Published in final edited form as:

Bioorg Med Chem Lett. 2009 May 1; 19(9): 2429–2434. doi:10.1016/j.bmcl.2009.03.067.

Induction of Apoptosis Promoted by Bang52; a Small Molecule that Downregulates Bcl-x_L

Matteo Rossi^a, Jeong-kyu Bang^a, Sharlyn Mazur^a, Jaclyn A. Iera^b, Darren C. Phillips^c, Gerard P. Zambetti^c, and Daniel H. Appella^b

^a Laboratory of Cell Biology, NCI, NIH, Bethesda, MD, 20892

^b Laboratory of Bioorganic Chemistry, NIDDK, NIH, Bethesda, MD 20892

^c Department of Biochemistry, St. Jude Children's Research Hospital, Memphis, Tennessee 38105

Abstract

Cancer cells evade death by over-producing specific proteins that inhibit apoptosis. One such group of proteins is the Bcl-2 family, of which Bcl-x_L is an important member. This protein binds and inhibits BAK, another protein that promotes apoptosis. While the development of chemical inhibitors that block Bcl-x_L – BAK association have been the focus of intense research efforts, we demonstrate in this manuscript an alternative strategy to downregulate Bcl-x_L. We have identified a small molecule (Bang52) that induces apoptosis in a lymphoblast-derived cell line by lowering levels of Bcl-x_L. Since Bang52 bears no resemblance to any chemical binder of Bcl-x_L we believe that degradation of the protein is stimulated by a new type of pathway. These findings highlight a novel approach to the development of small molecules that promote apoptosis.

Approaches in the development of new anticancer therapeutics are focusing on the selective induction of tumor cell death through the activation of apoptotic pathways. Apoptosis is a highly controlled cellular process that is essential for the maintenance of normal tissue homeostasis and embryonic development. However, a failure to appropriately induce apoptosis often results in the accumulation of defective cells that are symptomatic of disease states such as cancer.^{1,2} Therefore, small molecules that selectively induce apoptosis in tumor cells are of considerable interest in the clinical management of cancer. The identification of several key protein targets that regulate apoptosis has resulted in the development of molecules that bind to one (or more) of these proteins and activate apoptotic pathways.³ At the same time, cell-based screens have also identified interesting compounds that induce apoptosis. Some of these studies have reported molecules with the unique ability to restore activity to mutant forms of the protein p53.⁴

p53 functions as an important guardian of cellular function. If a healthy cell is damaged, p53 is able to inhibit cell growth or induce apoptosis. Mutated forms of p53 lose these functions, and therefore allow the uncontrolled growth of damaged cells. Mutant forms of p53 are present in about half of all cancers.^{5–7} Restoration of activity to mutant p53 by small molecules has been a recent discovery, and by now, a small collection of molecules have been reported to selectively induce apoptosis in cancer cells that possess mutant p53.^{8–11} Two of the most studied molecules in this area are PRIMA-1 and the related mono-methyl ether derivative PRIMA-1^{Met} (Fig. 1).^{12–19} Both PRIMA molecules are reported to restore the proper function to many different mutant forms of p53, although the exact targets of these molecules is not clear. In our own research, we previously reported a very simple chemical scaffold to probe for reactivation of mutant p53 and we identified a molecule (**1**) that was able to induce apoptosis in cells that had been engineered to express different forms of mutant p53.²⁰ While these results served as a model for selectively targeting cells with mutant p53, we later found that **1** did not

reactivate p53 in a human B-lymphoblast-derived cell line that expresses a specific form of mutant p53, M237I.

To test whether the original molecular scaffold could be modified to achieve reactivation of p53, we developed a new synthetic approach to make a small library of molecules. The ability of each molecule to induce apoptosis was investigated in three cell-lines that differ in their p53 status (cells were either p53 positive, negative, or expressed the M237I mutant). In this manuscript, we describe the chemistry to make the library, the identification of an active molecule (Bang 52) from this library, and the effects of Bang52 on apoptosis in comparison to PRIMA-1. Surprisingly, both molecules kill cells independently of the p53 status, but Bang52 induces apoptosis in a unique manner involving reduction of the pro-survival factor Bcl-x_L.

Chemistry

The initial goal for modification of **1** was to increase the stability in cells. To achieve this, we replaced the ester in the sidechain of **1** with an amide. The synthesis of these molecules (i.e. Bang52 and its analogs) was developed on a solid support so that numerous derivatives could be rapidly prepared (Fig. 2). First, the Weinreb amide derivative of Alloc-protected diaminopropionic acid (Alloc-Dap) was coupled to p-nitrophenylcarbonate Wang resin. Reaction with an excess of a phenyl Grignard reagent was then performed, followed by Alloc deprotection and acylation of the primary amine. Cleavage from the resin then afforded the crude products, which were purified by HPLC. Using this procedure, 54 different analogs were prepared with variations at the aromatic position (Ar) and the sidechain (R¹). Ultimately, Bang52 was identified as the only molecule from the library that induces apoptosis in our new cell-based assay. For comparison to Bang52, PRIMA-1 was also prepared by following published procedures in the literature.

Biological Results

In the studies described below, the activities of Bang52 and PRIMA-1 were compared across three human B lymphoblast cell lines: TK6 (wild-type p53), NH-32 (null for p53),²¹ and WTK1 (M237I mutant p53).^{22,23} These cell lines were derived from the same progenitor, WIL2,^{21, 24,25} and differ only in their p53 status. Therefore, results obtained across these three cell lines directly show whether p53 is a crucial component for induction of apoptosis. As a control, doxorubicin (DOX) was also included in the studies because there is a clear mechanism of action for this molecule that involves activation of p53.

The effect of p53 status on cellular responses to Bang52, PRIMA-1, and DOX was examined in TK6, NH-32, and WTK1 cells by flow cytometric analysis of DNA cell cycle profiles.^{26, 27} An indication of the amount of cell death was obtained by analysis of the sub-G0/G1 DNA content of each cell cycle histogram. As shown in Figure 3, all three cell lines treated with 25 μM of Bang52 or PRIMA-1 showed considerable increases in sub-G0/G1 phase DNA after 72 hours as compared to untreated cells. As a control, the same cells were treated with 0.1 μM of DOX. Since DOX is known to induce DNA damage and stimulate apoptosis through a p53-mediated pathway, results with this molecule demonstrated how each cell line responds according to its p53 status. As expected, DOX treatment produced a large fraction of cells with sub-G0/G1 DNA content in the TK6 cells, which express wild-type p53. This effect was abrogated in NH-32 and WTK1 cells, which are null for p53 or contain a mutant p53, respectively. In contrast to DOX, Bang 52 and PRIMA-1 induced significant amounts of sub-G0/G1 DNA in all three cells lines (Figure 3).

The requirement for fully functional p53 in the B lymphoblast cell lines was confirmed by Western Blot analysis using antibodies to detect: p53 expression, p53 activation via

phosphorylation of Ser15, and p53 transcriptional activity through detection of p21^{Cip1/Waf1}. In TK6 cells, which express wild-type p53, phospho-p53 (at Ser15) and p21 expression was observed following DOX treatment. In the WTK1 cells, which express mutant p53, DOX promotes formation of phospho-p53 without induction of p21. This result is consistent with loss of p53 transcriptional activity in this cell line. In contrast with DOX, Bang52 and PRIMA-1 did not induce phospho-p53 or p21 expression. Collectively, these data indicate that Bang52 and PRIMA-1 induce apoptosis in the B lymphoblast cells lines independently of the p53 status.

Several additional methods were used to examine the mechanism of PRIMA-1 or Bang52-induced cell death. Apoptosis is accompanied by loss of organization in the plasma membrane, and a marker of this event is the translocation of the phospholipid phosphatidylserine (PS) from the inner to the outer portion of the membrane. Annexin V is a PS-binding protein that can be conjugated to fluorescent groups and used in FACS analysis to determine cell viability. Annexin V is typically used in conjunction with 7-amino-actinomycin (7-ADD), a fluorescent molecule that is impermeable to cells with intact membranes but permeable to dead cells. In the FACS analysis of untreated TK6 cells (Figure 5A), the majority of cells appeared negative for staining with Annexin V and 7-ADD, indicating that the cells were healthy. Upon treatment with Bang52 for 8 hours, FACS analysis showed significant shifts in the cell population. Specifically, the percentage of healthy cells decreased significantly from 68% to 24% and, in addition, two new populations of cells were observable. The most significant new population of cells was positive for both Annexin V and 7-ADD, indicating these cells had already died either by necrosis or apoptosis. An additional cell population that is more positive for Annexin V than for 7-ADD was also observable, indicating that these cells were undergoing apoptosis. Under the same conditions, PRIMA-1 had no effect on the cells populations compared to the untreated cells.

The mitochondria act as a point of convergence for numerous apoptotic signaling pathways. While the precise mechanisms of mitochondrial involvement in apoptosis are still the subject of ongoing research, the loss of the inner mitochondrial membrane potential is associated with the release of pro-apoptotic factors, such as cytochrome c, into the cytosol. Furthermore, loss of mitochondrial function is linked to the activation of caspases and caspase-dependent nucleases that induce DNA fragmentation, which are key features of apoptosis.^{28,29} Consistent with the previous results, a probe for mitochondrial depolarization showed that Bang52 significantly impacts the mitochondria. The release of pro-apoptotic factors from the mitochondria is often associated with mitochondrial depolarization. The cationic dye JC-1 selectively accumulates in mitochondria such that its fluorescence properties shift from green (JC-1 monomer, FL1H) to orange (JC-1 aggregates in the mitochondria, FL2H). Mitochondrial depolarization reduces JC-1 concentration within the cell, resulting in decreased orange fluorescence.^{30,31} In the treatment of TK6 cells with Bang52, significant depolarization of the mitochondria is observed after 8 hours relative to a positive control (carbonyl cyanide 3-chlorophenylhydrazone (CCCP)). In contrast, PRIMA-1 caused no observable depolarization under the same conditions (Figure 6).

The presence of additional apoptotic indicators were examined for all three cell lines under conditions where the cells were treated with Bang52, PRIMA-1, or DOX. During apoptosis, several caspase proteins are converted from the inactive (pro-form) to the active form. Treatment of the three cell lines with Bang52, produced a strong increase in the level of cleaved caspase-3, and a simultaneous decrease in the level of pro-caspase-3 (Figure 7). Cleavage of poly(ADP-ribose) polymerase (PARP) by activated caspases is another indicator of apoptosis. Consistent with caspase-3 activation, enhanced PARP cleavage was also observed. In contrast, PRIMA-1 induced only modest changes in the levels of pro-caspase-3, caspase-3, and PARP under the same conditions.

Several additional derivatives were examined for their ability to induce cell death in a manner similar to Bang52. In general, we wanted to determine the impact of small alterations in Bang52 on the activity of the molecule and also probe whether degradation of Bang52 could account for the activity of the parent molecule. The importance of the difluoroacetamide group is highlighted by comparing Bang3, 53, and 44 (Figure 8). Replacement of the two fluorines with hydrogens (Bang3) eliminated the ability of the molecule to induce cell death, whereas monofluoro or trifluoroacetamide derivatives exhibited diminished activity. The difluoroacetamide group is not the only portion of the molecule responsible for the activity. Removal of the phenyl ketone or the primary amine (as seen in Bang56–57) completely eliminated all activity, resulting in molecules that are inactive and non-toxic (See supporting information for results of cellular assays).

Bcl-2 and Bcl-x_L are anti-apoptotic members of the Bcl-2 family that maintain mitochondrial integrity by preventing the release of pro-apoptotic factors.^{28,29} Cancer cells expressing elevated levels of Bcl-2 and/or Bcl-x_L possess a survival advantage and are in some cases “addicted” to these oncogenes for survival. Therefore, loss of functions associated with these proteins leads to apoptosis.³² Furthermore, enhanced expression of Bcl-2 and Bcl-x_L are commonly associated with drug resistance and poor prognosis in several types of cancer.^{1,2,33–35} Strategies that antagonize the function of Bcl-2 proteins are therefore the focus of therapeutic development.^{36–38} Western blot analysis showed that Bang52 significantly reduced the amount of Bcl-x_L in all three cell types of this study (Figure 9). Similarly, PRIMA-1 also reduced the amount of this protein, although much less dramatically and in variable amounts. For instance, PRIMA-1 was most effective at reducing Bcl-x_L in the WTK1 cells, while it was moderately effective in the TK6 cells and minimally effective in the NH32 cells. In contrast, Bang52 was highly effective in all three cell lines. The effect seems highly specific to Bcl-x_L because the level of Bcl-2 is unchanged. Quantitative RT-PCR demonstrated that Bcl-x_L and Bcl-x_S mRNA levels remained unchanged following treatment with Bang52 (Supporting Information), indicating that Bang52 does not affect Bcl-x_L at the transcriptional level. Taken together, these results suggest that Bang52 affects Bcl-x_L at the protein level.

To confirm the specificity of action, the effects of Bang52 were compared in regular HeLa cells and a derivative HeLa cell line that stably expresses Bcl-x_L at elevated levels (called HeLa-X_L).³⁹ If Bang52 targets Bcl-x_L for degradation, then over-expression of Bcl-x_L should protect the HeLa-X_L cells from death. This effect was clearly observed, especially at higher concentrations of Bang52 (Figure 11). Treatment with 100 μM Bang52 substantially increased the percentage of sub-G1 DNA content in HeLa cells while producing no effect in HeLa-X_L cells.

Bcl-x_L is a well-known antiapoptotic factor that has been a target for inhibition by several research groups.³ The association of Bcl-x_L with BAK is an example of a protein-protein interaction that has provided a structural basis for the design of inhibitors.^{40–43} These efforts have led to a well-known set of Bcl-x_L inhibitors that define the molecular feature for inhibition of this protein with BAK.^{38,44} The compound Bang52 does not share any chemical features with these known inhibitors of Bcl-x_L. Therefore, it is likely that Bang52 exerts its effects by a novel pathway that does not involve direct targeting of the protein. Recently, two other groups have independently reported the isolation of natural products that similarly eliminate Bcl-x_L. Wang and coworkers reported that a pyrrolizidine alkaloid (clivorine) derived from plants induced apoptosis in hepatocytes by downregulation of Bcl-x_L.⁴⁵ Similarly, Imoto and colleagues found that incednine, a molecule isolated from the culture of a *Streptomyces*, induced apoptosis in HEK293T cells also by downregulation of Bcl-x_L.⁴⁶ As with Bang52, the natural products also have no obvious similarity with the known binders of Bcl-x_L. At the present time, the exact target of Bang52 (as well as clivorine and incednine) remains unknown.

With the development of chemical biology, small molecules that selectively perturb cellular pathways can be valuable research tools. The recent emergence of p53 as a target for small molecules has fueled the possibility of designing molecules to induce apoptosis in some of the most resistant cancers. However, a number of the p53-targeting molecules possess very complex mechanisms of action that are difficult to fully characterize. In this regard, there is increasing evidence that PRIMA-1 targets multiple entities within a cell^{15,47} and the results from our study indicate that p53 is not required for PRIMA-1 to induce cell death. Since cancer is a highly heterogeneous disease, we feel that it is necessary to develop a chemical arsenal of molecules that may induce apoptosis by a number of different cellular pathways. While such molecules may not developed into drugs to treat cancer, well-defined chemical probes for apoptosis may provide valuable insight into the biological functions of tumor cells and highlight the weaknesses of cancers, thus stimulating ideas to develop treatments. We feel that Bang52 may provide such a tool to probe the activity of Bcl-x_L. Bang52 results in the selective elimination of this protein by a mechanism that is likely to be distinct from that used by the known chemical inhibitors of this protein. Additional research to discover the protein target (or targets) of this molecule along with its mode of action may provide new directions for the induction of apoptosis in chemoresistant tumor cells.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

The authors gratefully acknowledge financial support from the Intramural Research Program of NIH, CA6320 and NIH/NCI Cancer Center Support Grant CA21765. We are also grateful for the support of the American Lebanese Syrian Associated Charities (ALSAC) of St. Jude Children's Research Hospital. We also want to thank Tom Chittenden (Apoptosis Technology, Inc., Boston, MA) for providing the HeLa-x_L cells, and Dr. Ana Robles (NCI) for providing the WTK1, NH32, and TK6 cells.

References and Notes

1. Danial NN, Korsmeyer SJ. Cell 2004;116:205. [PubMed: 14744432]
2. Hanahan D, Weinberg RA. Cell 2000;100:57. [PubMed: 10647931]
3. Fesik SW. Nat Rev Cancer 2005;5:876. [PubMed: 16239906]
4. Selivanova G, Wiman KG. Oncogene 2007;26:2243. [PubMed: 17401433]
5. Romer L, Klein C, Dehner A, Kessler H, Buchner J. Angew Chem Int Ed Engl 2006;45:6440. [PubMed: 16983711]
6. Vogelstein B, Lane D, Levine AJ. Nature 2000;408:307. [PubMed: 11099028]
7. Zambetti GP. J Cell Physiol 2007;213:370. [PubMed: 17671971]
8. Bykov VJ, Issaeva N, Shilov A, Hultcrantz M, Pugacheva E, Chumakov P, Bergman J, Wiman KG, Selivanova G. Nat Med 2002;8:282. [PubMed: 11875500]
9. Foster BA, Coffey HA, Morin MJ, Rastinejad F. Science 1999;286:2507. [PubMed: 10617466]
10. Bykov VJ, Issaeva N, Zache N, Shilov A, Hultcrantz M, Bergman J, Selivanova G, Wiman KG. J Biol Chem 2005;280:30384. [PubMed: 15998635]
11. North S, Pluquet O, Maurici D, El-Ghissassi F, Hainaut P. Mol Carcinog 2002;33:181. [PubMed: 11870884]
12. Bykov VJ, Issaeva N, Selivanova G, Wiman KG. Carcinogenesis 2002;23:2011. [PubMed: 12507923]
13. Bykov VJ, Zache N, Stridh H, Westman J, Bergman J, Selivanova G, Wiman KG. Oncogene 2005;24:3484. [PubMed: 15735745]
14. Charlot JF, Nicolier M, Pretet JL, Mougin C. Apoptosis 2006;11:813. [PubMed: 16554962]
15. Cory AH, Chen JM, Cory JG. Anticancer Res 2006;26:1289. [PubMed: 16619536]

16. Nahi H, Merup M, Lehmann S, Bengtzen S, Mollgard L, Selivanova G, Wiman KG, Paul C. Br J Haematol 2006;132:230. [PubMed: 16398657]
17. Shi H, Lambert JMR, Hautefeuille A, Bykov VJN, Wiman KG, Hainaut P, de Fromental CC. Carcinogenesis 2008;29:1428. [PubMed: 18048389]
18. Supiot S, Zhao H, Wiman K, Hill RP, Bristow RG. Radiotherapy and Oncology 2008;86:407. [PubMed: 18237796]
19. Wang T, Lee K, Rehman A, Daoud SS. Biochem Biophys Res Commun 2007;352:203. [PubMed: 17113036]
20. Myers MC, Wang J, Iera JA, Bang JK, Hara T, Saito S, Zambetti GP, Appella DH. J Am Chem Soc 2005;127:6152. [PubMed: 15853303]
21. Chuang YY, Chen Q, Brown JP, Sedivy JM, Liber HL. Cancer Res 1999;59:3073. [PubMed: 10397247]
22. Little JB, Nagasawa H, Keng PC, Yu Y, Li CY. J Biol Chem 1995;270:11033. [PubMed: 7744731]
23. Xia F, Wang X, Wang YH, Tsang NM, Yandell DW, Kelsey KT, Liber HL. Cancer Res 1995;55:12. [PubMed: 7805021]
24. Skopek TR, Liber HL, Penman BW, Thilly WG. Biochem Biophys Res Commun 1978;84:411. [PubMed: 214074]
25. Benjamin MB, Potter H, Yandell DW, Little JB. Proc Natl Acad Sci USA 1991;88:6652. [PubMed: 1677771]
26. Nicoletti I, Migliorati G, Pagliacci MC, Grignani F, Riccardi C. J Immunol Methods 1991;139:271. [PubMed: 1710634]
27. Ormerod, MG. Flow Cytometry: A Practical Approach. Oxford University Press; 2000.
28. Donovan M, Cotter TG. Biochim Biophys Acta 2004;1644:133. [PubMed: 14996498]
29. Youle RJ, Strasser A. Nat Rev Mol Cell Biol 2008;9:47. [PubMed: 18097445]
30. Cossarizza A, Baccarani-Contri M, Kalashnikova G, Franceschi C. Biochem Biophys Res Commun 1993;197:40. [PubMed: 8250945]
31. Cossarizza A, Ceccarelli D, Masini A. Exp Cell Res 1996;222:84. [PubMed: 8549677]
32. Certo M, Del Gaizo Moore V, Nishino M, Wei G, Korsmeyer S, Armstrong SA, Letai A. Cancer Cell 2006;9:351. [PubMed: 16697956]
33. Reed JC. Semin Hematol 1997;34:9. [PubMed: 9408956]
34. Tsujimoto Y, Finger LR, Yunis J, Nowell PC, Croce CM. Science 1984;226:1097. [PubMed: 6093263]
35. Wei MC. Int J Hematol 2004;80:205. [PubMed: 15540893]
36. Becattini B, Kitada S, Leone M, Monosov E, Chandler S, Zhai D, Kipps TJ, Reed JC, Pellecchia M. Chem Biol 2004;11:389. [PubMed: 15123268]
37. Kitada S, Leone M, Sareth S, Zhai D, Reed JC, Pellecchia M. J Med Chem 2003;46:4259. [PubMed: 13678404]
38. Tse C, Shoemaker AR, Adickes J, Anderson MG, Chen J, Jin S, Johnson EF, Marsh KC, Mitten MJ, Nimmer P, Roberts L, Tahir SK, Xiao Y, Yang X, Zhang H, Fesik S, Rosenberg SH, Elmore SW. Cancer Res 2008;68:3421. [PubMed: 18451170]
39. Goldmacher VS, Bartle LM, Skaletskaya A, Dionne CA, Kedersha NL, Vater CA, Han JW, Lutz RJ, Watanabe S, Cahir McFarland ED, Kieff ED, Mocarski ES, Chittenden T. Proc Natl Acad Sci USA 1999;96:12536. [PubMed: 10535957]
40. Stauffer SR. Curr Top Med Chem 2007;7:961. [PubMed: 17508927]
41. Verdine GL, Walensky LD. Clin Cancer Res 2007;13:7264. [PubMed: 18094406]
42. Walensky LD. Cell Death Differ 2006;13:1339. [PubMed: 16763614]
43. Zhang L, Ming L, Yu J. Drug Resist Updat 2007;10:207. [PubMed: 17921043]
44. Domling A, Antuch W, Beck B, Schauer-Vukasinovic V. Bioorg Med Chem Lett 2008;18:4115. [PubMed: 18583128]
45. Ji L, Chen Y, Liu T, Wang Z. Toxicol Appl Pharmacol 2008;231:393. [PubMed: 18586292]
46. Futamura Y, Sawa R, Umezawa Y, Igarashi M, Nakamura H, Hasegawa K, Yamasaki M, Tashiro E, Takahashi Y, Akamatsu Y, Imoto M. J Am Chem Soc 2008;130:1822. [PubMed: 18205364]

47. Cory AH, Chen J, Cory JG. Anticancer Res 2008;28:681. [PubMed: 18507007]

Figure 1.
Molecules Tested for p53 Reactivation.

Figure 2. Synthetic Strategy to Make Bang52 and Associated Derivatives. The synthesis was performed on solid support.

Figure 3.

Bang52 and PRIMA-1 induced apoptosis independently of p53 function in human B lymphoblast cell lines. TK6 (WTp53), NH32 (p53^{-/-}) and WTK1 (MTp53) cells treated with Vehicle (lane 1), Bang 52 (25μM; lane 2), PRIMA-1 (25μM; lane 3) or Doxorubicin (0.1μM; lane 4) for 72 hours. Cells were harvested, washed twice in ice cold PBS and resuspended in hypotonic fluorochrome solution (50μg/ml propidium iodide, 25μg/ml RNase, 0.1% sodium citrate, 0.1% Triton X-100). Cell samples were incubated 1.5 hours at 4°C in the dark prior to DNA cell cycle analysis by flow cytometry. The percentage of cells in each phase of the cell cycle and the percentage of apoptotic cells was subsequently determined from 10,000 events per histogram.

Figure 4.

Bang52 does not induce p53 phosphorylation or mediate p53-dependent transcription. TK6, NH32 and WTK1 cells were treated with Vehicle (lane 1), Bang 52 (25 μ M; lane 2), PRIMA-1 (25 μ M; lane 3) or Doxorubicin (0.1 μ M; lane 4) for 8 hours prior to protein extraction and Western Blot analysis as described in Materials and Methods. Samples were probed for p53, phospho-p53 (Ser15), p21^{Cip1/Waf1} and β -actin.

Figure 5.

Bang52 induces apoptosis in TK6 cells. TK6 cells were treated with vehicle, Bang52 (25μM) or PRIMA-1 (25μM) for 8 hours. Cells were harvested, washed twice with ice cold PBS and then stained with Annexin-V-PE and 7-ADD as per the manufacturers instructions (BD Biosciences). Samples were analyzed by flow cytometry to determine Annexin-V and/or 7-ADD positive populations. The flow cytometry histograms are representative of three independent experiments. Numbers represent the percentage of the total cell population present in each quadrant.

Figure 6.

Bang52 induces depolarization of the inner mitochondrial membrane potential ($\Delta\Psi_m$). TK6 cells were treated with vehicle, Bang52 (25μM), PRIMA-1 (25μM) or CCCP (100μM) for 8 hours. Twenty minutes prior to the end of the treatment period, cells were loaded with JC-1 (5 μg/mL). Cells were harvested, washed twice with ice cold PBS and resuspended in 250μl of PBS. JCL-1 fluorescence was determined on dual parameter histograms of FL1H (JC-1 monomers) vs FL2 H (JC-1 aggregates) by flow cytometry. Data depicts flow cytometry histograms that are representational of three independent experiments.

Figure 7.

Biochemical evidence that Bang52 induced cell death is independent of p53 status. TK6, NH32 and WTK1 cells treated with Vehicle (lane 1), Bang 52 (25 μ M; lane 2), PRIMA-1 (25 μ M; lane 3) or Doxorubicin (0.1 μ M; lane 4) for 8 hours prior to protein extraction and Western Blot analysis as described in Experimental Procedures. Samples were probed for cleaved PARP, caspase-3 and β -actin.

Figure 8.

Analogues of Bang52 that were tested to determine the molecular requirements for elimination of Bcl-x_L . None of these molecules had the activity of Bang52 based on FACS analysis using PI staining.

Figure 9.

Bang52 induces degradation of Bcl-x_L. TK6, NH32 and WTK1 cells treated with Vehicle (lane 1), Bang 52 (25 μM; lane 2), PRIMA-1 (25 μM; lane 3) or Doxorubicin (0.1 μM; lane 4) for 8 hours prior to protein extraction and Western Blot analysis as described in Experimental Procedures. Samples were probed for Bcl-x_L, Bcl-2 and β-actin.

Figure 10.

Bcl-x_L hyperexpression inhibits Bang52-mediated cell death. HeLa cells hyperexpressing Bcl-x_L (HeLa-x_L)³⁹ and the parental cell line transfected with the empty vector (HeLa) were treated for 48 hours with 0, 25, 50 or 100 μM Bang52. The degree of apoptosis was determined from the sub-G0/G1 DNA content fraction in cell cycle histograms analyzed by flow cytometry as described in figure 3. Data are presented as the means ± s.e.m. of three independent experiments.