect analysis was performed using CompuSyn program from ComboSyn, Inc. (Paramus,NJ). The software computes combination index (CI) values based on the following equation: CI = (D)1/(Dx)1 + (D)2/(Dx)2 + (D)1(D)2/(Dx)1(Dx)2, where(D)1 and (D)2 are the doses of drug 1 and drug 2 that have x effect when used in combination and (Dx)1 and (Dx)2 are the doses of drugs 1 and 2 that have the same x effect when used alone. The CI values indicate synergism (<1), additivity (1), or antagonism (>1). The CIs of 0.1−0.3, 0.3−0.7, and 0.7−0.85 are considered strong synergism, synergism, and moderate synergism, respectively.

Statistical Analysis. All biological experiments, including in vitro cytotoxicity assay, caspase-3/7 activation assay, and synergism assay, were performed at least twice with triplicates in each experiment. Representative results are depicted in this report. Data are presented as means ± SD, and comparisons were made using Student's t test. A probability of 0.05 or less was considered statistically significant.

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Supporting Information Available: Purity results of final compounds and fluorescent based binding assay procedures and results are provided. This material is available free of charge via the Internet at http://pubs.acs.org.

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