Cell Death and Differentiation (2006) 13, 1419–1421

www.nature.com/cdd

Letter to the Editor

Comparison of chemical inhibitors of antiapoptotic Bcl-2-family proteins

Cell Death and Differentiation (2006) **13,** 1419–1421. doi:10.1038/sj.cdd.4401937; published online 28 April 2006

Dear Editor,

Bcl-2-family proteins are critical regulators of cell life and death, with homologs found in most animal species. In humans, six antiapoptotic members of the Bcl-2 family have been identified and characterized, including Bcl-2, Bcl-X_L, Mcl-1, Bcl-W, Bfl-1, and Bcl-B.[1] Overexpression of antiapoptotic Bcl-2 family proteins occurs in many cancers, generating interest in these proteins as possible drug discovery targets.[2] A favored strategy for Bcl-2 antagonism is based on mimicking the actions of endogenous inhibitors that bind Bcl-2 and its relatives via BH3 domains (reviewed in Reed[3] and Huang[4]). The BH3 domain is a protein interaction motif found in numerous proapoptotic members of the Bcl-2 family that consists of a $\sim$16–25 amino-acid amphipathic alpha-helix.[1] Peptides corresponding to the BH3 domain bind a hydrophobic crevice found on the surface of antiapoptotic Bcl-2 family proteins, thereby nullifying their cytoprotective activity and promoting cell death.[5]

Several chemicals have been identified or generated synthetically that occupy the same binding site on Bcl-2 or Bcl-X_L as BH3 peptide, promoting apoptosis. These chemical inhibitors of Bcl-2 and Bcl-X_L are now commonly employed as research tools for interrogating the function of antiapoptotic Bcl-2 family proteins, and some may also enter human clinical trials as potential cancer treatments.[6] However, the specificity of these chemicals for the six human antiapoptotic Bcl-2 family proteins has not been heretofore described and the relative affinities of these compounds for the BH3-binding site have not been compared.

Here, we describe fluorescence polarization assays (FPAs) for monitoring the binding of fluorochrome-conjugated BH3 peptides to all six antiapoptotic human Bcl-2 family proteins and we use these FPAs to compare the known small-molecule inhibitors of Bcl-2 and Bcl-X_L. The findings provide a framework for utilizing these compounds as tools for cell death research and for possibly interpreting their activities in clinical trials.

For these experiments, we expressed the six human antiapoptotic Bcl-2 family proteins in bacteria as glutathione *S*-transferase (GST)-fusion proteins, in which the C-terminal transmembrane domains were deleted for purposes of enhancing protein solubility. Previous structural studies of Bcl-2, Bcl-X_L, Bcl-W, and Mcl-1 have been performed using proteins in which the C-terminal membrane-insertion domain was similarly omitted from the recombinant protein.[7–9] Proteins were purified by one-step affinity chromatography using glutathione-Sepharose. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis with Coomassie staining demonstrated that the resulting proteins were $>$85% intact (Figure 1a).

Various synthetic BH3 peptides with N-terminal fluoroscein isothiocynate (FITC) were tested for binding to the six antiapoptotic Bcl-2 family proteins by FPA. BH3 peptide sequences derived from Bid or Bim bound to all six proteins, in a concentration-dependent and saturable manner, whereas other BH3 peptides derived from Bad or Bak were more selective in their binding (Figures 1b, c and not shown), consistent with prior reports.[10] The relative affinities (EC_{50}) of FITC-Bid BH3 peptide of Bfl-1, Mcl-1, Bcl-X_L, Bcl-B, Bcl-W, and Bcl-2 were 4.6, 11.3, 7.9, 16, 30.3, and 83 nM, respectively (Figures 1b, c), based on experiments where various concentrations of GST-Bcl-2-family fusion proteins were titrated into reactions with a fixed concentration of FITC-Bid peptide. Binding of FITC-labeled BH3 peptides was competed by unlabeled Bid BH3 peptides, confirming competitive, reversible binding. The relative competitive affinities (IC_{50}) against GST-Bfl-1, Mcl-1, Bcl-X_L, Bcl-B, Bcl-W, and Bcl-2 were 0.55, 0.67, 1.53, 3.32, 1.29, and 2.29 μM, respectively (Figures 1d and e). The stoichiometry of antiapoptotic Bcl-2-family proteins *in vivo* is unknown, but because GST-fusion proteins tend to be dimeric, we sought to compare results with monomeric proteins. Cleavage of GST from the proteins using thrombin was successful only for Bfl-1, owing to internal sites of cleavage in the other proteins. Expression of the proteins with His6-tags instead of GST was successful for Bcl-X_L, resulting in soluble protein with good yield and providing an alternative approach to monomeric protein production. Indeed, gel-sieve chromatography confirmed that thrombin cleaved Bfl-1 and His6-Bcl-X_L were monomeric proteins (not shown). When using these monomeric proteins and FITC-Bid BH3 peptide for FPAs, results comparable to the GST-fusion proteins were obtained in competition studies using unlabeled Bid BH3 peptide ($IC_{50} = 2.3\,\mu$M for Bcl-X_L and 0.73 μM for Bfl-1) (Figure 1f). Thus, GST does not significantly affect the results of these FPAs.

We then used the FITC-Bid peptide to profile various Bcl-2 inhibitory compounds against the six human antiapoptotic members of the Bcl-2 family expressed as GST-fusions, evaluating a range of concentrations of the compounds with respect to their ability to compete with FITC-Bid peptide for binding. An example of competitive BH3 peptide displacement is shown in Figure 1g for the green tea compound, (−)-Epigallocatechin gallate (EGCG), and an inactive control compound (−)-Catechin (−C). Note that EGCG reduced the binding of FITC-Bid to all six human antiapoptotic Bcl-2 family

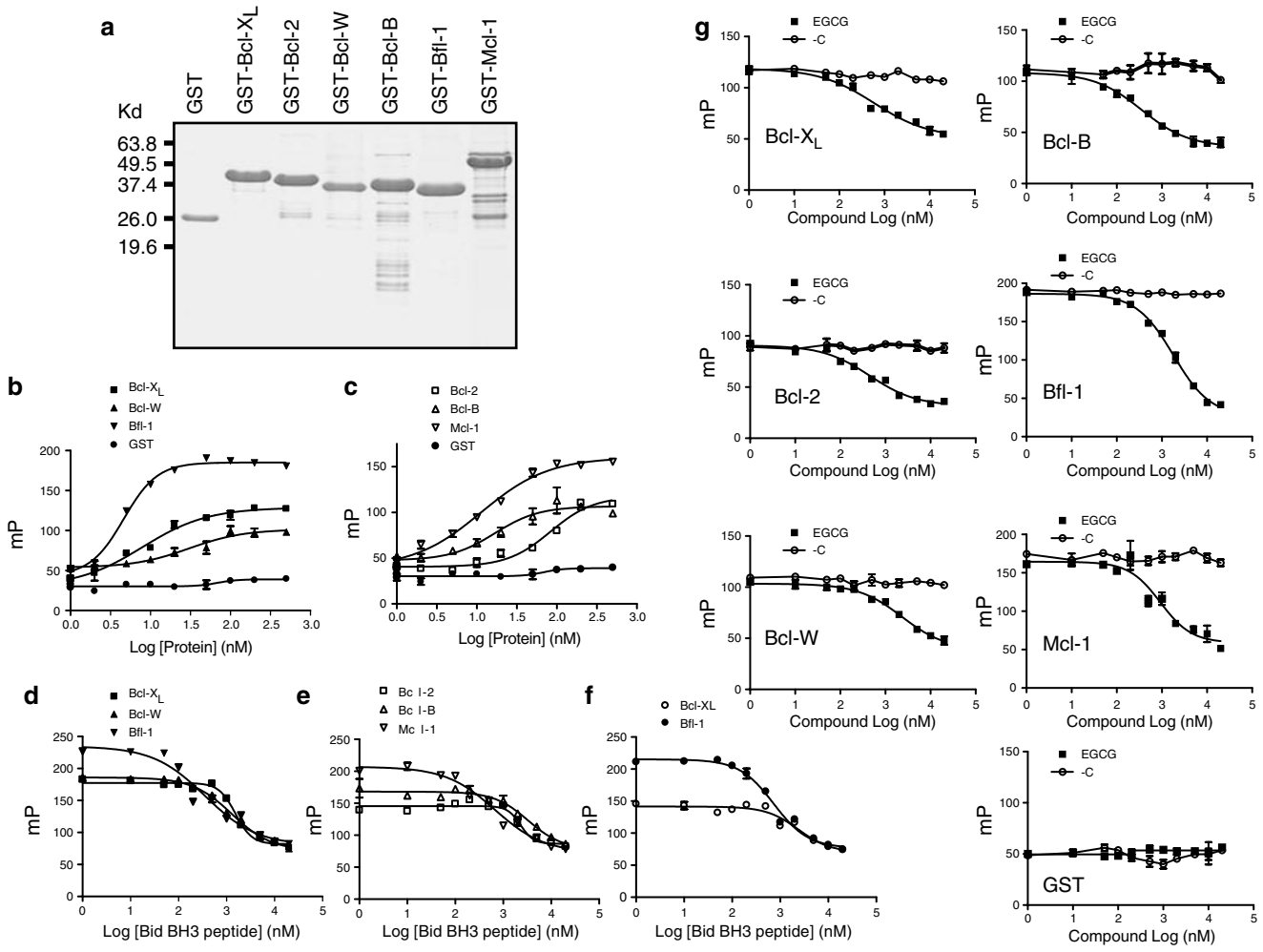

Figure 1 Development of FPAs for monitoring BH3 peptide binding to antiapoptotic human Bcl-2-family proteins. (**a**) Expression and purification of Bcl-2 family proteins. GST-fusion proteins were produced in bacteria and purified by affinity chromatography using glutathione-sepharose. Ten micrograms of each purified protein was analyzed by SDS-PAGE followed by Coomassie Blue staining. GST protein was used as control. Molecular weight markers are shown in kilo-Daltons. GST-fusion proteins containing Bcl-X_L, Bcl-2, Bcl-W, Bcl-B, Bfl-1, and Mcl-1 lacking their C-terminal transmembrane domains ($\sim$ last 20 amino acids) ('ΔTM') were expressed from pGEX 4T-1 plasmid in XL-1 Blue cells (Stratagene, Inc.). Briefly, cells were grown in 2 l of LB with 50 μg/ml ampicillin at 37°C to an OD 600 nm of 1.0., then IPTG (0.5 mM) was added, and the cultures were incubated at 25°C for 6 h. Cells were then recovered in 20 mM phosphate buffer (pH 7.4), 150 mM NaCl, 1 mM DTT, 1 mM EDTA, 1 mM PMSF, followed by sonication. Cellular debris were sedimented by centrifugation at 27 500 $\times$ *g* for 20 min, and the resulting supernatants were incubated with 10 ml of glutathionine-sepharose (Pharmacia) at 4°C for 2 h. The resin was washed three times with 20 mM phosphate buffer (pH 7.4), 150 mM NaCl, and 1 mM DTT, and then 10 mM of reduced glutathione dissolved in 50 mM Tris-HCl (pH 8.0) was used to elute the GST-fusion proteins. (**b**, **c**) FPA analysis of Bcl-2-family proteins using FITC-Bid BH3 peptide. Binding of FITC-conjugated Bid BH3 peptide to GST-Bcl-2-family fusion proteins was monitored by FPAs, performed according to published procedures.[20] To optimize binding assays, various concentrations of Bcl-2 proteins were incubated with 5 nM of FITC-conjugated synthetic Bid BH3 peptide (FITC-Ahx-EDIIRNIARHLAQVGDSMDR) in phosphate-buffered saline (PBS) using 96-well black plates (Greiner bio-one), thus identifying the approximately optimal concentration of target protein (generally 100 nM). Fluorescence polarization was measured after 10 min using an Analyst TM AD Assay Detection System (LJL Biosystem, Sunnyvale, CA, USA) in PBS (pH 7.4). Data are representative of multiple experiments ($n = 3$). FITC-Ahx-Bid BH3 peptide was synthesized using Fmoc solid-phase synthesis on an ACT 350 multiple peptide synthesizer. The peptides were deprotected and cleaved from the resin by treatment with 94% TFA/2.5% H_2O/2.5% EDT/1% TIS for 2 h at room temperature. The crude peptides were purified with a Gilson HPLC instrument and analyzed by MALDI-TOF mass analysis with an Applied Biosystems Voyager System 6264. (**d**–**g**) Competition assay analysis of unlabeled Bid BH3 peptide and green tea compound EGCG. 100 nM of GST-Bcl-2 fusion proteins (for **d**, **e,** and **g**) or His6-Bcl-X_L ΔTM or Bfl-1 without GST tag (cleaved by thrombin) (for **f**) were incubated with various concentrations of unlabeled Bid BH3 peptide, EGCG, or control compound $-$C for 10 min in 50 μL PBS buffer. Then, 5 nM FITC-conjugated-Bid BH3 peptide was added, bringing final volume to 100 μl. Fluorescence polarization was measured after 20 min. Data are representative of multiple experiments ($n = 4$)

proteins in a concentration-dependent manner, whereas the control compound had little effect.

Table 1 summarizes the results for the natural products EGCG, Gossypol, Antimycin A, and Chelerythrine, for Apogossypol, a semisynthetic derivative of Gossypol, and for the synthetic compounds ABT-737, GX-15 (an analog of the clinical candidate GX-15-070), BH3I-1, and YC137.[11–19] Because the conditions of the FPAs were set to accommodate a variety of Bcl-2-family proteins with different affinities for the Bid BH3 peptide, we report data as the inhibitory concentration at which 50% reduction in fluorescence polarization was achieved (IC_{50}), rather than inhibitory constants (Ki). Given the protein concentrations employed, the resulting measurements should be considered upper limits of the IC_{50} values. EGCG, GX-15, and BH3I-1 were the only compounds that displaced BH3 peptide from all six Bcl-2 antiapoptotic proteins

Table 1 Summary of IC_{50} of small molecule Bcl-2 antagonists assessed using FPAs for all six antiapoptotic human Bcl-2-family proteins

	Compound IC_{50} (μM)								
Proteins	**EGCG**	**Gossypol**	**ApoG**	**Antimycin A**	**Chelerythrine**	**ABT-737**	**GX 15**	**BH3I-1**	**YC137**
Bcl-X_L	0.59	3.03	2.80	2.70	~10	0.064	4.69	5.86	>20
Bcl-2	0.45	0.28	0.64	2.95	~10	0.12	1.11	1.14	6.43
Bcl-W	2.33	1.4	2.10	4.57	>10	0.024	7.01	2.33	2.21
Bcl-B	0.36	0.16	0.37	1.83	>10	>10	2.15	1.08	3.10
Bfl-1	1.79	>10	>10	>10	~10	>20	5.00	4.65	>20
Mcl-1	0.92	1.75	3.35	2.51	>10	>20	2.90	2.17	2.47

For competition experiments, various concentrations of chemical inhibitors were incubated with ~100 nM GST-Bcl-2-family fusion proteins in 50 μl PBS for 10 min, then 5 nM of FITC-Bid BH3 peptide was added and fluorescence polarization was measured after 20 min. IC_{50} determinations were generated by fitting the experimental data using a sigmoidal dose-response nonlinear regression model using GraphPad Prism software (GraphPad, Inc., San Diego, CA, USA). Data represent upper limit estimates. Chemicals: (−)-Epigallocatechin gallate (EGCG), (−)-Catechin (−C), Gossypol, Antimycin A, and Chelerythrine were purchased from Sigma-Aldrich. BH3I-1, YC137, and HA14-1 were obtained from Calbiochem. ABT 737 was from Abbott, GX15 was from Gemin X Biotechnologies Inc., Apogossypol was from Jia Lee (NCI). Compounds were dissolved in DMSO at 10 mM concentration and stored at −20°C

with IC_{50}s <10 μM. EGCG was approximately 2–10-fold more potent than GX-15 and BH3I-1, depending on the Bcl-2-family member, with the exception of Bcl-W where EGCG and BH3I-1 displayed similar competitive activity. Gossypol, Apogossypol, and Antimycin A competed for BH3 peptide binding with IC_{50} <10 μM for five of the six proteins, displaying little affinity for Bfl-1. Gossypol and Apogossypol displayed similar affinities for Bcl-2, Bcl-X_L, Bcl-W, Mcl-1, and Bcl-B, and were comparable to or slightly greater in potency compared to Antimycin A. Attempts to obtain reproducible data for HA14-1 were unsuccessful owing to poor solubility and instability of this compound (not shown). ABT-737 was the most potent and most selective compound of those available for testing. ABT-737 competed for BH3 peptide binding to Bcl-X_L, Bcl-2, and Bcl-W with IC_{50}s that were typically 1–2 logs lower than other compounds, but displayed little reactivity with Bcl-B, Bfl-1, and Mcl-1. YC137 was semiselective, competing for BH3 peptide binding to Bcl-2, Bcl-W, Bcl-B, and Mcl-1 with $IC_{50}<10$ μM, but displaying little activity against Bcl-X_L and Bfl-1. Insignificant competition ($\geqslant 10$ μM) was observed for Chelerythrine (Table 1). In contrast to their ability to inhibit in the BH3 displacement assay, none of the compounds tested here displayed inhibitory activity in FPAs involving other types of proteins, including an assay using fluorochrome-conjugated tetrapeptide from SMAC binding to the BIR3 domain of XIAP (not shown), thus excluding nonspecific mechanisms.

The data provided here should serve as a resource of designing experiments using chemical inhibitors of antiapoptotic Bcl-2-family proteins. The spectrum of activities of the chemical inhibitors reveals several classes of antagonists, ranging from broad-spectrum antagonists that inhibit all six human antiapoptotic Bcl-2-family members to semiselective antagonists that inhibit subsets of the antiapoptotic Bcl-2 family. The structures of the compounds are shown as Supplementary data (Figure 1s). Pharmacokinetics studies are required to assess the suitability of these compounds for *in vivo* work. It should also be noted that while the FPA format for comparing compound affinity offers many advantages, particularly the ability to perform homogenous competitive binding assays, results may differ somewhat if other types of binding assays are employed, such as solid-phase binding assays using proteins immobilized on plastic or other solid supports. Use of different BH3 peptides with different affinities for each of the six antiapoptotic Bcl-2 family proteins would also alter the results, and should be taken into consideration – particularly if higher affinity than the Bid BH3 peptide employed here. These caveats notwithstanding, the comparison of chemical inhibitors of antiapoptotic Bcl-2-family proteins provided here provides a framework for designing experiments that utilize these compounds for discovery research and drug target validation.

Acknowledgements

We thank Saul Rosenberg (ABBOTT), Gemin X Biotechnologies Inc., and Jia Lee (NCI) for compounds, Melanie Hanaii for manuscript preparation, and the NIH for generous support (U01-CA113318).

D Zhai[1], C Jin[1], AC Satterthwait[1] and JC Reed[*,1]*

[1] Burnham Institute for Medical Research, La Jolla, CA 92037, USA
* Corresponding author: JC Reed, Burnham Institute for Medical Research, 10901 N. Torrey Pines Road, La Jolla, CA 92037, USA. Tel: +1-858-646-3100x3368; Fax: +1-858-646-3194; E-mail: reedoffiece@burnham.org

1. Reed JC (2000) Am. J. Pathol. 157: 1415–1430.
2. Kitada S *et al.* (2002) Oncogene 21: 3459–3474.
3. Reed JC (2003) Cancer Cell 3: 17–22.
4. Huang Z (2000) Oncogene 19: 6627–6631.
5. Sattler M *et al.* (1997) Science 275: 983–986.
6. Reed JC and Pellecchia M (2005) Blood 106: 408–418.
7. Day CL *et al.* (2005) J. Biol. Chem. 280: 4738–4744.
8. Denisov AY *et al.* (2003) J. Biol. Chem. 278: 21124–21128.
9. Muchmore SW *et al.* (1996) Nature 381: 335–341.
10. Chen L *et al.* (2005) Mol. Cell 17: 393–403.
11. Oltersdorf T *et al.* (2005) Nature 435: 677–681.
12. Real PJ *et al.* (2004) Cancer Res. 64: 7753–7947.
13. Becattini B *et al.* (2004) Chem. Biol. 11: 389–395.
14. Leone M *et al.* (2003) Cancer Res. 63: 8118–8121.
15. Kitada S *et al.* (2003) J. Med. Chem. 46: 4259–4264.
16. Chan SL *et al.* (2003) J. Biol. Chem. 278: 20453–20456.
17. Tzung SP *et al.* (2001) Nat. Cell. Biol. 3: 183–191.
18. Degterev A *et al.* (2001) Nat. Cell. Biol. 3: 173–182.
19. Wang JL *et al.* (2000) Proc. Natl. Acad. Sci. USA 97: 7124–7129.
20. Zhai D *et al.* (2003) Biochem. J. 376: 229–236.

Supplementary Information accompanies the paper on Cell Death and Differentiation website (http://www.nature.com/cdd)