Structure−Activity Relationship Studies of Ethyl 2-Amino-6-bromo-4-(1-cyano-2-ethoxy-2-oxoethyl)-4*H*-chromene-3-carboxylate (HA 14-1), an Antagonist for Antiapoptotic Bcl-2 Proteins To Overcome Drug Resistance in Cancer

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The structure−activity relationship studies of ethyl 2-amino-6-cyclopentyl-4-(1-cyano-2-ethoxy-2-oxoethyl)-4*H*-chromene-3-carboxylate (**1**, HA 14-1), an antagonist of the antiapoptotic Bcl-2 proteins, are reported. A series of analogues of **1** with varied functional groups at the 6-position of the chromene ring were synthesized. These candidates were evaluated for their binding interactions with three antiapoptotic proteins: Bcl-2, Bcl-X_L, and Bcl-w. They were also assayed for their in vitro cytotoxicities against a set of Jurkat cells with varied levels of Bcl-2 and Bcl-X_L proteins and a non-small-cell lung carcinoma cell line (NCI-H460). It was found that the 6-bromo of **1** was not essential for its bioactivity and the 6-position can accommodate a variety of alkyl groups. **1** and its analogues bind to all of the three antiapoptotic Bcl-2 proteins tested. Positive correlations were observed between the binding affinities of these candidates to the antiapoptotic Bcl-2 proteins and their in vitro cytotoxicities, suggesting that the antiapoptotic Bcl-2 proteins are likely to be the cellular targets of **1** and its analogues. (In this study, the binding interactions of the small molecules to antiapoptotic Bcl-2 proteins were studied by assaying their abilities to compete against a Bak peptide binding to the antiapoptotic Bcl-2 proteins. Inhibitory constants, instead of dissociation constants, were obtained in such assays. The term "binding affinity" is used in this article for simplicity.) The most active compound, **3g**, had a >3-fold increase of binding affinity to the antiapoptotic Bcl-2 proteins and a >13-fold increase of in vitro cytotoxicity over **1**. Though Jurkat cells with transgenic overexpression of Bcl-2 or Bcl-X_L protein can develop resistance to standard cancer therapies, such cells failed to develop resistance to **1** based candidates. **1** also sensitizes Jurkat cells to cisplatin. These studies provide further support that **1** and its analogues function as antagonists for antiapoptotic Bcl-2 proteins and that they have the potential, either as a single agent or as a combination therapy with other anticancer agents, to treat cancers with the overexpression of antiapoptotic Bcl-2 proteins.

Introduction

Drug resistance is one major barrier in the fight against cancer.[1] At the molecular level, drug resistance can be acquired through the overexpression of antiapoptotic Bcl-2 proteins which protect cancer cells from apoptosis induced by cancer therapies.[2−4] Overexpression of the antiapoptotic Bcl-2 proteins is expected to be a general mechanism for the development of drug resistance because the level of Bcl-2 protein has been observed to be elevated in over 60% of all of the major malignancies.[5] Antagonizing antiapoptotic Bcl-2 proteins therefore holds promise for overcoming drug resistance for cancer treatment. This concept was proved to be valid by the preclinical and clinical studies of an antisense of Bcl-2 protein, Genasense.[6−8] Because of the intrinsic problems of the antisense approach (cost of preparation, delivery, stability, and toxicity), small-molecule antagonists for antiapoptotic Bcl-2 proteins are desirable.[9]

Bcl-2 proteins are a protein family with over 20 members.[10−13] Some Bcl-2 protein members are antiapoptotic, such as Bcl-2, Bcl-X_L, and Bcl-w, and the other members are proapoptotic, such as Bax, Bak, and Bid. Mechanistically, antiapoptotic Bcl-2 proteins are proposed to protect cells from apoptosis by antagonizing proapoptotic Bcl-2 proteins through dimerization.[10,11] Structural studies of a complex of Bcl-X_L protein and a peptide from the BH3 domain of Bak protein have revealed a hydrophobic cleft on the Bcl-X_L protein.[14] The hydrophobic cleft is believed to be the binding pocket for proapoptotic Bcl-2 proteins, and molecules binding to this hydrophobic cleft may antagonize the protective effect of the antiapoptotic Bcl-2 proteins.[15] These discoveries have stimulated the recent identification of small molecules that bind to the antiapoptotic Bcl-2 proteins at the hydrophobic cleft. Figure 1 shows a partial list of such candidates discovered over the past 6 years.[16−24]

Ethyl 2-amino-6-cyclopentyl-4-(1-cyano-2-ethoxy-2-oxoethyl)-4*H*-chromene-3-carboxylate (**1**, HA 14-1), the first reported small-molecule antagonist for Bcl-2 protein, was identified by Huang et al. in 2000.[16] Compound **1** competed against the BH3 domain peptide of Bak protein binding to a recombinant Bcl-2 protein. Compound **1** also induced apoptosis in vitro. Later, Lickliter et al. and Dai et al. respectively observed that **1** could selectively induce apoptosis in malignant cells that overexpress Bcl-2 protein.[25,26] Especially in the study by Dai et al., leukemia cells developed resistance to imatinib by overexpressing Bcl-2 protein during the course of imatinib treatment. Such imatinib-resistant tumor cells were more sensitive to **1** than the parent leukemia cells. These studies demonstrated the potential of **1** in selectively eliminating drug-resistant tumor cells. More excitingly, numerous laboratories have established that **1** can sensitize different cancer cells to various cancer therapies, possibly by antagonizing the protective effect of Bcl-2 protein.[25−38] These studies demonstrated the potential of **1** as a part of combination therapy for cancer treatment.

Because **1** was discovered as a single lead through virtual screening, little information about the pharmacophores of **1** is

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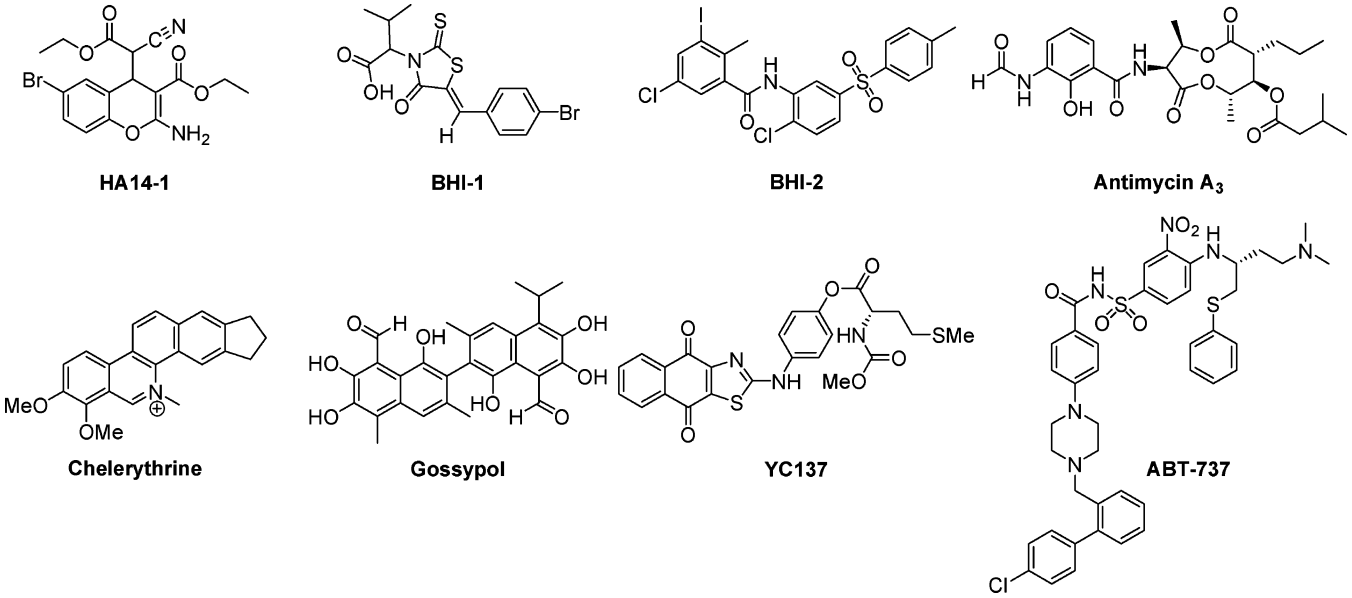
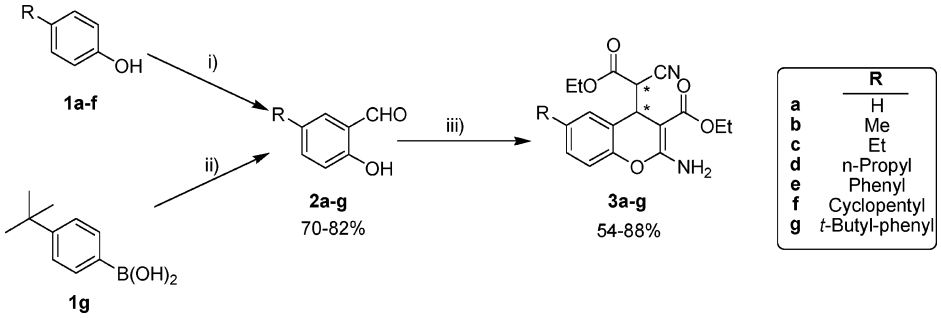


Figure 1. Partial list of the reported small-molecule antagonists for antiapoptotic Bcl-2 proteins.

Scheme 1. Synthesis of **1** Based Analogues[a]



[a] Reagents and conditions: (i) $MgCl_2$, Et_3N, paraformaldehyde, dry CH_3CN, reflux; (ii) 5-bromosalicylaldehyde, $Pd(OAc)_2$, K_2CO_3, H_2O, room temp; (iii) ethyl cyanoacetate, EtOH, 3 Å molecular sieves, room temp. The asterisk (∗) represents chiral centers.

known. Since its discovery in 2000, no further effort has been reported to modify **1** to improve its bioactivity. Because of the relatively low in vitro cytotoxicity ($GI_{50} \approx 20\ \mu M$) and the not-well-defined binding affinity of **1** to Bcl-2 protein (only IC_{50} was reported), we initiated structure−activity relationship (SAR) studies to search for more potent candidates and to define the pharmacophores of **1**. Such SAR studies are also expected to help determine whether Bcl-2 protein would be the target of **1**.

In this study, we modified the 6-position of **1** with functional groups of varied lipophilicity, size, and electronic properties. These candidates were evaluated for their binding interactions with Bcl-2 protein. Because the antiapoptotic Bcl-2, Bcl-X_L, and Bcl-w proteins are highly conserved and all these proteins contain the hydrophobic cleft,[14,39−41] we speculate that **1** may have cross activities among these antiapoptotic Bcl-2 proteins. We therefore evaluated the binding interactions of **1** and its analogues with Bcl-X_L and Bcl-w proteins as well. To explore whether the binding interactions of **1** and its analogues to antiapoptotic Bcl-2 proteins are critical to their in vitro activities, these candidates were evaluated for their in vitro cytotoxicities against a hematologic tumor cell line: Jurkat cell (a T cell leukemia) and a solid tumor cell line, NCI-H460 (a non-small-cell lung carcinoma). The effect of overexpressing Bcl-2 or Bcl-X_L proteins on the in vitro activities of **1** and its analogues was studied by evaluating the cytotoxicities of these candidates in two Jurkat cells overexpressing the Bcl-2 or Bcl-X_L protein. The capability of **1** in sensitizing Jurkat cells to cisplatin treatment was also evaluated because no studies have yet explored whether **1** can synergize the anticancer activity of cisplatin.

Synthesis and Characterization of the Stereochemistry of 1 and Its Analogues

1 and a group of its analogues (compounds **3a**−**g**), possessing a variety of alkyl groups at the 6-position of the chromene ring, were synthesized with a 45−55% overall yield through a route similar to that previously reported by Yu et al. (Scheme 1).[42] In brief, 5-substituted salicylaldehydes **2a**−**f** were prepared from the corresponding phenol through formylation using paraformaldehyde.[43] Salicylaldehyde **2g** was prepared from 5-bromosalicylaldehyde through Suzuki coupling with *tert*-butylphenylboronic acid. These salicylaldehydes (**2a**−**g**) and 5-bromosalicylaldehyde were condensed with ethyl α-cyanoacetate in ethanol to give **3a**−**g** and **1**.

Because there are two chiral centers on **1** and its analogues, the stereochemistry of **1** and its analogues can be critical to their biological activities. The stereochemistry of **1**, however, has not been characterized before. Since a set of four diastereomers could be generated in our synthesis of **1**, to ensure that the synthetic **1** would have the same chirality as the reported **1**, we purchased **1** from Maybridge (U.K.) and compared the purchased **1** with the synthesized **1** by NMR, TLC, and in vitro cytotoxicity assays. No difference was detected between the two samples, suggesting that our synthesized **1** has the same

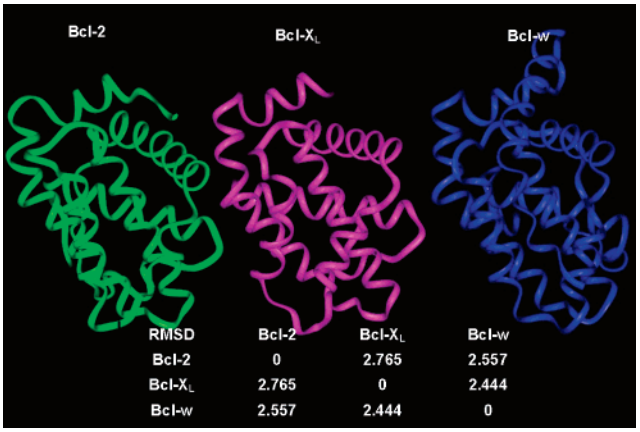
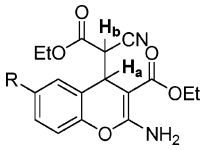


Figure 2. Structural comparison of Bcl-2, Bcl-X_L, and Bcl-w proteins (rmsd calculated by using Insight II).

Table 1. Chemical Shifts of the Two Protons on the Chiral Centers of **1** and Its Analogues



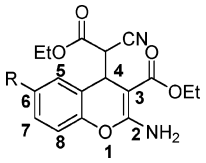
	chemical shift δ (ppm)		
compd	H_a	H_b	J_{H_a}-H_b (Hz)
1	3.98	4.68	3.6
3a	3.98	4.72	3.6
3b	3.97	4.67	3.6
3c	3.98	4.70	3.6
3d	3.97	4.69	3.9
3e	4.02	4.79	3.9
3f	3.96	4.68	3.6
3g	4.02	4.78	3.6

stereochemistry as the reported **1**. On the basis of the ^1H and ^{13}C NMR spectra of **1**, both the commercial and the synthetic **1** contain only one set of enantiomers instead of four diastereomers because only one set of NMR peaks (^1H and ^{13}C) was detected. The relative stereochemical assignment of **3a** was studied by X-ray crystallography. The X-ray characterization established that **3a** is a set of enantiomers with the relative chirality as (*R*,*R*) and (*S*,*S*). We have not characterized the relative stereochemistry of **1** and **3b**−**g** by X-ray crystallography. However, on the basis of the similarity of the chemical shifts of H_a and H_b (the two protons directly attached to the two chiral centers (Table 1)), we propose that **1** and **3b**−**g**, the same as **3a**, are sets of enantiomers with (*R*,*R*) and (*S*,*S*) as the relative chirality. The X-ray data of **3a** are reported in Supporting Information.

Binding Interactions of 1 and Its Analogues to Antiapoptotic Bcl-2 Proteins

As shown in Figure 2, the structures of the antiapoptotic Bcl-2, Bcl-X_L, and Bcl-w proteins are highly conserved. **1**, initially identified as an antagonist for Bcl-2 protein, may antagonize Bcl-X_L and Bcl-w protein as well. Therefore, the binding interactions of **1** and its analogues to the three antiapoptotic Bcl-2 proteins (Bcl-2, Bcl-X_L, and Bcl-w) were evaluated in this study. The binding interactions of these small molecules to the antiapoptotic Bcl-2 proteins were studied by assaying their abilities to compete against an Oregon Green fluorescein-labeled Bak BH3 domain peptide (Flu-Bak) binding to the antiapoptotic Bcl-2 proteins. The competition was monitored by following the decrease of fluorescence polarization (FP).[17] The inhibitory constants of **1** and its analogues against the binding of Flu-Bak to antiapoptotic Bcl-2 proteins were obtained by fitting the change of FP to the concentration of **1** or its analogues based on a single-site competition binding model. (The competition as a single-site competition is based on the original report of **1** by Huang et al.,[16] and the derivation of the mathematical equation for this model is detailed in Supporting Information.) The results are summarized in Table 2. It was found that modification of the 6-position of **1** induced changes to the binding affinity of **1** to the Bcl-2 protein. In general, candidates with functional groups of increased lipophilicity had better binding affinities. As we hypothesized, **1** and its analogues also bound to Bcl-X_L and Bcl-w proteins with moderate improvement of the binding affinities compared to Bcl-2 protein. Interestingly, the binding profile of **1** and its analogues to Bcl-2 protein matches their binding profiles to Bcl-X_L and Bcl-w proteins (Figure 3).

Table 2. Binding Affinities of **1** and its Analogues among Bcl-2, Bcl-X_L, and Bcl-w Proteins



		$K_i \pm$ SEM (μM)[a]		
compd	R	Bcl-2	Bcl-X_L	Bcl-w
1	Br	169 ± 18.9	58.9 ± 5.3	59.32 ± 5.32
3a	H	531 ± 41.8	570 ± 159	696.61 ± 50.15
3b	Me	309 ± 33.3	191 ± 21	198.98 ± 49.12
3c	ethyl	210 ± 30.2	135 ± 20	120.52 ± 18.31
3d	*n*-propyl	128 ± 13.8	70.7 ± 6.2	56.69 ± 2.80
3e	phenyl	112 ± 13.9	52.9 ± 6.2	41.18 ± 5.56
3f	cyclopentyl	128 ± 15.5	36.5 ± 0.3	30.81 ± 2.87
3g	*tert*-butylphenyl	ND[b]	21.7 ± 0.8	20.31 ± 4.17

[a] Results are given as the mean of two independent experiments with triplicate determinations in each experiment. [b] ND: not determined.

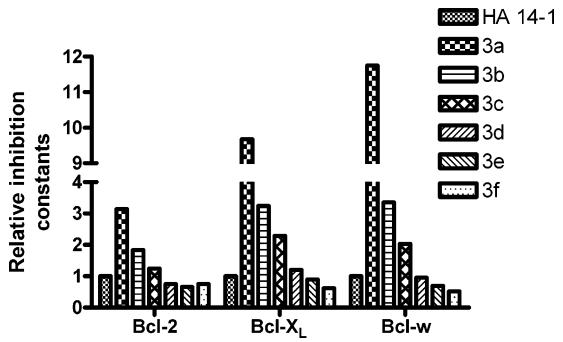


Figure 3. Binding profiles of **1** and its analogues to Bcl-2, Bcl-X_L, and Bcl-w proteins.

Characterization of the Bcl-2 or Bcl-X_L Overexpressing Jurkat Cells

In order to ensure that the Bcl-2 or Bcl-X_L transgenetic Jurkat cells have elevated levels of Bcl-2 or Bcl-X_L protein relative to the normal Jurkat cells and have no other variations, several proteins in the three Jurkat cell lines were characterized by Western blot. As shown in Figure 4, among the proteins that have been studied, only Bcl-2 protein is elevated in Bcl-2 overexpressing Jurkat cells and only Bcl-X_L protein is elevated in Bcl-X_L overexpressing Jurkat cells.

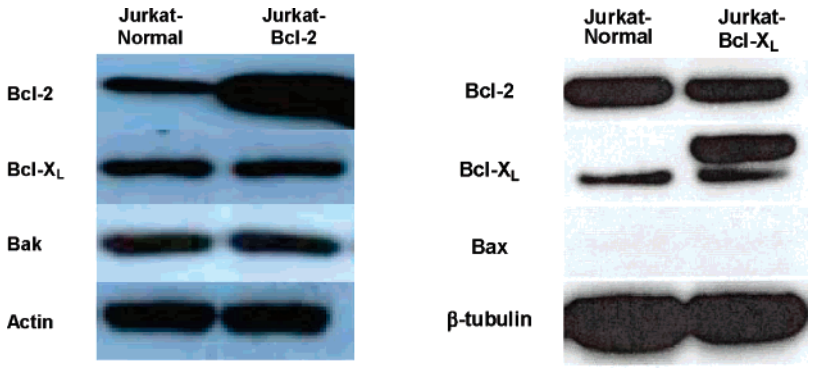


Figure 4. Levels of various proteins in normal Jurkat cell, Bcl-2 overexpressing Jurkat cell, and Bcl-X_L overexpressing Jurkat cell.

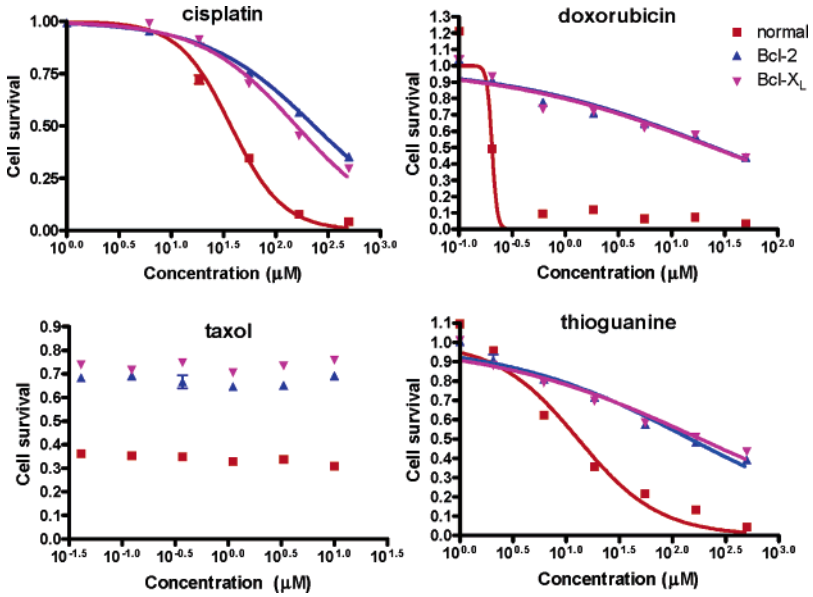


Figure 5. Dose response curves of the growth inhibition induced by standard cancer chemotherapies in the three Jurkat cell lines.

Table 3. GI_{50} Values of Standard Chemotherapies against the Three Jurkat Cell Lines

	$GI_{50} \pm$ SEM (μM)[a]			
	cisplatin	doxorubicin	paclitaxel	thioguanine
normal	37 ± 9.5	0.21	<0.01	13 ± 3.6
Bcl-2	232 ± 21	26 ± 11	>10	147 ± 30
Bcl-X_L	158 ± 28	24 ± 11	>10	183 ± 65
ratio[b]	>4	>100	>100	>11

[a] Results are given as the mean of two independent experiments with triplicate determinations in each experiment. [b] Ratio refers the ratio of the GI_{50} for Jurkat cells overexpressing Bcl-2 or Bcl-X_L to the GI_{50} for the normal Jurkat cells.

We next examined whether the transgenetically overexpressed Bcl-2 or Bcl-X_L protein in Jurkat cells would induce resistance to cancer therapy. To achieve this objective, the cytotoxicities of cisplatin, doxorubicin, paclitaxel, and thioguanine to the three Jurkat cells (normal Jurkat cell, Jurkat cell overexpressing Bcl-2, and Jurkat cell overexpressing Bcl-X_L) were evaluated. These four anticancer agents were selected for testing because they have different mechanisms of action for their anticancer activities. As shown in Figure 5 and summarized in Table 3, compared to the normal Jurkat cells, Jurkat cells overexpressing Bcl-2 or Bcl-X_L protein are at least over 4-fold less sensitive to any of the four cancer therapies tested. These results establish that overexpression of Bcl-2 or Bcl-X_L protein induces Jurkat cells resistant to general cancer therapies.

In Vitro Assay of the Cytotoxicities of 1 and its Analogues

The objective of this experiment was to evaluate the in vitro cytotoxicities of **1** and its analogues and their sensitivities to the drug resistance induced by the overexpression of Bcl-2 or Bcl-X_L protein. In brief, the cytotoxicities of **1** and its analogues to the three Jurkat cells were evaluated. As shown in Figure 6 and summarized in Table 4, in contrast to the standard chemotherapies, all three Jurkat cells have very similar sensitivity to **1** and its analogues, with the largest GI_{50} difference being <1.9-fold. These results suggest that the overexpression of Bcl-2 protein or Bcl-X_L protein cannot induce drug resistance to **1** and its candidates. It was also observed that in each cell system, the in vitro cytotoxicities of **1** and its analogues correlate positively with their capabilities of inhibiting the binding of Flu-Bak to the antiapoptotic Bcl-2 proteins (Figure 7). The in vitro cytotoxicities of **1** and its analogues was also evaluated in NCI-H460, a non-small-cell lung solid carcinoma. **1** and its analogues inhibit the cell growth in NCI-H460 with GI_{50} values comparable to those in Jurkat cells. The in vitro cytotoxicities of **1** and its analogues in NCI-H460 also correlate positively with their binding affinities to the antiapoptotic Bcl-2 proteins (data in Supporting Information).

Compound 1 Sensitizing Jurkat Cells to Cisplatin Treatment

The goal of this study was to determine whether **1** could synergize the anticancer activity of cisplatin in Jurkat cells. In

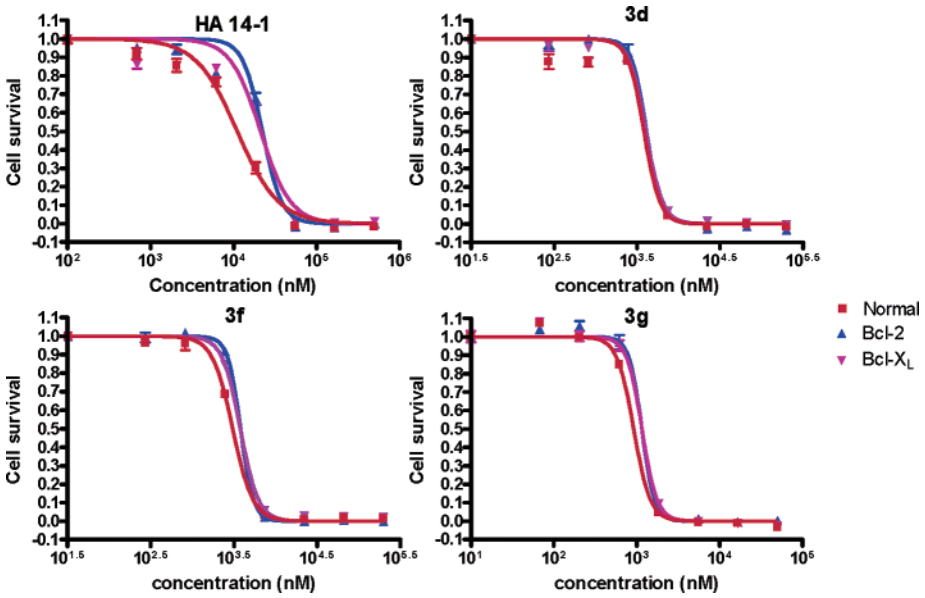


Figure 6. Dose response curves of the growth inhibition induced by **1** and its analogues in the three Jurkat cell lines.

Table 4. GI_{50} of **1** and Its Analogues against the Three Jurkat Cell Lines

	$GI_{50} \pm SEM$ (μM)[a]							
	1	**3a**	**3b**	**3c**	**3d**	**3e**	**3f**	**3g**
normal	11 ± 0.1	66 ± 0.8	29 ± 2.7	13 ± 1.1	5.2 ± 1.3	3.9 ± 0.4	3.5 ± 0.5	0.80 ± 0.1
Bcl-2	21 ± 1.0	94 ± 20	37 ± 4.9	12 ± 3.3	5.7 ± 1.4	4.2 ± 0.2	3.8 ± 0.1	0.99 ± 0.1
Bcl-X_L	18 ± 3.2	99 ± 18	41 ± 8.3	13 ± 2.3	6.3 ± 2.3	4.1 ± 0.3	3.9 ± 0.1	0.99 ± 0.1
ratio[b]	<1.9	<1.50	<1.41	<1.0	<1.21	<1.07	<1.1	<1.24

[a] Results are given as the mean of two independent experiments with triplicate determinations in each experiment. [b] Ratio refers the ratio of the GI_{50} for Jurkat cells overexpressing Bcl-2 or Bcl-X_L to the GI_{50} for the normal Jurkat cells.

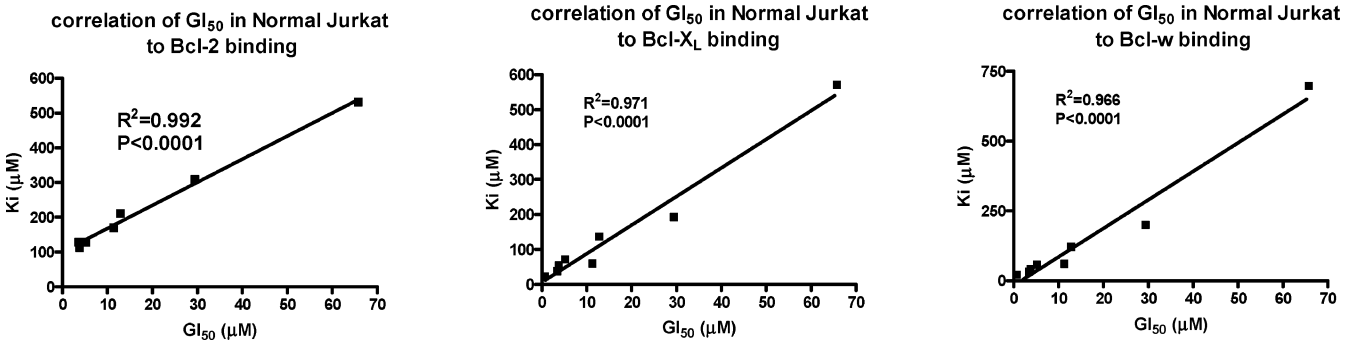


Figure 7. Correlations of the binding affinities of **1** and its analogues to antiapoptotic Bcl-2 proteins and their in vitro cytotoxicities.

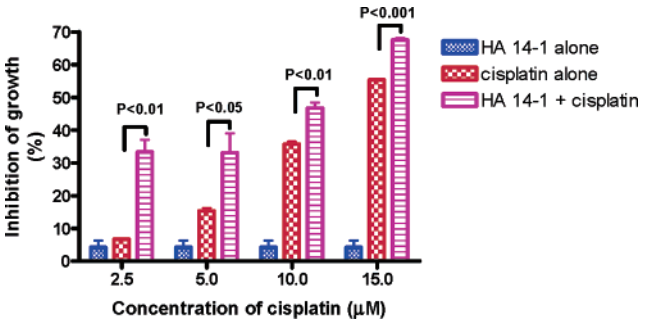


Figure 8. Inhibition of growth in Jurkat cells induced by **1** alone, cisplatin alone, and the combination of both.

brief, Jurkat cells were treated with **1** at a nontoxic concentration of 2 μM (at this concentration, **1** alone induced <5% growth inhibition) and simultaneously treated with cisplatin of varied concentrations. As shown in Figure 8, **1** sensitized Jurkat cells to cisplatin, especially at the lower concentrations of cisplatin. This experiment suggested that **1** had the potential to lower the effective dose of cisplatin for cancer treatment. It remains to be determined whether the **1** analogues of improved binding affinities and in vitro cytotoxicities would have enhanced capability of sensitizing cells to anticancer therapies.

Discussion and Conclusion

We have synthesized a series of analogues of **1** with different substitutions at the 6-position. Both the binding assay and the in vitro cytotoxicity assay established that the 6-bromo on **1** is not essential for its bioactivity and the 6-position of **1** can accommodate a variety of alkyl and aryl functional groups. The most potent candidate, 3**g**, has a >3-fold increase in the binding affinity to the three antiapoptotic Bcl-2 proteins and a >13-fold increase of in vitro cytotoxicity among the four tumor cells tested. As we have hypothesized, **1** and its analogues bind to all three antiapoptotic Bcl-2 proteins tested with moderate preference to recombinant Bcl-X_L and Bcl-w proteins. Overall, the binding profile of **1** and its analogues to Bcl-2 protein

matches their binding profiles to Bcl-X_L and Bcl-w proteins. More importantly, the SAR studies reveal nice positive correlations between the in vitro cytotoxicities of **1** and its analogues in the four cell lines and their binding affinities to the three antiapoptotic Bcl-2 proteins. Such positive correlations suggest that the antiapoptotic Bcl-2 proteins are likely to be the cellular targets of **1**. On the basis of the studies in the Jurkat cell systems, although the overexpression of Bcl-2 or Bcl-X_L protein can induce resistance to general cancer therapies, the overexpression of Bcl-2 or Bcl-X_L protein cannot induce resistance to **1** and its analogues. This study further supports that **1** and its analogues can be used as a single agent to treat tumors with the overexpression of antiapoptotic Bcl-2 proteins. Finally, as has been established for various cancer therapies, **1** can synergize the anticancer activity of cisplatin.

Overall, the studies reported here suggest that the antiapoptotic Bcl-2 proteins, instead of Bcl-2 alone, are the cellular targets of **1**. Compound **1** and its analogues have the potential to overcome the drug resistance induced by the overexpression of antiapoptotic Bcl-2 proteins.

Experimental Section

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 (TLC) was performed on EM Science silica gel 60 F_{254} (0.25 mm). Compounds were visualized by UV light and/or stained with *p*-anisaldehyde, potassium permanganate, or cerium molybdate solutions followed by heating. Flash column chromatography was performed on Fisher Scientific silica gel (230−400 mesh). Melting points were determined on a Thomas-Hoover Unimelt 6406-K melting point apparatus and were uncorrected. IR spectra were recorded on a Nicolet Portege 460 FT-IR instrument. NMR (^{1}H) spectra were recorded on a Varian 300 MHz spectrometer and calibrated using an internal reference. ESI mode mass spectra were recorded on a BrukerBioTOF II mass spectrometer. Elemental analyses were performed by M-H-W Laboratories, Phoenix, AZ.

General Procedure for the Synthesis of the Salicylaldehydes (2a−f). The syntheses of the salicylaldehydes **2a**−**f** were carried out by formylation of the corresponding phenols using the procedure similar to that reported by Skattebol et al.[43] In brief, in a round-bottom flask equipped with a reflux condenser and a magnetic stirrer, the 4-substituted phenol (1.6 mmol), anhydrous magnesium dichloride (2.4 mmol), and dry triethylamine (6.1 mmol) were taken in dry acetonitrile (4 mL). To this mixture dry (P_2O_5) paraformaldehyde (11 mmol) was added, and the reaction mixture was heated under reflux for 5−8 h. The reaction was monitored by TLC, and upon consumption of the phenol, the mixture was cooled and the reaction was quenched by the addition of water. The reaction mixture was then acidified using HCl (5.5 N) and extracted using ethyl acetate (3 × 30 mL). The extracts were combined and dried ($MgSO_4$), and the solvent was removed under vacuum to furnish an oil, which on purification by flash chromatography afforded the pure salicylaldehyde (**2a**−**f**, 70−82%). Salicylaldehydes **2a**−**f** are known compounds with no characterization reported herein.

Synthesis of 5-*tert*-Butylphenylsalicylaldehyde (2g). In a round-bottom flask equipped with a magnetic stirrer, 5-bromosalicylaldehyde (0.100 g, 0.49 mmol), K_2CO_3 (0.206 g, 1.49 mmol), *tert*-butylphenylboronic acid (0.0884 g, 0.49 mmol), and $Pd(OAc)_2$ (0.5 mol %) were taken in water (3 mL). The mixture was stirred at room temperature under an atmosphere of nitrogen for 16 h. The reaction mixture was then diluted with water (25 mL), followed by acidification using HCl (5.5 N) and extraction with ethyl acetate (3 × 30 mL). The extracts were combined and dried ($MgSO_4$), and the solvent was removed under vacuum. The crude solid was purified by flash chromatography to isolate the desired 5-*tert*-butylphenylsalicylaldehyde (71%). TLC (EtOAc/hexane = 1:6), R_f = 0.73. ^{1}H NMR ($CDCl_3$): δ 10.98 (1H, s, OH), 9.97 (1H, s, CHO), 7.76 (2H, m, 3,4-H), 7.49 (4H, d, J = 1.2 Hz, Ph), 7.06 (1H, d, J = 8.1 Hz, 5-H), 1.36 (9H, s, C(C**H**$_3$)$_3$).

General Procedure for the Synthesis of 3a−g and 1. In a round-bottom flask equipped with a magnetic stirrer, the 5-substituted salicylaldehyde (**2a**−**g** and 5-bromosalicylaldehyde, 0.157 mmol) was dissolved in absolute ethanol (5 mL). To this, 3 Å molecular sieves (0.6 g) was added. The resulting reaction mixture was stirred for 5 min, and ethyl α-cyanoacetate (0.3454 mmol) was then added. The reaction mixture was stirred overnight at 23 °C. The molecular sieves were then filtered off, and the mixture was washed with THF (3 × 5 mL) and methylene chloride (3 × 5 mL). The combined filtrate then was concentrated under vacuum. The final compounds (**3a**−**g** and **1**) were obtained in pure crystalline form by recrystallization using methylene chloride and hexanes as the solvents (52−68%). **1** and **3a** are known compounds with no characterization reported herein.[44]

Ethyl 2-Amino-6-methyl-4-(1-cyano-2-ethoxy-2-oxoethyl)-4*H*-chromene-3-carboxylate (3b). Yield: 65%. TLC (EtOAc/hexane = 1:2), R_f = 0.13. Mp: 110−112 °C. IR (KBr): 3436, 3301, 2246, 1746, 1680, 1637 cm^{-1}. ^{1}H NMR ($CDCl_3$): δ 7.11 (1H, dd, J = 1.8, 8.1 Hz, 7-H), 6.97 (1H, d, J = 8.1 Hz, 8-H), 6.89 (1H, d, J = 1.2 Hz, 5-H), 4.25 (4H, m, 2 × COOC**H**$_2$CH$_3$), 4.67 and 3.97 (1H, d, J = 3.9 Hz; 1H, d, J = 3.6 Hz, CNC**H**COOC$_2$H$_5$ and 4-H), 2.28 (3H, s, C**H**$_3$), 1.32 (6H, m, 2 × COOCH$_2$C**H**$_3$). ESI-MS (positive): m/z 254.1 (M − CNCH$_2$COOEt + Na)$^+$, 232.1 (M − CNCH$_2$COOEt + H)$^+$. Anal. ($C_{18}H_{20}N_2O_5$·0.144C_6H_{14}) C, H, N. Calcd: C, 63.55; H, 6.22; N, 7.85. Found: C, 63.55; H, 5.98; N, 8.09.

Ethyl 2-Amino-6-ethyl-4-(1-cyano-2-ethoxy-2-oxoethyl)-4*H*-chromene-3-carboxylate (3c). Yield: 63%. TLC (EtOAc/hexane = 1:2), R_f = 0.18. Mp: 112−114 °C. IR (KBr): 3395, 3295, 2248, 1738, 1672, 1617 cm^{-1}. ^{1}H NMR ($CDCl_3$): δ 7.13 (1H, dd, J = 1.5, 8.4 Hz, 7-H), 7.00 (1H, d, J = 8.7 Hz, 8-H), 6.92 (1H, d, J = 1.5 Hz, 5-H), 4.26 (4H, m, 2 × COOC**H**$_2$CH$_3$), 4.70 and 3.98 (1H, d, J = 3.6 Hz; 1H, d, J = 3.9 Hz, CNC**H**COOC$_2$H$_5$ and 4-H), 2.58 (2H, q, J = 7.5 Hz, C**H**$_2$CH$_3$), 1.32 (6H, m, 2 × COOCH$_2$C**H**$_3$), 1.19 (3H, t, J = 7.5 Hz, CH$_2$C**H**$_3$). ESI-MS (positive): m/z 268.1 (M − CNCH$_2$COOEt + Na)$^+$, 246.1 (M − CNCH$_2$COOEt + H)$^+$. Anal. ($C_{19}H_{22}N_2O_5$·0.01CH_2Cl_2) C, H, N. Calcd: C, 63.16; H, 6.15; N, 7.74. Found: C, 63.16; H, 6.17; N, 7.71.

Ethyl 2-Amino-6-*n*-propyl-4-(1-cyano-2-ethoxy-2-oxoethyl)-4*H*-chromene-3-carboxylate (3d). Yield: 62%. TLC (EtOAc/hexane = 1:2), R_f = 0.13. Mp: 112−114 °C. IR (KBr): 3400, 3297, 2248, 1736, 1671, 1617 cm^{-1}. ^{1}H NMR ($CDCl_3$): δ 7.11 (1H, dd, J = 2.1, 8.4 Hz, 7-H), 6.99 (1H, d, J = 8.7 Hz, 8-H), 6.90 (1H, d, J = 2.1 Hz, 5-H), 4.25 (4H, m, 2 × COOC**H**$_2$CH$_3$), 4.69 and 3.97 (1H, d, J = 3.9 Hz; 1H, d, J = 3.9 Hz, CNC**H**COOC$_2$H$_5$ and 4-H), 2.51 (2H, t, J = 6.9 Hz, C**H**$_2$CH$_2$CH$_3$), 1.58 (2H, m, CH$_2$C**H**$_2$CH$_3$), 1.34 (3H, t, J = 7.5 Hz, COOCH$_2$C**H**$_3$), 1.28 (3H, t, J = 6.9 Hz, COOCH$_2$C**H**$_3$), 0.91 (3H, t, J = 7.5 Hz, CH$_2$-CH$_2$C**H**$_3$). ESI-MS (positive): m/z 282.11 (M − CNCH$_2$COOEt + Na)$^+$, 260.13 (M − CNCH$_2$COOEt + H)$^+$. Anal. ($C_{20}H_{24}N_2O_5$) C, H, N. Calcd: C, 64.50; H, 6.50; N, 7.52. Found: C, 64.81; H, 6.60; N, 7.90.

Ethyl 2-Amino-6-phenyl-4-(1-cyano-2-ethoxy-2-oxoethyl)-4*H*-chromene-3-carboxylate (3e). Yield: 62%. TLC (EtOAc/hexane = 1:2), R_f = 0.19. Mp: 134−136 °C. IR (KBr): 3429, 3316, 2247, 1734, 1681, 1623 cm^{-1}. ^{1}H NMR ($CDCl_3$): 7.42 (7H, m, 5-H, 7-H, and 6-C$_6$**H**$_5$), 7.15 (1H, d, J = 8.7 Hz, 8-H), 4.23 (4H, m, 2 × COOC**H**$_2$CH$_3$), 4.79 and 4.02 (1H, d, J = 3.9 Hz; 1H, d, J = 3.9 Hz, CNC**H**COOC$_2$H$_5$ and 4-H), 1.36 (3H, t, J = 7.2 Hz, COOCH$_2$C**H**$_3$), 1.17 (3H, t, J = 7.5 Hz, COOCH$_2$C**H**$_3$). ESI-MS (positive): m/z 429.2 (M + Na)$^+$, 316.1 (M − CNCH$_2$COOEt + Na)$^+$, 294.1 (M − CNCH$_2$COOEt + H)$^+$. Anal. ($C_{23}H_{22}N_2O_5$·0.22C_6H_{14}) C, H, N. Calcd: C, 68.69; H, 5.95; N, 6.57; Found: C, 68.69; H, 5.78; N, 6.84.

Ethyl 2-Amino-6-cyclopentyl-4-(1-cyano-2-ethoxy-2-oxoethyl)-4*H*-chromene-3-carboxylate (3f). Yield: 52%. TLC (EtOAc/hexane = 1:2), R_f = 0.23. Mp: 113−115 °C. IR (KBr): 3397, 3296, 2247, 1736, 1672, 1617 cm^{-1}. ^{1}H NMR ($CDCl_3$): δ 7.15

(1H, dd, $J = 2.1$, 8.4 Hz, 7-H), 6.97 (1H, d, $J = 8.4$ Hz, 8-H), 6.94 (1H, d, $J = 2.1$ Hz, 5-H), 4.23 (4H, m, 2 × $COOCH_2CH_3$), 4.68 and 3.96 (1H, d, $J = 3.6$ Hz; 1H, d, $J = 3.9$ Hz, $CNCHCOOC_2H_5$ and 4-H), 2.92 (1H, m, $CH(CH_2)_4$), 2.01, 1.71, and 1.49 (8H, m, $CH(CH_2)_4$), 1.35 (6H, 2 × t,$COOCH_2CH_3$). ESI-MS (positive): *m*/*z* 286.2 $(M - CNCH_2COOEt + H)^+$, 308.2 $(M - CNCH_2COOEt + Na)^+$. Anal. ($C_{22}H_{26}N_2O_5$) C, H, N. Calcd: C, 66.32; H, 6.58; N, 7.03. Found: C, 66.92; H, 6.49; N, 7.40.

Ethyl 2-Amino-6-(4-*tert*-butylphenyl)-4-(1-cyano-2-ethoxy-2-oxoethyl)-4*H*-chromene-3-carboxylate (3g). Yield: 68%. TLC (EtOAc/hexane = 1:2), $R_f = 0.46$. Mp: 116−118 °C. IR (KBr): 3465, 3317, 2250, 1738, 1682, 1652 cm^{-1}. ^1H NMR ($CDCl_3$): δ 7.51 (1H, dd, $J = 2.1$, 8.4 Hz, 7-H), 7.44 (4H, s, Ph), 7.33 (1H, d, $J = 2.4$ Hz, 5-H), 7.13 (1H, d, $J = 9.0$ Hz, 8-H), 4.24 (4H, m, 2 × $COOCH_2CH_3$), 4.78 and 4.02 (1H, d, $J = 3.6$ Hz; 1H, d, $J = 3.9$ Hz, $CNCHCOOC_2H_5$ and 4-H), 1.36 (3H, t, $J = 7.2$ Hz, $COOCH_2CH_3$), 1.36 (9H, s, $C(CH_3)_3$), 1.19 (3H, t, $J = 7.2$ Hz, $COOCH_2CH_3$). ESI-MS (positive): *m*/*z* 350.13 $(M - CNCH_2COOEt + H)^+$, 372.11 $(M - CNCH_2COOEt + Na)^+$. Anal. ($C_{27}H_{30}N_2O_5$) C, H, N. Calcd: C, 70.11; H, 6.54; N, 6.06. Found: C, 69.86; H, 6.63; N, 6.05.

Structural Comparison of Bcl-2, Bcl-X_L, and Bcl-w Proteins by Insight II. The structural comparison of these three antiapoptotic Bcl-2 proteins was conducted on a SGI Octane2 workstation using Insight II (Accelrys, Inc., CA) with the NMR energy-minimized average structures of Bcl-2, Bcl-X_L, and Bcl-w proteins taken from Protein Data Bank (PDB codes 1G5M, 1LXL, and 1MK3).

Preparation of Recombinant Bcl-2, Bcl-X_L, and Bcl-w Proteins. A pET-25b(+) vector inserted with the DNA sequence encoding Bcl-2ΔC21 fused with a His_6 tag was a generous gift from Jialing Lin at the University of Okalahoma. pET-29b(+) vectors inserted with the DNA sequence encoding Bcl-wΔC22 fused with a His_6 tag and the DNA sequence encoding Bcl-X_LΔloopΔC40 fused with a His_6 tag were generous gifts from Kalle Gehring at McGill University. The plasmids were transformed into the *Escherichia coli* strain ER2566 (New England Biolabs, MA). The expression of the fusion proteins was induced by 1 mM IPTG, and the fusion proteins were purified by Ni-NTA resin by following a native protein purification protocol provided by the manufacture (Qiagen, CA). The purity of the recombinant proteins was characterized by SDS−PAGE and Commassie blue staining (Supporting Information). Recombinant proteins were concentrated using Centrifugal Filter Devices (Millipore, MA) and dialyzed against PBS buffer containing 15% glycerol and 1 mM DTT. The concentration of the recombinant protein was determined by the Bradford method using BSA as a standard, and the sample was stored at −20 °C.

Fluorescence Polarization (FP) Assays. The Bak BH3 (GQVGRQLAIGDDINR) peptide was synthesized at the Oligonucleotide & Peptide Synthesis Facility at the University of Minnesota, purified by HPLC. The purified peptide was labeled with Oregon Green 488 fluorescein at the N terminus following the manufacture's protocol (Promega, CA), purified by HPLC, characterized by MS, and named as Flu-Bak. Flu-Bak was dissolved in ddH_2O and stored at −20 °C as aliquots. FP assay was conducted using a GENios Pro plate reader (Tecan US, NC) with all assays performed in triplicate, each assay performed twice.

To determine the binding affinity of the Flu-Bak peptide with an antiapoptotic Bcl-2 protein, 3-fold dilutions of the antiapoptotic Bcl-2 protein were prepared in a PBS solution, pH 7.0, with 45 nM Flu-Bak peptide and 1 mM DTT. The solutions were incubated at 23 °C for 1 h (the time-course study of binding process of Flu-Bak peptide to all the antiapoptotic Bcl-2 proteins demonstrated that the binding interaction reached equilibrium within 5 min). To each well in a 96-well half-area black plate (Corning, NY) the solution (50 μL) was added and fluorescence polarization (in mP unit) was measured. The binding affinity of Flu-Bak to the antiapoptotic Bcl-2 protein was determined by fitting the FP changes to the concentrations of the protein using a single-site saturation binging model in GraphPad (GraphPad, CA).

To determine the binding interactions of small molecules to an antiapoptotic Bcl-2 protein, a series of 3-fold dilutions of small molecules were prepared in DMSO, i.e., 10, 3.33, 1.11, 0.37, 0.123, 0.041, 0.014, and 0 mM. To each well in a 96-well half-area black plate, 5 μL of the small molecule stock solution was added. A PBS solution (pH 7.0, 1 mM DTT) containing 50 nM Flu-Bak peptide and the amount of the antiapoptotic Bcl-2 protein that resulted in 60% of Flu-Bak peptide binding to the antiapoptotic Bcl-2 protein was prepared and incubated at 23 °C for 1 h. To each well the solution of Flu-Bak peptide and the antiapoptotic Bcl-2 protein (45 μL) was added by autoinjection at a rate of 200 μL/s. The sample was incubated at 23 °C for 1 h, and the fluorescence polarization (in millipolarization units (mP)) was measured. (The 10% DMSO in the final solution was studied to have no interference to the binding interaction of Flu-Bak to the antiapoptotic Bcl-2 proteins.) Small-molecule controls included dose response measurements in the absence of proteins to assess for any interactions between the compounds and the Flu-Bak peptide. Eventual effects were taken into account by subtraction. The inhibitory constant (K_i) was determined by fitting the FP values to the concentrations of the small molecule using a single-site competition model for fluorescence polarization based competition assay established in this laboratory in GraphPad. The derivation of the mathematical equation used for data fitting is detailed in Supporting Information.

Western Blot Analysis. Jurkat cells and NCI-H460 cells were cultured in RPMI-1640 media supplemented with 10% FBS at 37 °C and 5% CO_2. For Western blot studies, Jurkat cells (2.5×10^7 cells) were pelleted by centrifugation. The cell pellet was suspended in 200 μL of RIPA buffer supplemented with protease inhibitor cocktail from Sigma. The cell suspension was incubated on ice for 30 min and centrifuged on a benchtop centrifuge at 12000*g* for 15 min. The supernatant was collected, and the concentration of proteins in the supernatant was quantified by the Bradford method with BSA as the standard. The proteins in the supernatant were separated by 12% SDS−PAGE. The proteins were then blotted to PVDF membrane from Millipore and analyzed by following standard Western blot procedures. Anti-Bcl-2, anti-Bcl-X_L, anti-Bak, anti-Bax, antiactin, and antitubulin were purchased from Santa Cruz (CA). Secondary antibodies, antigoat and antimouse IgGs, were purchased from Sigma (MO).

In Vitro Cytotoxicity Assay. The in vitro cytotoxicity of the small molecules was assayed by determining the GI_{50} values (the concentration of the small molecules to reduce the cell growth by 50%). In brief, the cells were plated in a 96-well plate (2.5×10^3 cells/well for NCI-H460 and 10^4 cells/well for Jurkat cells). The cells were treated with the small molecules with a series of 3-fold dilution with 1% DMSO in the final cell media (cells treated with media of 1% DMSO served as a control). After 24 h of treatment, the relative cell viability in each well was determined by using CellTiter-Blue cell viability assay kit (a fluorescence assay that measures the reduction of a dye (resazurin) into a fluorescent end product (resorufin) by metabolically active cells—viable cells) (Promega, CA). The GI_{50} of each agent was determined by fitting the relative viability of the cells to the drug concentration by using a dose response model in GraphPad. For the synergistic study, the cells were cotreated with cisplatin and **1** simultaneously for 24 h and the relative cell viability was determined.

Statistical Analysis. All biological experiments, including the binding assays and the in vitro cytotoxicity assays, were performed at least twice with triplicates in each experiment. Representative results are depicted in this report. Data were analyzed and presented using the GraphPad software. The Student's *t*-test was applied for comparison between groups using the GraphPad software. The correlation study was performed using a linear fitting model in GraphPad. Significance was set at $P < 0.05$.

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encoding Bcl-wΔC22 or DNA sequence encoding Bcl-X_LΔloopΔC40 inserted. We also thank Dr. Daniel Johnson at the University of Pittsburgh for providing Bcl-X_L overexpressing Jurkat cells and Dr. Claus Belka at University of Tuebingen for providing the Bcl-2 overexpressing Jurkat cells.

Supporting Information Available: The SDS−PAGE characterization of recombinant Bcl-2, Bcl-X_L, and Bcl-w proteins; dose response curves of the in vitro cytotoxicity of some compounds in three Jurkat cells; dose response curves of the in vitro cytotoxicities of **1** and its analogues against NCI-H460 cells; the correlation of the binding affinities to the in vitro cytotoxicities of the compounds in NCI-H460 cells; the mathematical derivations of the equation for the calculation of K_i based on the fluorescence polarization single-site competition binding assay; and X-ray crystallographic data for **3a**. This material is available free of charge via the Internet at http://pubs.acs.org.

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