# Targeting Multiple Arms of the Apoptotic Regulatory Machinery

Yun Dai[1] and Steven Grant[1,2,3]

Departments of [1]Medicine, [2]Biochemistry, and [3]Pharmacology, Virginia Commonwealth University and Massey Cancer Center, Richmond, Virginia

## Abstract

**ABT-737 targets Bcl-2/Bcl-xL but not Mcl-1, which confers resistance to this novel agent. Here, we summarize recent findings indicating that Mcl-1 represents a critical determinant of ABT-737 sensitivity and resistance, and that Mcl-1 down-regulation by various pharmacologic agents or genetic approaches dramatically increases ABT-737 lethality in diverse malignant cell types. These findings also show that the multidomain proapoptotic proteins Bax and Bak play important functional roles in ABT-737–mediated apoptosis, and that Bak activation is essential in potentiation of ABT-737 lethality by agents that down-regulate Mcl-1. Collectively, these findings suggest a novel therapeutic strategy targeting multiple arms of the apoptotic machinery.** [Cancer Res 2007;67(7):2908–11]

Cell survival is determined by the complex interplay between proapoptotic and antiapoptotic Bcl-2 family proteins. Antiapoptotic proteins (e.g., Bcl-2, Bcl-xL, Bcl-w, Mcl-1, and Bfl-1/A1), which display sequence homology in all BH1-BH4 domains, promote cell survival, whereas proapoptotic proteins mediate receptor-, mitochondria-, or endoplasmic reticulum (ER) stress–dependent apoptosis. The latter group is subdivided into multidomain or BH3-only proteins. The former consists of Bax and Bak, which are essential for apoptosis (1). The BH3-only proteins are further divided into two subclasses: "activators" (e.g., Bim and tBid), which directly activate Bax/Bak to induce mitochondrial outer membrane permeabilization (MOMP; ref. 2), and "sensitizers/derepressors" (e.g., Bad, Bik, Bmf, Hrk, Noxa, and Puma), which do not activate Bax/Bak directly but instead neutralize antiapoptotic proteins (2, 3). The central role that Bax/Bak play in apoptosis is supported by evidence that BH3-only proteins fail to trigger apoptosis in Bax/Bak–deficient cells (4, 5). Antiapoptotic proteins block death signaling by antagonizing the actions of Bax/Bak through yet-to-be-defined mechanisms. As recently summarized (6), antiapoptotic proteins prevent Bax/Bak activation by sequestering/inhibiting "activator" BH3-only proteins and/or directly inhibiting Bax/Bak activation. "Sensitizer" BH3-only proteins displace "activator" BH3-only proteins from antiapoptotic proteins, leading to Bax/Bak activation. Alternatively, BH3-only proteins may directly neutralize/inhibit antiapoptotic proteins, releasing their inhibition of Bax/Bak.

In human malignancies, increased expression of antiapoptotic proteins (e.g., Bcl-2, Bcl-xL, or Mcl-1) commonly occurs and is associated with disease maintenance and progression, resistance to chemotherapy, and poor clinical outcome. These findings have prompted the development of anticancer strategies targeting such proteins, including small molecule Bcl-2 inhibitors (e.g., HA14-1; ref. 7), antisense oligodeoxynucleotides (e.g., G3139; ref. 7), or BH3 peptidomimetics (8), among others. Recently, a novel Bcl-2/Bcl-xL/Bcl-w inhibitor (ABT-737) has been developed, which in preclinical studies markedly enhances the activity of chemotherapeutic agents or ionizing radiation and displays impressive activity against certain human tumors including follicular lymphoma, chronic lymphocytic leukemia, and small cell lung cancer (SCLC) *in vitro* and *in vivo* (9). ABT-737 mimics the BH3-only protein Bad by docking to the hydrophobic groove of antiapoptotic proteins, thereby disabling their capacity to antagonize the actions of proapoptotic proteins. ABT-737 is highly potent (i.e., at nanomolar concentrations) in killing tumor cells displaying high levels of Bcl-2 and which are dependent upon Bcl-2 for survival (10). However, ABT-737 exhibits a relatively low affinity for other antiapoptotic proteins (e.g., Mcl-1 and A1; refs. 9, 11) and is considerably less efficient in killing tumor cells expressing high Mcl-1 levels (10). Although A1 is rarely expressed in tumor cells (11), Mcl-1 is relatively highly expressed in various malignant cell types and may theoretically limit the therapeutic effect of ABT-737 or related agents. One plausible approach to this problem would be to combine ABT-737 with agents capable of down-regulating/inhibiting nontargeted proteins, particularly Mcl-1, thereby circumventing resistance (12).

Very recently, several groups (11, 13–15), including our own (16), put this strategy into practice by reporting, almost simultaneously, that Mcl-1 down-regulation by various means dramatically enhances ABT-737 lethality in diverse human tumor cells. For example, Konopleva et al. (13) and Chen et al. (16) observed that the sensitivity of leukemia cells to ABT-737 correlated closely with endogenous levels of Mcl-1 and/or the ratio of Bcl-2/Mcl-1. Specifically, HL-60 cells, which exhibit low Mcl-1 but high Bcl-2 expression, were much more sensitive to ABT-737 than U937 and OCI-AML3 cells displaying higher levels of Mcl-1. A similar phenomenon was also noted in SCLC cells (15). To determine whether Mcl-1 represents a critical factor conferring resistance to ABT-737, van Delft et al. (11) and Chen et al. (16) showed that Mcl-1 overexpression in lymphoma cells (both *in vitro* and *in vivo*) or leukemia cells largely diminished ABT-737 activity. Conversely, knocking down Mcl-1 by small interfering RNA (siRNA)/short hairpin RNA dramatically increased ABT-737 sensitivity of epithelial tumor cells (e.g., HeLa and MCF-7; ref. 11), leukemia cells (e.g., U937 and OCI-AML3; ref. 13, 16), and SCLC cells (e.g., NCI-H196 and NCI-H146; refs. 14, 15). This notion was confirmed by the observation that Mcl-$1^{-/-}$ mouse embryonic fibroblasts (MEF) were exquisitely sensitive to ABT-737 (16). Finally, all five groups used clinically relevant pharmacologic approaches to down-regulate/inhibit Mcl-1. For example, van Delft et al., Konopleva et al., and Tahir et al. showed that anticancer synergism between ABT-737 and chemotherapeutic agents (e.g., 1-β-D-arabinofuranosylcytosine, doxorubicin, etoposide, and carboplatin/etoposide) in all

**Requests for reprints:** Steven Grant, Division of Hematology/Oncology, Medical College of Virginia, 1101 East Marshall Street, P.O. Box 980230, Richmond, VA 23298-0230. Phone: 804-828-5211; Fax: 804-225-3788; E-mail: stgrant@hsc.vcu.edu.



doi:10.1158/0008-5472.CAN-07-0082

likelihood reflected a reduction in Mcl-1 levels by p53-mediated up-regulation of Noxa (11, 15), which binds to Mcl-1 and triggers its proteasomal degradation. Konopleva et al. (13) also observed that the mitogen-activated protein kinase kinase 1 inhibitor PD98059 synergistically enhanced ABT-737–induced apoptosis at least in part by down-regulating Mcl-1 expression. This occurred despite the fact that PD98059 also inhibited Bcl-2 phosphorylation, which opposes the proapoptotic actions of ABT-737 by antagonizing its suppression of Bcl-2 dimerization with Bax. Moreover, Lin et al. (14) showed that BAY43-9006 (sorafenib), originally developed as a Raf inhibitor but subsequently found to down-regulate Mcl-1 at the translational level, effectively overcame ABT-737 resistance in SCLC cells. Chen et al. (16) primarily focused on cyclin-dependent kinase (CDK) inhibitors, as such compounds, including flavopiridol and CYC202 (Seliciclib), efficiently down-regulate expression of the short-lived protein Mcl-1 at the transcriptional level by inhibiting the CDK9/cyclin T, positive transcription elongation factor-b complex. Specifically, CDK inhibitors (e.g., roscovitine and R-roscovitine) markedly reduce Mcl-1 expression by blocking Mcl-1 transcription via inhibition of phosphorylation of the COOH-terminal domain of RNA polymerase II, an event mediated by CDK9. Significantly, coadministration of these agents synergistically potentiated ABT-737 lethality in human leukemia cells including those ectopically expressing Bcl-2 or Bcl-xL (16). Synergistic interactions between ABT-737 and other CDK inhibitors (e.g., CYC202) were also reported by van Delft et al. (11) and Lin et al. (14).

The question naturally arises how Mcl-1 down-regulation enhances ABT-737 lethality. Initial studies focused on the roles of the "activator" BH3-only protein Bim, inasmuch as Bim binds to and is sequestered by multiple antiapoptotic proteins (e.g., Bcl-2, Bcl-xL, and Mcl-1). However, ABT-737 has been shown to enhance imatinib-induced apoptosis in Bim knockout Bcr/Abl$^+$ hematopoietic cells (17). Moreover, Konopleva et al. (13) reported that Bim knockout did not diminish ABT-mediated apoptosis, although this agent did disrupt the Bcl-2/Bim association. Together, these findings argue against a major functional role for Bim in mediating apoptotic signaling triggered by ABT-737. Moreover, Konopleva et al. and Chen et al. noted that Bid, another "activator" BH3-only protein that requires activated caspase-8 for processing into a truncated (active) form (tBid), is also unlikely to be involved because caspase-8 deficiency or transfection with a dominant-negative caspase-8 failed to modify ABT-737 lethality alone or in combination with CDK inhibitors.

In view of these negative findings, attention focused on multidomain proapoptotic proteins Bax and Bak, as their activation leads directly to MOMP (1), a critical cell death decision point (18, 19). Under normal conditions, Bax is localized to the cytoplasm as a soluble monomeric protein but, upon stimulation, undergoes a conformational change to an activated form and translocates to mitochondrial and ER membranes. In contrast, Bak resides in complexes on the mitochondrial and the ER membrane in healthy cells. Following noxious stimuli, Bak changes conformation to an active form. The active forms of both Bax and Bak then form homooligomers, resulting in MOMP and release of proapoptotic factors including cytochrome *c*, Smac/Diablo, and AIF, thus triggering apoptosis. Konopleva et al. (13) observed that ABT-737 induced Bax conformational change in ABT-737–sensitive HL-60 cells probably by disrupting Bcl-2/Bax heterodimerization by binding to Bcl-2, as ABT-737 does not directly bind to Bax (11). This group also showed that Bax$^{-/-}$ colon carcinoma cells (HCT116) were completely resistant to ABT-737 even at high concentration (e.g., 10 μmol/L), whereas knockdown of Bak by siRNA conferred partial resistance to this agent. Van Delft et al. (11) found that ectopic expression of Noxa, which triggers Mcl-1 degradation, sensitized wild-type MEFs to ABT-737, whereas Bax/Bak double knockout MEFs were entirely resistant. Moreover, killing of Noxa-expressing cells required either Bax or Bak but was much more efficient in the presence of both proteins. However, these studies did not address the functional roles of Bax and particularly Bak in potentiation of ABT-737 lethality by Mcl-1 down-regulation.

Possible answers to these questions have emerged from recent studies suggesting that multiple antiapoptotic protein cooperate to sequester multidomain proapoptotic proteins and prevent their activation. In particular, it has been reported that Bak is sequestered by both Bcl-xL and Mcl-1 but not Bcl-2 (20). Furthermore, apoptosis is fully induced only when Bak is released from both Mcl-1 (i.e., through displacement by Noxa) and Bcl-xL (i.e., through the actions of another proapoptotic BH3-only protein, e.g., Bad). However, displacing Bak from either Mcl-1 or Bcl-xL is relatively inefficient in triggering apoptosis because Bak remains tethered to the other protein and thus inactive. To address the roles of Bax and Bak activation in coordinately mediating the lethality of simultaneous disruption of the Bcl-2/Bcl-xL and Mcl-1 axes, Chen et al. (16) used several approaches. First, they found that ABT-737 alone triggered Bax conformational change in ABT-737–resistant U937 cells, consistent with findings of Konopleva et al. (13), but it failed to (*a*) induce Bak conformational change, a marker of activation, or (*b*) Bax translocation to organellar membranes. Konopleva et al. (13) noted that Bak associated tightly with Mcl-1, an interaction that ABT-737 was unable to disrupt. Moreover, the findings of Chen et al. indicate that the capacity of CDK inhibitors (e.g., roscovitine) to potentiate ABT-737 lethality stems from cooperativity between these agents in disrupting the association between Bak with Bcl-xL and Mcl-1, respectively, resulting in pronounced Bak activation. Significantly, this event was accompanied by a marked Bax conformational change and translocation to the mitochondria. Notably, ectopic expression of Mcl-1, but not Bcl-2 or Bcl-xL, blocked Bak activation and diminished the interaction between roscovitine and ABT-737. Furthermore, Mcl-1$^{-/-}$ MEFs exhibited exquisite sensitivity to ABT-737–mediated Bak activation, whereas roscovitine failed to enhance ABT-737 lethality and Bak activation in such cells presumably because Mcl-1 expression was already absent. Finally, studies employing Bax$^{-/-}$, Bak$^{-/-}$, and double knockout MEFs showed that Bax is necessary for the lethality of ABT-737 either alone or in combination, whereas Bak is primarily required for enhancement of ABT-737 lethality by roscovitine. Collectively, these findings indicate that Mcl-1 down-regulation markedly potentiates ABT-737 lethality through a mechanism involving two distinct levels of cooperation between multidomain antiapoptotic and proapoptotic proteins: (*a*) simultaneous untethering of Bak from Bcl-xL (by ABT-737) and Mcl-1 (e.g., by roscovitine) and (*b*) the resulting activation of both Bax (i.e., conformational change and translocation) and Bak. These findings suggest that such pharmacologic approaches may mimic interactions between more physiologic mediators of the apoptotic machinery, for example, Noxa and Bad (20) whose capacity to release Bak from Mcl-1 and Bcl-xL are recapitulated by strategies targeting Mcl-1 (e.g., CDK inhibitors) and ABT-737, respectively. A model summarizing these concepts is shown in Fig. 1.

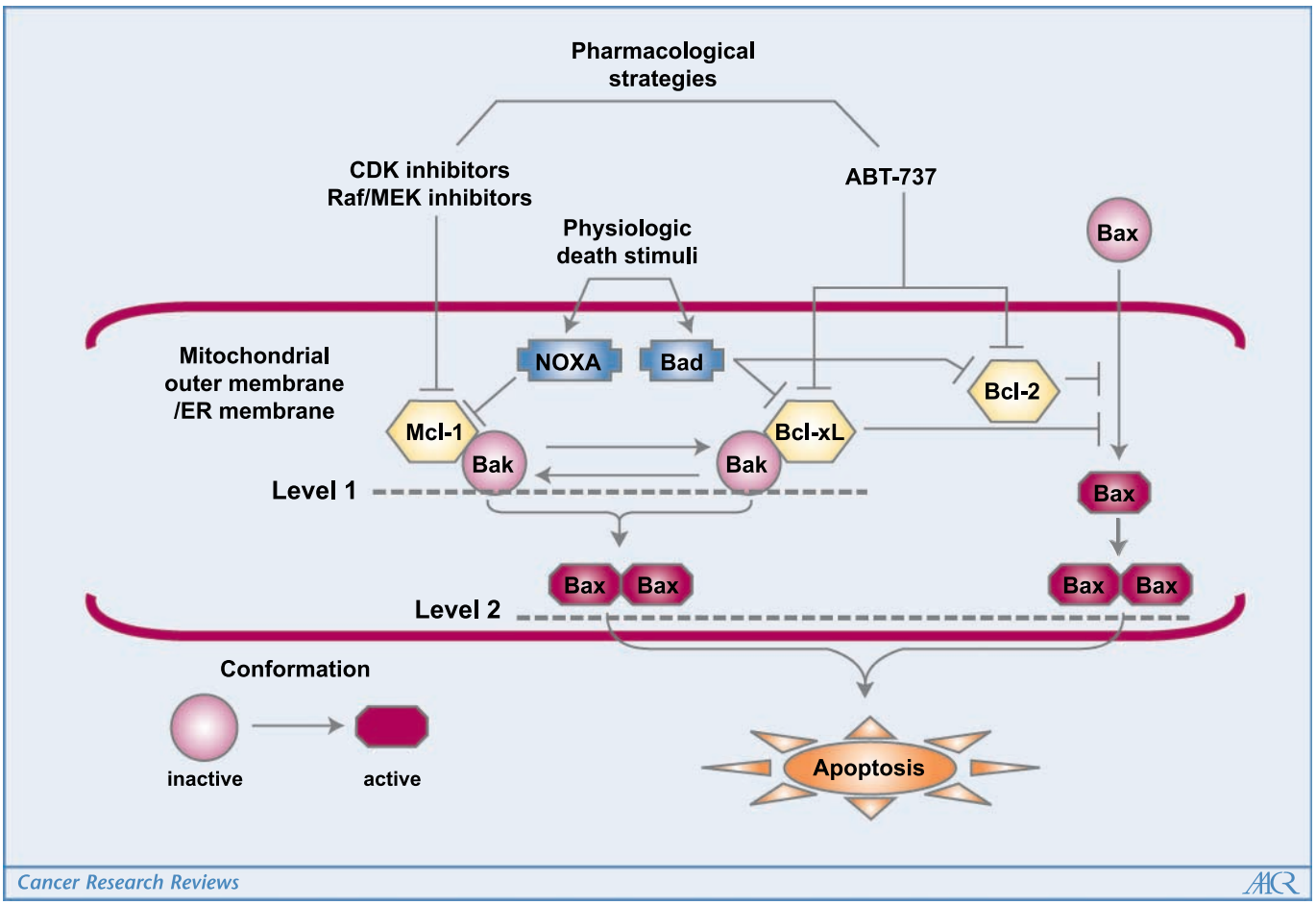


**Figure 1.** Mechanisms of potentiation of ABT-737 lethality by strategies targeting Mcl-1. Physiologic death stimuli act through signaling intermediaries (e.g., Noxa and Bad) to trigger cell death pathways. ABT-737 mimics the actions of Bad by neutralizing Bcl-2/Bcl-xL, thus activating Bax and untethering Bak from Bcl-xL. However, these events do not efficiently trigger apoptosis, particularly in cells with high levels of Mcl-1. In such cells, ABT-737 is unable to free Bak from Mcl-1 and to activate Bak. On the other hand, Mcl-1 down-regulation by various pharmacologic agents recapitulates the more physiologic effects of Noxa in diminishing the availability of Mcl-1. When pharmacologic agents that inhibit Bcl-2/Bcl-xL are combined with those that down-regulate Mcl-1, Bak is released from both Mcl-1 and Bcl-xL and activated. Activated Bak then cooperates with activated Bax (i.e., exhibiting conformational change and mitochondrial translocation) to evoke the apoptotic cascade. Thus, compounds like ABT-737 and agents targeting Mcl-1 may cooperate at two related levels: (*a*) simultaneous dissociation of Bak from Mcl-1 and Bcl-xL and (*b*) activation of both Bax and Bak, thus mimicking the physiologic death process.

Notably, ABT-737 itself exhibits impressive anticancer effects *in vivo* in murine tumor models (9, 11, 13), kills primary tumor cells (e.g., acute myelogenous leukemia/AML; ref. 13) and AML stem cells (13), and potentiates the activity of standard cytotoxic agents (13). However, whereas it is tempting to propose strategies in which ABT-737 or similar compounds are combined with established agents, the significance of the studies described above is that an alternative approach involving the rational combination of agents that trigger separate arms of the apoptotic machinery deserves serious consideration. More specifically, these newer findings suggest that pharmacologic agents targeting Mcl-1 may cooperate with agents targeting Bcl-2/Bcl-xL to mimic the actions of more physiologic regulators of apoptosis, for example, Noxa and Bad, which are normally activated under conditions of stress (e.g., growth factor deprivation). Thus, by neutralizing Mcl-1, which compensates for the loss of Bcl-2/Bcl-xL function, barriers to apoptosis induction by agents such as ABT-737 are effectively eliminated. In this way, it may be possible to recapitulate, by pharmacologic means, the signaling events responsible for activation of the apoptotic cascade that regularly occur in normal cells. The recent introduction into the clinical arena of agents potentially capable of down-regulating Mcl-1 expression could serve as an impetus for such efforts. Whether such strategies will lead to enhanced therapeutic activity will depend upon multiple factors, including the capacity of such agents to diminish Mcl-1 expression *in vivo* and whether these regimens selectively target transformed versus normal cells. Notably, in all of the preclinical studies cited, ABT-737 and compounds that down-regulated Mcl-1 expression were administered simultaneously. The effect of sequential administration of these agents needs to be determined, as this could have significant implications for the optimal translation of this approach into the clinical arena. In any case, this promising new strategy clearly warrants further attention.

## Acknowledgments

Received 1/8/2007; revised 2/8/2007; accepted 2/13/2007.

**Grant support:** National Cancer Institute grants CA63753, CA 93738, and CA 100866; Leukemia and Lymphoma Society of America award 6045-03; V. Foundation; and Department of Defense.

## References


1. Reed JC. Proapoptotic multidomain Bcl-2/Bax-family proteins: mechanisms, physiological roles, and therapeutic opportunities. Cell Death Differ 2006;13:1378–86.
2. Letai A. BCL-2: found bound and drugged! Trends Mol Med 2005;11:442–4.
3. Kim H, Rafiuddin-Shah M, Tu HC, et al. Hierarchical regulation of mitochondrion-dependent apoptosis by BCL-2 subfamilies. Nat Cell Biol 2006;8:1348–58.
4. Wei MC, Zong WX, Cheng EH, et al. Proapoptotic BAX and BAK: a requisite gateway to mitochondrial dysfunction and death. Science 2001;292:727–30.
5. Zong WX, Lindsten T, Ross AJ, MacGregor GR, Thompson CB. BH3-only proteins that bind pro-survival Bcl-2 family members fail to induce apoptosis in the absence of Bax and Bak. Genes Dev 2001;15:1481–6.
6. Green DR. At the gates of death. Cancer Cell 2006;9:328–30.
7. Reed JC, Pellecchia M. Apoptosis-based therapies for hematologic malignancies. Blood 2005;106:408–18.
8. Walensky LD, Kung AL, Escher I, et al. Activation of apoptosis *in vivo* by a hydrocarbon-stapled BH3 helix. Science 2004;305:1466–70.
9. Oltersdorf T, Elmore SW, Shoemaker AR, et al. An inhibitor of Bcl-2 family proteins induces regression of solid tumours. Nature 2005;435:677–81.
10. Certo M, Moore VG, Nishino M, et al. Mitochondria primed by death signals determine cellular addiction to antiapoptotic BCL-2 family members. Cancer Cell 2006;9:351–65.
11. van Delft MF, Wei AH, Mason KD, et al. The BH3 mimetic ABT-737 targets selective Bcl-2 proteins and efficiently induces apoptosis via Bak/Bax if Mcl-1 is neutralized. Cancer Cell 2006;10:389–99.
12. Cory S, Adams JM. Killing cancer cells by flipping the Bcl-2/Bax switch. Cancer Cell 2005;8:5–6.
13. Konopleva M, Contractor R, Tsao T, et al. Mechanisms of apoptosis sensitivity and resistance to the BH3 mimetic ABT-737 in acute myeloid leukemia. Cancer Cell 2006;10:375–88.
14. Lin X, Morgan-Lappe S, Huang X, Li L, et al. 'Seed' analysis of off-target siRNAs reveals an essential role of Mcl-1 in resistance to the small-molecule Bcl-2/Bcl-X(L) inhibitor ABT-737. Oncogene. Epub 2006 Dec 18.
15. Tahir SK, Yang X, Anderson MG, et al. Influence of Bcl-2 family members on the cellular response of small-cell lung cancer cell lines to ABT-737. Cancer Res 2007;67:1176–83.
16. Chen S, Dai Y, Harada H, Dent P, Grant S. Mcl-1 down-regulation potentiates ABT-737 lethality by cooperatively inducing Bak activation and Bax translocation. Cancer Res 2007;67:782–91.
17. Kuroda J, Puthalakath H, Cragg MS, et al. Bim and Bad mediate imatinib-induced killing of Bcr/Abl$^{+}$ leukemic cells, and resistance due to their loss is overcome by a BH3 mimetic. Proc Natl Acad Sci U S A 2006;103:14907–12.
18. Green DR. Apoptotic pathways: ten minutes to dead. Cell 2005;121:671–4.
19. Lucken-Ardjomande S, Martinou JC. Newcomers in the process of mitochondrial permeabilization. J Cell Sci 2005;118:473–83.
20. Willis SN, Chen L, Dewson G, et al. Proapoptotic Bak is sequestered by Mcl-1 and Bcl-xL, but not Bcl-2, until displaced by BH3-only proteins. Genes Dev 2005;19:1294–305.

# Targeting Multiple Arms of the Apoptotic Regulatory Machinery

Yun Dai and Steven Grant

*Cancer Res* 2007;67:2908-2911.