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Development of selective inhibitors for anti-apoptotic Bcl-2 proteins from BHI-1

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Abstract—A series of inhibitors for anti-apoptotic Bcl-2 proteins based on BHI-1 were synthesized and their binding interactions with Bcl-2, Bcl-X_L, and Bcl-w were evaluated. It was found that modification of BHI-1 resulted in varied binding profiles among Bcl-2, Bcl-X_L, and Bcl-w, and a set of inhibitors with varied selectivity to Bcl-2, Bcl-X_L, and Bcl-w proteins have been identified. Molecular modeling of the interaction of the BHI-1 based analogues with the anti-apoptotic Bcl-2 proteins suggested that the binding site for the BHI-1 based inhibitor was the least conserved section among Bcl-2, Bcl-X_L, and Bcl-w: targeting the non-conserved section may account for the observed selectivity of the BHI-1 based inhibitors among the anti-apoptotic Bcl-2 proteins. The validity of the model was supported by a strong correlation between the model-calculated binding energy and the experimental binding affinity. In summary, our studies suggest that most of the reported inhibitors for anti-apoptotic Bcl-2 proteins are nonselective and BHI-1 is a promising template to distinguish among Bcl-2, Bcl-X_L, and Bcl-w by targeting the non-conserved domain among the anti-apoptotic Bcl-2 proteins. Molecular-modeling-aided rational development of BHI-1 based selective inhibitor for anti-apoptotic Bcl-2 proteins is underway.
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1. Introduction

Drug resistance is one major barrier in the fight against cancer.[1] At the molecular level, drug resistance can be acquired through the over-expression of anti-apoptotic Bcl-2 proteins which protect cancer cells from apoptosis induced by cancer therapy.[2–4] Inhibiting anti-apoptotic Bcl-2 proteins, therefore, holds promise as a way to overcome drug resistance for cancer treatment. This concept has been proved valid through anti-sense approach.[5–7]

Anti-apoptotic Bcl-2 proteins are a subgroup of the Bcl-2 protein family, including Bcl-2, Bcl-X_L, Bcl-w, Mcl-1, and A1. In some cancers, more than one anti-apoptotic Bcl-2 protein is co-over-expressed.[8–10] Under such circumstance, a non-selective inhibitor for multiple anti-apoptotic Bcl-2 proteins may be needed to antagonize these proteins simultaneously to overcome the drug resistance. Some cancers, however, exhibit exclusive over-expression of one specific anti-apoptotic Bcl-2 protein.[11–15] For instance, in a study of head and neck carcinoma by Thompson et al., 52% of the patients had up-regulated Bcl-X_L and 17% of the patients had up-regulated Bcl-2. There was no overlap between these two groups.[12] In Kaposi's sarcoma, the expression level of Bcl-X_L protein was elevated, while Bcl-2 protein remained at the basal level.[13] In mastocytosis, the expression of Bcl-2 significantly increased while no up-regulation of Bcl-X_L was detected.[16] In gastric and colorectal adenocarcinomas, only Bcl-w was up-regulated.[14,15] Besides their selective over-expression in cancer tissues, anti-apoptotic Bcl-2 proteins are expressed in healthy tissues in a tissue-selective manner[17–19] and function to protect healthy cells.[19] The cancer/tissue-selective expression of anti-apoptotic Bcl-2 proteins suggests that as therapeutic agents, member-selective inhibitors for anti-apoptotic Bcl-2 proteins could be safer than the non-selective counterparts in those tumors with the selective elevation of one single anti-apoptotic Bcl-2 protein. However, it is still debatable whether the selective inhibitor will be as effective as the non-selective ones in overcoming drug resistance. To address these questions, selective inhibitors and non-selective inhibitors of the same class need to be developed. In addition, the selective inhibitors for the anti-apoptotic Bcl-2 proteins can be useful chemical probes to help

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define the biological functions of the individual anti-apoptotic Bcl-2 proteins.

Mechanistically, anti-apoptotic Bcl-2 proteins protect cells from apoptosis by antagonizing pro-apoptotic Bcl-2 proteins through dimerization.[20,21] Structural studies of a complex of Bcl-X_L protein and a pro-apoptotic peptide (Bak BH3) have revealed a hydrophobic cleft on Bcl-X_L protein as the binding pocket for the pro-apoptotic peptide[22] and molecules binding to that hydrophobic cleft may overcome the protective effect of the Bcl-X_L protein.[23] These observations have stimulated the recent discovery of several small molecules targeting the hydrophobic cleft of Bcl-X_L or Bcl-2 proteins with a partial list shown in Figure 1.[24–32] As the hydrophobic cleft is very conserved among the anti-apoptotic Bcl-2, Bcl-X_L, Bcl-w, and Mcl-1 proteins,[22,33–36] these reported inhibitors could have cross activities among these anti-apoptotic Bcl-2 proteins as has been established for ABT-737.[30] However, it is possible to develop selective inhibitors among the anti-apoptotic Bcl-2 proteins as the anti-apoptotic Bcl-2 proteins can differentiate the peptides derived from the BH3 domain of the pro-apoptotic Bcl-2 proteins with selectivity up to 10 fold.[37,38] In addition, a small-molecule inhibitor (YC-137) was recently discovered by Wang et al. to selectively target Bcl-2 protein over Bcl-X_L protein.[29] Biological evaluation of YC-137 demonstrated that YC-137 can selectively eliminate the tumors with the over-expression of Bcl-2 protein over those with the over-expression of Bcl-X_L protein. More excitingly, YC-137 showed minimal toxicity to the normal healthy cells with no elevation of Bcl-2 protein, further underscoring the need to develop selective inhibitors for anti-apoptotic Bcl-2 proteins.

Since there is no obvious structural similarity among the reported inhibitors, we hypothesized that their respective interactions with anti-apoptotic Bcl-2 proteins involve distinct sections along the long hydrophobic cleft. Inhibitors targeting the less conserved portion of the hydrophobic cleft would be more promising templates in the search for member-selective inhibitors. With initial NMR and molecular modeling study suggesting that BHI-1 targets the less conserved section of the hydrophobic cleft,[25] BHI-1 was selected for study to investigate its potential as lead templates for developing member-selective inhibitors for anti-apoptotic Bcl-2 proteins.

2. Results and discussion

2.1. Chemistry

A group of BHI-1 analogues (compounds **3a**–**o**) possessing variation at the amino acid and alkylidene sections were synthesized with a 20–40% overall yield by first cyclizing respective amino acids with CS_2 and α-chloroacetate to form rhodanines by using a procedure similar to that previously reported,[39] followed by Knoevenagel condensation of rhodanines with varied aldehydes or ketones to form the final analogues (Scheme 1). The condensation generated two isomers (E and Z) and the major product was the thermodynamically stable Z isomer as characterized by the down-field shift of the methylene proton of the Z isomer compared to that of the E isomer. No racemerization was detected in the synthesis of the BHI-1 analogues as demonstrated in the Supplementing Materials.

2.2. Biology

The binding affinities of the synthesized small molecules and several reported inhibitors to three anti-apoptotic Bcl-2 proteins, Bcl-2, Bcl-X_L, and Bcl-w, were determined by using a fluorescence polarization (FP) based competition assay against an Oregon Green fluorescein-labeled Bak BH3 domain peptide (Flu-Bak).[25] First, the binding affinities of the Flu-Bak peptide to the three Bcl-2 proteins were determined by using a Saturation binding model with a constant concentration of Flu-Bak peptide (45 nM) and varied concentrations of the proteins. The binding affinity of Flu-Bak peptide to the three Bcl-2 proteins is summarized in Table 1. The binding affinity of Flu-Bak peptide to Bcl-X_L protein

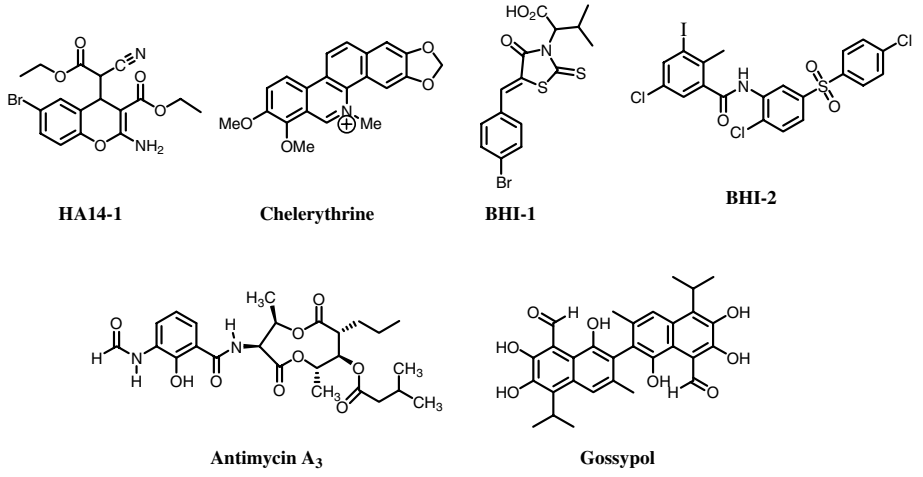
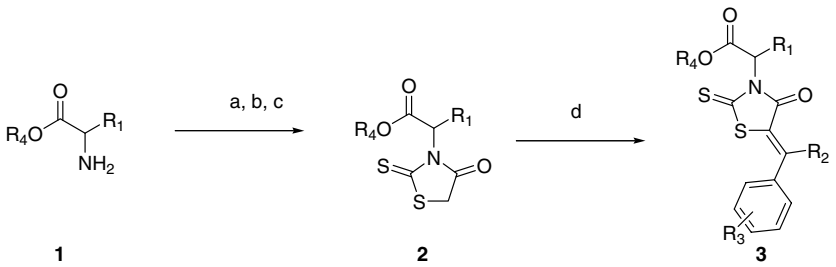


Figure 1. Reported inhibitors for Bcl-2 or Bcl-X_L proteins.



Scheme 1. Synthesis of BHl-1 based BCl-2 inhibitors **3a**–**o**. Reagents and conditions: (a) NaOH, CS_2, 23 °C, overnight; (b) $ClCH_2CO_2Na$, 23 °C, 3 h; (c) HCl, reflux, 12 h; (d) benzaldehyde/ketone, NH_4OAc, toluene, reflux, 1–12 h.

Table 1. The binding affinities of Flu-Bak peptide to Bcl-2, Bcl-X_L, and Bcl-w proteins

Compound	$K_d \pm$ SD (nM)		
	Bcl-2	Bcl-X_L	Bcl-w
Flu-Bak	990 ± 100	362 ± 36	555 ± 41

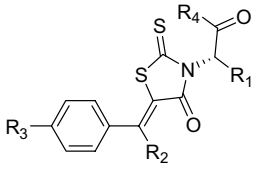
(K_d = 362 ± 36 nM) is comparable to the binding affinity determined by Sattler et al., K_d = 340 nM.[22] While the binding affinity of Flu-Bak peptide to Bcl-2 determined in this study (K_d = 6200 ± 1500 nM) is quite different from the one determined by Gemperli et al. (K_d = 900 ± 100 nM),[37] it is possible that the difference is because of the different forms of recombinant Bcl-2 proteins used in these two studies. The recombinant Bcl-2 protein used in this study is transmembrane-domain deleted. With the binding affinities of Flu-Bak peptide to the proteins determined, the interactions of BHI-1 based small molecules to the Bcl-2 proteins were indirectly evaluated by assaying their capabilities of competing against Flu-Bak peptide binding to the Bcl-2 proteins. The capabilities of the small molecules to compete against Flu-Bak peptide binding to Bcl-2 proteins (K_i) are summarized in Table 2 for reported inhibitors and Table 3 for BHI-1 based inhibitors.

Table 2. The inhibitory capability of reported inhibitors against Flu-Bak binding to Bcl-2, Bcl-X_L, and Bcl-w proteins

Compound	$K_i \pm$ SD (μM)		
	Bcl-2	Bcl-X_L	Bcl-w
Antimycin A_3	124 ± 10.6	127 ± 16.3	127 ± 11.9
Chelerythrine	171 ± 24.3	96 ± 8.9	106 ± 6.3
Gossypol	10.1 ± 0.9	24.7 ± 1.8	17.7 ± 1.3
HA14-1	254 ± 17.2	86.2 ± 7.9	210 ± 18
BHI-1	43.4 ± 3.9	133 ± 7.6	124 ± 12.5

As we have hypothesized, the reported inhibitors bound to all the three anti-apoptotic Bcl-2 proteins tested without much selectivity. In our effort to identify an optimal template for the search of selective inhibitors, a series of analogues of BHI-1 were synthesized. Of note, modifications resulted in a wider range of binding affinities

Table 3. The inhibitory capability of BHI-1 based candidates against Flu-Bak binding to Bcl-2, Bcl-X_L, and Bcl-w proteins



Compound	R_1	R_2	R_3	R_4	$K_i \pm$ SD (μM) (selectivity)		
					Bcl-2	Bcl-X_L	Bcl-w
BHI-1	2-Propyl	H	Br	OH	43.4 ± 3.9 (**2.85**)	133 ± 7.6	124 ± 12.5
3a	Me	H	Br	OH	115 ± 8.4 (**2.11**)	374 ± 16.3	243 ± 14.7
3b	$CH_3S(CH_2)_2$	H	Br	OH	69.6 ± 4.9 (**1.26**)	134 ± 20.3	88 ± 14.2
3c	3-Indolyl-CH_2	H	Br	OH	2.85 ± 0.21 (**11.51**)	32.8 ± 2.7	54.2 ± 5.5
3d	$C(O)OH(CH_2)_2$	H	Br	OH	320 ± 21.7 (**1.91**)	848 ± 39.0	610 ± 31.4
3e	$PhCH_2$	H	Br	OH	14.2 ± 1.2	5.35 ± 0.39 (**2.65**)	21.5 ± 1.8
3f	$PhCH_2$	*n*-Propyl	H	OH	12 ± 0.8 (**2.33**)	27.9 ± 2.7	41.1 ± 3.7
3g	$(CH_3)_2CHCH_2$	H	Br	OH	7.43 ± 0.65	2.99 ± 0.18 (**2.48**)	31.2 ± 3.8
3h	$(CH_3)_2CHCH_2$	*n*-Propyl	H	OH	13.5 ± 0.6 (**1.79**)	41.3 ± 4.2	24.1 ± 1.7
3i	$(CH_3)_2CHCH_2$	Me	H	OH	21.2 ± 1.6 (**2.74**)	128.2 ± 5.9	58.3 ± 4.2
3j	$(CH_3)_2CHCH_2$	Ph	H	OH	57.8 ± 4.6 (**1.07**)	68.8 ± 5.5	61.9 ± 6.9
3k	$(CH_3)_2CHCH_2$	Ph	Ph	OH	1.95 ± 0.13 (**3.58**)	8.7 ± 0.46	6.98 ± 0.38
3l	Me	H	Br	*O*-2-Propenyl	192 ± 12.4 (**1.40**)	268 ± 18.4	>1000
3m	Me	H	Br	*N*-Morpholinyl	70.0 ± 4.4 (**2.01**)	228 ± 19.7	146 ± 17.6
3n	$PhCH_2$	H	Br	*O*-2-Propenyl	>1000	244 ± 20.1	56.8 ± 2.9 (**4.29**)
3o	$PhCH_2$	H	Ar	*O*-2-Propenyl	260 ± 19.7	176 ± 14.6	116 ± 11.8 (**1.52**)

(1.95–848 μM) and modification also changed the binding profiles of these inhibitors for Bcl-2, Bcl-X_L, and Bcl-w proteins. For instance, **3e** and **3g** are more selective for Bcl-X_L, L-Phe esters (**3n** and **3o**) are more selective for Bcl-w, while **3c** and the ketone analogues (**3f**, **3h**, **3i**, **3j**, and **3k**) are selective for Bcl-2 with **3c** > 10-fold selective for Bcl-2 over both Bcl-X_L and Bcl-w. The distinct binding profiles of BHI-1 based inhibitors among Bcl-2, Bcl-X_L, and Bcl-w suggest that BHI-1 is a promising template for the development of selective inhibitors.

2.3. Molecular modeling

To understand the observed binding selectivity of BHI-1 based inhibitors for Bcl-2, Bcl-X_L, and Bcl-w, molecular modeling of the binding interaction by InsightII was performed to define the binding section in the hydrophobic cleft on the proteins for these small molecules. The validity of the model first was evaluated by correlating (1) the model-estimated binding energies of the inhibitors to the protein with (2) experimentally determined binding affinities; the estimated binding energy correlates highly with the experimental binding affinity (Fig. 2 shows the correlation for seven BHI-1 based inhibitors for Bcl-X_L protein, $R^2 = 0.88$; similar correlations were obtained when Bcl-2 and Bcl-w homology models were used for docking as well). The high correlation between modeled and experimental results suggested that the binding model likely reflects the actual binding interactions.

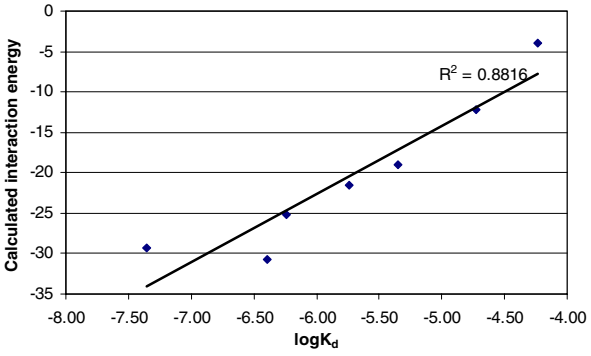


Figure 2. Correlation between the calculated binding energy and experimental binding affinity of BHl-1 based BCl-2 inhibitors.

Table 4. Non-conserved amino acids in the hydrophobic cleft of Bcl-2, Bcl-X_L, and Bcl-w

	104	108	122
Bcl-X_L	Ala	Leu	Ser
Bcl-2	Asp	Met	Arg
Bcl-w	Thr	Leu	Arg

Figure 3A shows the modeled three-dimensional structure of one BHI-1 candidate (**3e**) bound to Bcl-X_L protein. Based upon sequence alignment of Bcl-2, Bcl-X_L, and Bcl-w, the major distinctions among these three proteins in the hydrophobic cleft are amino acids 104, 108, and 122 (Table 4, Amino acids numbered based on Bcl-X_L sequence).[40] Amino acids 104 and 108 are located at the modeled binding site for BHI-1 based inhibitors, while amino acid 122 is ~2 to 6 Å from **3k** inhibitor (Fig. 1B). The modeling studies suggest that BHI-1 based inhibitors bind to the least conserved section of the hydrophobic cleft. The distinct D104 in Bcl-2 protein indicates that incorporating basic functional groups that will be protonated at physiological pH at the amino acid section of BHI-1 template will introduce binding preference to Bcl-2 and may account for the observed selective binding of **3c** to Bcl-2 protein. A104 and L108 on Bcl-X_L may be responsible for the moderate selectivity of **3e** and **3g** for Bcl-X_L because of steric effects and hydrophobic interactions. Interestingly, all of the analogues with a R2 substituent prefer Bcl-2 and we hypothesize that this selectivity trend is due to the relative wideness of the cleft among Bcl-2, Bcl-X_L, and Bcl-w; because Bcl-X_L has the narrowest cleft followed by Bcl-2, with Bcl-w the widest,[40] the analogues with a R2 substituent may fit well into the hydrophobic cleft of Bcl-2 while they are too bulky for Bcl-X_L and not big enough for Bcl-w. Incorporation of larger substituents is expected to result in analogues selective for Bcl-w. This spatial difference of the hydrophobic cleft also explains the selectivity of **3n** and **3o** for Bcl-w, while **3l** and **3m** do not bind Bcl-w selectively.

3. Conclusion

In this study, we evaluated the binding interactions of several reported inhibitors for anti-apoptotic Bcl-2 proteins with Bcl-2, Bcl-X_L, and Bcl-w proteins. None of

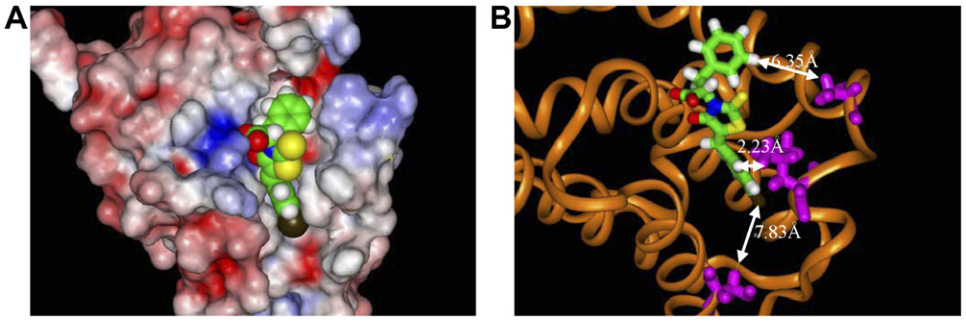


Figure 3. (A) Modeled interaction of BHI-1 based inhibitor **3e** with Bcl-X_L protein. (B) The relative position of amino acid 104, 108, and 122 (side chains are colored purple and distance in Å) to **3e**.

these inhibitors showed selectivity among the three anti-apoptotic Bcl-2 proteins. We also synthesized and evaluated a series of analogues based upon BHI-1 to search for selective inhibitors for the anti-apoptotic Bcl-2 proteins, as BHI-1 targets the less conserved section of the hydrophobic cleft. Our results suggest that modifications of BHI-1 can introduce dramatic binding affinity changes and varied binding profile among Bcl-2, Bcl-X_L, and Bcl-w proteins. Modeling of the binding interaction of this series of analogues with the three proteins supports that the binding site for BHI-1 is the least conserved section in the long hydrophobic cleft. The model also explains the observed binding selectivity of BHI-1 based inhibitors for Bcl-2, Bcl-X_L, and Bcl-w proteins. Molecular-modeling-aided rational design of member-selective inhibitors based on the BHI-1 template is in progress.

4. Experimental

4.1. Preparation of recombinant Bcl-2, Bcl-X_L, and Bcl-w proteins

The DNA sequence encoding Bcl-2ΔC21 inserted into a His_6 tag fused pET-25b(+) vector was a generous gift from Stanley Korsmeyer at Harvard University; the DNA sequence encoding Bcl-wΔC22 and the DNA sequence encoding Bcl-X_LΔloopΔC40 inserted into a His_6 tag fused pET-29b(+) vector were generous gifts from Kalle Gehring at McGill University. The plasmids were transformed into the Escherichia coli strain ER2566 (New England Biolab., MA). The expression of the fusion proteins was induced by 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) and the fusion proteins were purified by Ni–NTA resin following a native protein purification protocol provided by manufacturer (Qiagen, CA). Recombinant proteins were concentrated with Centrifugal Filter Devices (Millipore, MA) and dialyzed against phosphate-buffered saline (PBS) containing 15% glycerol, 1 mM dithiothreitol (DTT). The concentration of the recombinant protein was determined by the Bradford method with bovine serum albumin (BSA) as a standard, and stored at −20 °C.

4.2. Fluorescence polarization assays (FPA)

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 manufacturer's protocol (Promega, CA), purified by HPLC, analyzed by mass spectrometry (Calculated: 2119 Da; Observed: 2119.7 Da), and named as Flu-Bak. Flu-Bak was dissolved in double distilled water and stored at −20 °C as aliquot. FPA were conducted with the Flu-Bak peptide by using a GENios Pro plate reader (Tecan US, NC) with all assays performed in triplicate and each assay performed twice.

To determine the binding affinity of the Flu-Bak peptide for an anti-apoptotic Bcl-2 protein, a series of 3-fold dilutions of the anti-apoptotic Bcl-2 protein was prepared in a PBS solution, pH 7.0, with 45 nM Flu-Bak peptide and 1 mM DTT, and incubated at 23 °C for one hour (a time-course study of binding process of Flu-Bak peptide to all the anti-apoptotic Bcl-2 proteins demonstrated that binding interaction reaches 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 (FP, in mP unit) was measured. The binding affinity was determined by fitting the FP values to the concentrations of protein with a single-binding site saturation model, by using Prism software package (GraphPad, CA).[41]

To determine the binding affinity of small molecules for an anti-apoptotic Bcl-2 protein, a series of 3-fold dilutions of small molecules were prepared in dimethylsulfoxide, that is, 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 solution containing 50 nM Flu-Bak peptide and the anti-apoptotic Bcl-2 protein to be tested in PBS buffer, pH 7.0, 1 mM DTT was prepared and incubated at 23 °C for 1 h. The concentration of the anti-apoptotic Bcl-2 protein used corresponds to the one that resulted in 60% of Flu-Bak peptide complex with the anti-apoptotic Bcl-2 protein and such a concentration was determined in the Flu-Bak and Bcl-2 protein saturation binding experiment. There are two reasons that we chose the condition with 60% complex formation for the competing assay. First, with 60% complex formation, there will be a FP increase of >100 mP over the background. Such a FP increase would generate a considerable FP change in the presence of effective competition over background variation. Second, with 60% complex formation, relatively less competing ligand needs to be added to detect a considerable FP change compared to the competition with higher percentage of complex formation. The corresponding concentrations of Bcl-2, Bcl-X_L, and Bcl-w needed to achieve 60% complex formation under the assay conditions are 1000, 880, and 1200 nM, respectively. To each well the Flu-Bak peptide and anti-apoptotic Bcl-2 protein solution (45 μl) was added by auto-injection at a rate of 200 μl/s. The sample was incubated at 23 °C for 1 h and shaken for two seconds before fluorescence polarization (in mP unit) was measured. 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 changes to the concentration of the small molecule competing ligand by using an equation developed by Shaomeng Wang et al. with GraphPad Eq. 1.[42]

$$FP = \frac{FP_{max}}{1 + X * K_d/(2 * K_i * L_0 + K_i * P_0 + K_i * K_d)} \quad (1)$$

FP_{max}, the fluorescence polarization value when 60% of Flu-Bak binds to the protein; FP, the fluorescence polarization value with the addition of the small molecule; K_d, dissociation constant of Flu-Bak to the protein;

L_0, the total concentration of Flu-Bak peptide; P_0, the total concentration of protein; K_i, the inhibitory constant of the small molecule to the binding of Flu-Bak to protein.

4.3. Molecular modeling

Molecular modeling studies were conducted on a SGI Octane2 workstation using InsightII (Accelrys Inc., CA). The NMR energy-minimized average structure of Bcl-X_L was extracted from the complex of Bcl-X_L and Bak peptide from the protein data bank (PDB code: 1BXL). The structures of Bcl-2 and Bcl-w used for modeling studies were generated by homology-mimicking the structure of Bcl-X_L from the complex of Bcl-X_L and Bak peptide based on sequence alignments (PDB code: 1G5M, 1BXL, and 1MK3, respectively, for Bcl-2, Bcl-X_L, and Bcl-w) with side chains energy-minimized. The structures of all the inhibitors were created and energy-minimized within InsightII, followed by docking of the inhibitors into the Bak peptide binding site.[22] The energy minimization of the ligand–protein complex was carried out by keeping the binding site rigid using a dielectric constant of 80. The binding energy of the ligand to the protein was calculated by using $\varepsilon = 6$.

4.4. 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 either *p*-anisaldehyde, potassium permanganate, or cerium molybdate solutions followed by heating. Flash column chromatography was performed on Fischer Scientific silica gel (230–400 mesh). Melting points were determined by using a Thomas Hoover capillary melting point apparatus. 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. High-resolution mass spectra (HRMS) were recorded on a BrukerBio TOF II mass spectrometer. Reversed-phase high-performance liquid chromatography (RP-HPLC) was performed on a Beckman–Coulter System Gold 126 solvent module and 168 detector. Results were analyzed using the 32 Karat software package. Method MA1, MA2, MA3, and MB3 were used on a Celius C18 column, 4.6×150 mm. Method MB1 and MB2 were used on a Phenomenex Polar-RP column, 4.6×250 mm. Mobile phase A was 0.1%TFA in water, B1 was 0.1%TFA in acetonitrile, and B2 was 0.1%TFA in methanol. Flow rate was 1.0 mL/min. The run duration was 30 min. MA1 and MB1 were detected at 375 nm; MA2 and MB2 were detected at 355 nm; MA1 and MB3 were detected at 395 nm. The time program for MA1 was 100% A (0–5 min), 0–35% B1 (5–7 min), 35–95% B1 (7–22 min), 95% B1 (22–29 min), 95%–0B1 (29–30 min); MA2 was 100% A (0–5 min), 0–65% B1 (5–7 min), 65–75% B1 (7–22 min), 75–80% B1 (22–29 min), 80%–0B1 (29–30 min); MA3 was 100% A (0–5 min), 0–70% B1 (5–7 min), 70–80% B1 (7–22 min), 80–85% B1 (22–29 min), 85%–0B1 (29–30 min); MB1 was 100% A (0–5 min), 0–95% B2 (5–7 min), 95% B2 (7–29 min), 95%–0B2 (29–30 min); MB2 was 100% A (0–5 min), 0–85% B2 (5–7 min), 85% B2 (7–29 min), 85%–0B2 (29–30 min); MB3 was 100% A (0–5 min), 0–80% B2 (5–7 min), 80–90% B2 (7–22 min), 90–95% B2 (22–29 min), 95%–0B2 (29–30 min).

4.5. General procedure for synthesis of BHI-1 based inhibitors

In a round-bottomed flask equipped with a magnetic stirrer, the amino acid or ester (1 mmol) was dissolved with sodium hydroxide (80 mg, 2 mmol) in water (10 ml). Then, carbon disulfide (60 μl, 1 mmol) was added to the reaction mixture, which was stirred vigorously overnight. An aqueous solution of $ClCH_2CO_2Na$ (1 ml, 1 M, 1 mmol) was added and stirring was continued at 23 °C for 3 h. Then hydrochloric acid solution (3 ml, 5.5 N, 16.5 mmol) was added and the reaction mixture was refluxed overnight. The reaction mixture was neutralized with saturated $NaHCO_3$ solution. The solvent was removed under vacuum and the cyclized product was purified by flash chromatography. The cyclized intermediates are reported before and no characterization data are included herein.[39]

4.6. General procedure for condensation with aldehydes/ketones

To a round-bottomed flask equipped with a reflux condenser and a magnetic stirrer, cyclized rhodanine intermediate (1 mmol) in toluene (20 ml) was added. To this, the aldehyde/ketone (3 mmol) was added along with ammonium acetate (3 mmol). The mixture was refluxed overnight and the solvent was removed under vacuum. The final rhodanine candidate was purified by flash chromatography.

4.7. 2-[5-(4-Bromobenzylidene)-4-oxo-2-thioxothiazolidin-3-yl]-3-methylbutanoic acid (BHI-1)

Yield: 85%. TLC (CH_2Cl_2/MeOH = 5:1), R_f: 0.40. IR (KBr): 3700–2200, 2967, 2897, 1727, 1710, 1603, 1582, 1486, 1382, 1327, 1242, 1202, 1128, 1072, 1005, 815 cm^{-1}. ^{1}H NMR ($CDCl_3$): δ 7.62 (1H, s, 5′-dene proton), 7.57 (2H, d, $J = 8.5$ Hz, 2″ and 6″Ph protons), 7.30 (2H, d, $J = 8.5$ Hz, 3″ and 5″Ph protons), 5.34 (1H, m, 2-methine proton), 2.84 (1H, m, 3-methine proton), 1.25 (6H, d, $J = 7.4$ Hz, 3, 3-dimethyl protons); ESI-MS (negative): *m/z* 398, 400 (M^--H).

4.8. 2-[5-(4-Bromobenzylidene)-4-oxo-2-thioxothiazolidin-3-yl]propanoic acid (3a)

Yield: 81%. Yellow powder. TLC (CH_2Cl_2/MeOH = 10:1), R_f 0.11. Mp: 223–224 C°. IR (KBr): 3700–2200, 2922, 1714, 1602, 1583, 1486, 1400, 1342, 1249, 1125, 1071, 1006, 829 cm^{-1}. ^{1}H NMR ($CDCl_3$): δ 7.64 (1H, s, 5′-dene proton), 7.62 (2H, d, $J = 8.7$ Hz,

2″ and 6″ Ph protons), 7.35 (2H, d, J = 8.7 Hz, 3″ and 5″ Ph protons), 5.79 (1H, m, 2-methine proton), 1.68 (3H, d, J = 7.6 Hz, 3-methyl protons); ESI-MS (negative): m/z 370, 372 (M^-−H). HRMS ($C_{13}H_9BrNO_3S_2$) [M−H]$^-$: found m/z 369.9191, calcd m/z 369.9205; RP-HPLC method A1, minor isomer t_R = 19.150 min (2.19%) and major isomer t_R = 20.300 min (97.81%); method B1, minor isomer t_R = 15.083 min (2.12%) and major isomer t_R = 15.500 min (87.93%).

4.9. 2-[5-(4-Bromobenzylidene)-4-oxo-2-thioxothiazolidin-3-yl]-4-methylthiobutanoic acid (3b)

Yield: 81%. TLC (CH_2Cl_2/MeOH = 5:1), R_f 0.29. Mp: 165–166 C°. IR (KBr): 3700–2200, 2922, 1714, 1602, 1583, 1486, 1400, 1342, 1249, 1125, 1071, 1006, 829 cm^{-1}. ^{1}H NMR ($CDCl_3$): δ 7.62–7.45 (3H, m, 5′-dene proton and 2″ and 6″ Ph protons), 7.28–7.24 (2H, d, J = 8.4 Hz, 3″ and 5″ Ph protons), 5.76 (1H, m, 2-methine proton), 2.60–2.26 (4H, m, 3- and 4-methylene protons), 2.01 (3H, s, 4-methylthio methyl protons); ESI-MS (negative): m/z 430, 432 (M^-−H); 386, 388 (M^-−H–CO_2). HRMS ($C_{15}H_{13}BrNO_5$) [M−H]$^-$: found m/z 429.9250, calcd m/z 429.9239; RP-HPLC method MA1, minor isomer t_R = 20.367 min (12.07%) and major isomer t_R = 21.483 min (87.93%); method MB1, minor isomer t_R = 15.767 min (12.67%) and major isomer t_R = 16.300 min (87.33%).

4.10. 2-[5-(4-Bromobenzylidene)-4-oxo-2-thioxothiazolidin-3-yl]-3-(3-indolyl)-propanoic acid (3c)

Yield: 78%. Yellow powder. TLC (CH_2Cl_2/MeOH = 5:1), R_f 0.24. Mp: 158–159 C°. IR (KBr): 3700–2200, 2924, 1691, 1601, 1351, 1138 cm^{-1}. ^{1}H NMR ($CDCl_3$): δ 7.73 (2H, d, J = 8.4 Hz, 2″ and 6″ Ph protons), 7.62 (1H, d, J = 8.1 Hz, indole-7 proton), 7.60 (1H, s, 5′-dene proton), 7.48 (2H, d, J = 8.4 Hz, 3″ and 5″ Ph protons), 7.28 (1H, d, J = 8.1 Hz, indole-4 proton), 7.14–6.99 (2H, m, indole-5,6 protons), 7.00 (1H, s, indole-2 proton), 5.90 (1H, m, 2-methine proton), 4.04 (1H, dd, J_1 = 13.2 Hz, J_2 = 3.9 Hz, 3-methylene proton), 3.80 (1H, dd, J_1 = 13.2 Hz, J_2 = 3.9 Hz, 3-methylene proton); ESI-MS (negative): m/z 485, 487 (M^-−H); 441, 443 (M^-−H–CO_2); HRMS ($C_{21}H_{14}BrNO_3S_2$) [M−H]$^-$: found m/z 484.9634, calcd m/z 484.9627; RP-HPLC method MA1, minor isomer t_R = 19.467 min (1.23%) and major isomer t_R = 20.250 min (98.73%); method MB1, minor isomer t_R = 14.700 min (5.44%) and major isomer t_R = 15.100 min (94.56%).

4.11. 2-[5-(4-Bromobenzylidene)-4-oxo-2-thioxothiazolidin-3-yl]-glutaric acid (3d)

Yield: 76%. Yellow powder. TLC (CH_2Cl_2/MeOH = 1:1), R_f 0.28. Mp: 227–228 C°. IR (KBr): 3700–2200, 1700, 1602, 1487, 1401, 1345, 1252, 1174, 1073, 1008, 819 cm^{-1}. ^{1}H NMR (CD_3OD): δ 7.77 (2H, d, J = 8.4 Hz, 2″ and 6″ Ph protons), 7.70 (1H, s, 5′-dene proton), 7.57 (2H, d, J = 8.4 Hz, 3″ and 5″ Ph protons), 5.64 (1H, m, 2-methine proton), 2.30–2.04 (4H, m, 3 and 4-methylene protons); ESI-MS (negative): m/z 428, 430 (M^-−H). HRMS ($C_{15}H_{11}BrNO_5S_2$) [M−H]$^-$: found m/z 427.9268, calcd m/z 427.9260; RP-HPLC method MA1, minor isomer t_R = 16.417 min (15.06%) and major isomer t_R = 17.333 min (84.94%); method MB1, minor isomer t_R = 13.900 min (11.14%) and major isomer t_R = 14.433 min (92.53%).

4.12. 2-[5-(4-Bromobenzylidene)-4-oxo-2-thioxothiazolidin-3-yl]-3- phenylpropanoic acid (3e)

Yield: 89%. Yellow powder. TLC (CH_2Cl_2/MeOH = 10:1), R_f 0.13. Mp: 242–243 C°. IR (KBr): 3700–2200, 2923, 2361, 1700, 1600, 1487, 1400, 1342, 1239, 1174, 1073, 1008, 829, 700, 546 cm^{-1}. ^{1}H NMR ($CDCl_3$): δ 7.64–7.32 (3H, m, 5′-dene proton and 2″ and 6″ Ph protons), 7.26–7.00 (7H, m, 3″ and 5″ Ph and 3-Ph protons), 5.93 (1H, m, 2-methine proton), 3.55 (2H, m, 3-methylene protons); ESI-MS (negative): m/z 446, 448 (M^-−H); 402, 404 (M^-−H–CO_2). HRMS ($C_{19}H_{13}BrNO_3S_2$) [M−H]$^-$: found m/z 425.9534, calcd m/z 445.9518; RP-HPLC method MA1, minor isomer t_R = 21.050 min (5.42%) and major isomer t_R = 22.067 min (94.58%); method MB1, minor isomer t_R = 15.583 min (6.80%) and major isomer t_R = 16.167 min (93.20%).

4.13. 2-[5-(1-Phenylbutylidene)-4-oxo-2-thioxothiazolidin-3-yl]-3-phenylpropanoic acid (3f)

Yield: 43%. Orange oil. TLC (hexane/ethyl acetate = 1:1), R_f 0.38. IR (KBr): 3700–2200, 3027, 2959, 2929, 2870, 1711, 1589, 1569, 1455, 1441, 1382, 1332, 1230, 1175, 1029, 970, 920, 751, 698 cm^{-1}. ^{1}H NMR ($CDCl_3$): δ 7.43–7.18 (10H, m, 3-Ph and Ph″ protons), 6.05 (1H, m, 2-methine proton), 3.60 (2H, d, J = 7.2 Hz, 3-methylene protons), 3.17 (2H, m, ($CH_3CH_2CH_2$)″ protons), 1.41 (2H, m, ($CH_3CH_2CH_2$)″ protons), 0.94 (3H, t, J = 7.2 Hz, ($CH_3CH_2CH_2$)″ protons). ESI-MS (negative): m/z 410 (M^-−H). HRMS ($C_{22}H_{21}NO_3S_2$) [M−H]$^-$: found m/z 410.0892, calcd m/z 410.0883; RP-HPLC method MA2, minor isomer t_R = 23.233 min (35.97%) and major isomer t_R = 23.983 min (64.03%); method MB2, minor isomer t_R = 22.717 min (34.23%) and major isomer t_R = 23.833 min (65.77%).

4.14. 2-[5-(4-Bromobenzylidene)-4-oxo-2-thioxothiazolidin-3-yl]-4-methylpentanoic acid (3g)

Yield: 79%. Yellow powder. TLC (CH_2Cl_2/MeOH = 10:1), R_f 0.22. Mp: 155–156 C°. IR (KBr): 3700–2200, 2956, 1716, 1700, 1601, 1558, 1341, 1273, 1239, 1207 cm^{-1}. ^{1}H NMR ($CDCl_3$): δ 7.64 (1H, s, 5′-dene proton), 7.53 (2H, d, J = 8.1 Hz, 2″ and 6″ Ph protons), 7.23 (2H, d, J = 8.1 Hz, 3″ and 5″ Ph protons), 5.53 (1H, m, 2-methine proton), 2.00 (2H, m, 3-methylene protons), 1.23 (1H, m, 4-methine proton), 0.86 (6H, m, 4,4-dimethyl protons); ESI-MS (negative): m/z 412, 414 (M^-−H); 358, 360 (M^-−H–CO_2). HRMS ($C_{16}H_{16}NO_3S_2$) [M−H]$^-$: found m/z 411.9702, calcd m/z 411.9675; RP-HPLC method MA1, minor isomer t_R = 22.167 min (9.20%) and major isomer t_R = 23.433 min (90.80%); method MB1, minor isomer t_R = 15.317 min (13.02%) and major isomer t_R = 15.767 min (86.98%).

4.15. 2-[5-(1-Phenylbutylidene)-4-oxo-2-thioxothiazolidin-3-yl]-4- methylpentanoic acid (3h)

Yield: 46%. Orange oil. TLC (hexane/ethyl acetate = 1:1), R_f 0.17. IR (KBr): 3700–2200, 2959, 2930, 2870, 1709, 1590, 1332, 1232, 1213 cm^{-1}. ^1H NMR ($CDCl_3$): δ 7.51–7.20 (5H, m, Ph″ protons), 5.79 (1H, m, 2-methine proton), 3.21 (2H, m, $(CH_3CH_2CH_2)''$ protons), 2.20 (2H, m, $(CH_3CH_2CH_2)''$ protons), 2.01 (2H, m, 3-methylene protons), 1.60 (1H, m, 4-methine proton), 1.10–0.80 (9H, m, 4,4-dimethyl and $(CH_3CH_2CH_2)''$ protons). ESI-MS (negative): *m/z* 376 (M^-−H). HRMS ($C_{19}H_{22}NO_3S_2$) $[M-H]^-$: found *m/z* 376.1050, calcd *m/z* 376.1039; RP-HPLC method MA1, minor isomer t_R = 22.617 min (37.27%) and major isomer t_R = 22.083 min (62.73%); method MB2, minor isomer t_R = 22.733 min (34.89%) and major isomer t_R = 15.750 min (63.11%).

4.16. 2-[5-(1-Phenylethylidene)-4-oxo-2-thioxothiazolidin-3-yl]-4- methylpentanoic acid (3i)

Yield: 38%. Orange oil. TLC (hexane/ethyl acetate = 1:1), R_f 0.17. IR (KBr): 3700–2200, 2957, 2869, 1708, 1591, 1570, 1490, 1442, 1331, 1213, 1141, 1028, 990, 760, 698 cm^{-1}. ^1H NMR ($CDCl_3$): δ 7.59–7.20 (5H, m, Ph″ protons), 5.73 (1H, m, 2-methine proton), 2.72 (3H, s, 5-methyl″ protons), 2.14 (2H, m, 3-methylene protons), 1.55 (1H, m, 4-methine proton), 0.95 (6H, m, 4,4-dimethyl protons). ESI-MS (negative): *m/z* 348 (M^-−H). HRMS ($C_{17}H_{18}NO_3S_2$) $[M-H]^-$: found *m/z* 348.0756, calcd *m/z* 348.0726; RP-HPLC method MA2, minor isomer t_R = 17.800 min (23.19%) and major isomer t_R = 18.983 min (76.81%); method MB2, minor isomer t_R = 19.183 min (27.46%) and major isomer t_R = 20.133 min (72.54%).

4.17. 2-[5-(Diphenylmethylidene)-4-oxo-2-thioxothiazolidin-3-yl]-4- methylpentanoic acid (3j)

Yield: 30%. Orange oil. TLC (hexane/ethyl acetate = 1:1), R_f 0.26. IR (KBr): 3700–2200, 3056, 2956, 2869, 1712, 1616, 1579, 1558, 1444, 1330, 1212, 1133, 1093, 752, 697 cm^{-1}. ^1H NMR ($CDCl_3$): δ 7.51–7.20 (10H, m, Ph″, Ph″ protons), 5.76 (1H, m, 2-methine proton), 2.16 (2H, m, 3-methylene protons), 1.60 (1H, m, 4-methine proton), 1.10–0.93 (6H, m, 4,4-dimethyl protons). ESI-MS (negative): *m/z* 410 (M^-−H). HRMS ($C_{22}H_{20}NO_3S_2$) $[M-H]^-$: found *m/z* 410.0896, calcd *m/z* 410.0883; RP-HPLC method MA1, t_R = 22.700 min; method MB1, t_R = 16.083 min.

4.18. 2-[5-(Phenyl(4-biphenyl)methylidene)-4-oxo-2-thioxothiazolidin-3-yl]-4-methylpentanoic acid (3k)

Yield: 23%. Orange oil. TLC (hexane/ethyl acetate = 1:1), R_f 0.19. IR (KBr): 3700–2200, 3029, 2956, 2869, 1712, 1352, 1330, 1213, 1179, 752, 697 cm^{-1}. ^1H NMR ($CDCl_3$): δ 7.65-7.26 (14H, m, Ph″ and biphenyl″ protons), 5.77 (1H, m, 2-methine proton), 2.17 (2H, m, 3-methylene protons), 1.60 (1H, m, 4-methine proton), 1.00–0.90 (6H, m, 4,4-dimethyl protons). ESI-MS (negative): *m/z* 486 (M^-−H). HRMS ($C_{28}H_{24}NO_3S_2$) $[M-H]^-$: found *m/z* 486.1196, calcd *m/z* 486.1196; RP-HPLC method MA3, minor isomer t_R = 22.783 min (39.89%) and major isomer t_R = 23.750 min (60.11%); method MB3, minor isomer t_R = 23.317 min (42.08%) and major isomer t_R = 24.317 min (57.92%).

4.19. 2-Propenyl 2-[5-(4-bromobenzylidene)-4-oxo-2-thioxothiazolidin-3-yl]-3-propanoate (3l)

Yield: 66%. Yellow powder. TLC (Hexane/CH_2Cl_2 = 1:1), R_f 0.50. Mp: 105–106 C°. ^1H NMR ($CDCl_3$): δ 7.63 (1H, s, 5′-dene proton), 7.62 (2H, d, J = 8.4 Hz, 2″ and 6″ Ph protons), 7.35 (2H, d, J = 8.4 Hz, 3″ and 5″ Ph protons), 5.87 (1H, m, 2-methine proton), 5.73 (1H, m, $CH_2CH{=}CH_2$ proton), 5.30 and 5.23 (2H, d and d, J = 17.4 Hz and J = 9.6 Hz, $CH_2CH{=}CH_2$ protons), 4.66 (2H, m, $CH_2CH{=}CH_2$ protons), 1.69 (3H, d, J = 9.6 Hz, 3-methyl protons); ESI-MS (positive): *m/z* 412, 414 (M^++H); 368, 370 (M^++H–CO_2). HRMS ($C_{16}H_{14}BrNO_3S_2Na$) $[M+Na]^+$: found *m/z* 433.9504, calcd *m/z* 433.9495; RP-HPLC method MA1, minor isomer t_R = 23.083 min (3.45%) and major isomer t_R = 24.267 min (96.55%); method MB1, minor isomer t_R = 16.917 min (0.95%) and major isomer t_R = 17.70 min (99.05%).

4.20. 5-(4-Bromobenzylidene)-3-(1-morpholino-1-oxo-3-phenylpropan-2-yl)-4-oxo-2-thioxothiazolidine (3m)

Yield: 97%. Yellow oil. TLC (methanol/CH_2Cl_2 = 1:99), R_f 0.40. ^1H NMR ($CDCl_3$): δ 7.62 (1H, s, 5′-dene proton), 7.57 (2H, d, J = 8.4 Hz, 2″ and 6″ Ph protons), 7.35 (2H, d, J = 8.4 Hz, 3″ and 5″ Ph protons), 5.56 (1H, q, J = 7.5 Hz, 2-methine proton), 3.83 (4H, t, J = 5.4 Hz, $N(CH_2)_2(CH_2)_2O$ protons), 3.07 (4H, t, J = 5.4 Hz, $N(CH_2)_2(CH_2)_2O$ protons), 1.63 (3H, d, J = 7.2 Hz, 3-methyl protons); ESI-MS (positive): *m/z* 441, 443 (M^++H); 397, 399 (M^++H–CO_2). HRMS ($C_{17}H_{17}BrN_2O_3S_2Na$) $[M+Na]^+$: found *m/z* 462.9758, calcd *m/z* 462.9761; RP-HPLC method MA1, minor isomer t_R = 20.050 min (8.66%) and major isomer t_R = 21.150 min (91.34%); method MB1, minor isomer t_R = 16.600 min (8.71%) and major isomer t_R = 17.300 min (91.29%).

4.21. 2-Propenyl 2-[5-(4-bromobenzylidene)-4-oxo-2-thioxothiazolidin-3-yl]-3-phenylpropanoate (3n)

Yield: 82%. Yellow powder. TLC (CH_2Cl_2), R_f 0.54. Mp: 146–147 C°. ^1H NMR ($CDCl_3$): δ 7.61 (2H, d, J = 8.1 Hz, 2″ and 6″ Ph protons), 7.60 (1H, s, 5′-dene proton), 7.31 (2H, d, J = 8.1 Hz, 3″ and 5″ Ph protons), 7.20 (5H, m, 3-Ph protons), 5.92 (1H, m, $CH_2CH{=}CH_2$ proton), 5.88 (1H, m, 2-methine proton), 5.30 and 5.25 (2H, d and d, J = 18.5 Hz and J = 10.5 Hz, $CH_2CH{=}CH_2$ protons), 4.69 (2H, d, J = 4.8 Hz, $CH_2CH{=}CH_2$ protons), 3.63 (2H, d, J = 8.4 Hz, 3-methylene protons); ESI-MS (positive): *m/z* 488, 490 (M^++H); 444, 446 (M^++H–CO_2). HRMS ($C_{22}H_{18}BrNO_3S_2Na$) $[M+Na]^+$: found *m/z* 509.9834, calcd *m/z* 509.9808; RP-HPLC method MA1, minor isomer t_R = 24.700 min (12.28%) and major isomer t_R = 25.600 min (87.72%);

method MB1, minor isomer t_R = 17.983 min (9.07%) and major isomer t_R = 19.017 min (90.93%).

4.22. 2-Propenyl 2-[5-biphenylmethylidene-4-oxo-2-thioxo-thiazolidin-3-yl]-3- phenylpropanoate (3o)

Yield: 83%. Yellow oil. TLC (hexane/CH_2Cl2 = 6:4), R_f 0.20. ^1H NMR ($CDCl_3$): δ 7.64 and 7.60 (4H, d and d, J = 8.1 Hz and J = 8.1 Hz, $(C_6H_4C_6H_5)''$ protons), 7.62 (1H, s, 5′-dene proton),7.45 (5H, m, $(C_6H_4C_6H_5)''$ protons), 7.30 (5H, m, 3-Ph protons), 5.93 (1H, m, $CH_2CH{=}CH_2$ proton), 5.89 (1H, m, 2-methine proton), 5.30 and 5.25 (2H, d and d, J = 17.8 Hz and J = 10.5 Hz, $CH_2CH{=}CH_2$ protons), 4.69 (2H, d, J = 5.4 Hz, $CH_2CH{=}CH_2$ protons), 3.66 (2H, d, J = 7.8 Hz, 3-methylene protons). ESI-MS (positive): m/z 486 (M^++H); 442 (M^++H–CO_2). HRMS ($C_{28}H_{23}NO_3S_2Na$) $[M + Na]^+$: found m/z 508.1028, calcd m/z 508.1067; RP-HPLC method MA1, minor isomer t_R = 26.233 min (15.50%) and major isomer t_R = 27.083 min (84.50%); method MB1, minor isomer t_R = 19.167 min (13.53%) and major isomer t_R = 19.950 min (86.47%).

Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2006.12.020.

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