Corrections

CHEMISTRY. For the article “Tetrahedral structure or chains for liquid water,” by Teresa Head-Gordon and Margaret E. Johnson, which appeared in issue 21, May 23, 2006, of Proc Natl Acad Sci USA (103:7973–7977; first published May 12, 2006; 10.1073/pnas.0510593103), the authors note that there was a plotting error in Fig. 3a. The corrected figure and its legend are shown below. This error does not affect the conclusions of the article.

![Fig. 3](https://www.pnas.org/cgi/doi/10.1073/pnas.0608020103)

**Fig. 3.** Comparison of \( H_{\text{O}O}(Q) \) structure factors from experiments reported in ref. 12 (black) against the asymmetric water model (ref. 14; red) (a) and the classical polarizable TIP4P-pol2 model (ref. 16; red) (b). The simulated structure factors using the asymmetric and TIP4P-pol2 models show good agreement with x-ray scattering for \( Q > 6.5 \text{ Å}^{-1} \). However, the simulated structure factors for the asymmetric model show significant disagreement for \( Q < 6.5 \text{ Å}^{-1} \), whereas the TIP4P-pol2 model shows excellent agreement over the full \( Q \) range of the measured x-ray data.

www.pnas.org/cgi/doi/10.1073/pnas.0608020103

APPLIED BIOLOGICAL SCIENCES. For the article “Adeno-associated virus serotype 9 vectors transduce murine alveolar and nasal epithelia and can be readministered,” by Maria P. Limberis and James M. Wilson, which appeared in issue 35, August 29, 2006, of Proc Natl Acad Sci USA (103:12993–12998; first published August 22, 2006; 10.1073/pnas.0601433103), the authors note that on page 12995, right column, second full paragraph, the third sentence is incorrect in part. “For each vector the highest amount of vector in terms of vector per diploid genome was in the lung [approximately six and one vectors per diploid genome for AAV2/5 and AAV2/9, respectively (Table 1); note that \( 1.5 \times 10^5 \) vector genomes per 100 ng of cellular DNA is equivalent to one vector genome per diploid genome of the cell]” should read: “For each vector, the highest amount of vector in terms of vector per diploid genome was in the lung [approximately six and one vectors per diploid genome for AAV2/5 and AAV2/9, respectively (Table 1); note that \( 1.5 \times 10^4 \) vector genomes per 100 ng of cellular DNA is equivalent to one vector genome per diploid genome of the cell].” This error does not affect the conclusions of the article.

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GENETICS. For the article “A high-throughput gene knockout procedure for Neurospora reveals functions for multiple transcription factors,” by Hildur V. Colot, Gyungsoon Park, Gloria E. Turner, Carol Ringelberg, Christopher M. Crew, Liubov Litvinkova, Richard L. Weiss, Katherine A. Borkovich, and Jay C. Dunlap, which appeared in issue 27, July 5, 2006, of Proc Natl Acad Sci USA (103:10352–10357; first published June 26, 2006; 10.1073/pnas.0601456103), the authors note that on page 10354, in lines 13 and 14 of the last paragraph, right column, the gene mel-1 should be mld-1. These errors do not affect the conclusions of the article.

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MEDICAL SCIENCES. For the article “Bim and Bad mediate imatinib-induced killing of Bcr/Abl+ leukemic cells, and resistance due to their loss is overcome by a BH3 mimetic,” by Junya Kuroda, Hamsa Puthalakath, Mark S. Cragg, Priscilla N. Kelly, Philippe Bouillet, David C. S. Huang, Shinya Kimura, Oliver G. Ottmann, Brian J. Druker, Andreas Villunger, Andrew W. Roberts, and Andreas Strasser, which appeared in issue 40, October 3, 2006, of Proc Natl Acad Sci USA (103:14907–14912; first published September 22, 2006; 10.1073/pnas.0606176103), the authors note that the affiliation information for Andreas Villunger was incorrect in part. The correct institution name is “Innsbruck Medical University.”

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MICROBIOLOGY. For the article “Leishmania disease development depends on the presence of apoptotic promastigotes in the virulent inoculum,” by Ger van Zandbergen, Annalena Bollinger, Alexander Wenzel, Shaden Kamhawi, Reinhard Voll, Matthias Klinger, Antje Müller, Christoph Hölscher, Martin Herrmann, David Sacks, Werner Solbach, and Tamás Laskay, which appeared in issue 37, September 12, 2006, of Proc Natl Acad Sci USA (103:13837–13842; first published August 31, 2006; 10.1073/pnas.0600843103), due to a printer’s error, Fig. 1 was incorrect as shown. The corrected figure and its legend appear below.

**Fig. 1.** AnxA5 binding to *L. major* promastigotes. Populations of *L. major* promastigotes were stained with AnxA5-Fluos. Flow cytometry histogram profiles of stat. phase promastigotes (black line, a) and of stat. phase-derived metacyclic promastigotes (met, black line, b). The dotted lines (a and b) show the control staining in the absence of Ca²⁺. (c) Metacyclic promastigotes derived from *P. duboscqi* sandflies (black line) as described (22). The dotted line shows the unstained control. (d) Confocal micrograph (0.15-μm slice) of a promastigote stained positive with AnxA5 (arrow) and an AnxA5⁻ promastigote. (Scale bar, 5 μm.) (e) Flow cytometry densitoblot showing forward scatter (FSC-H) and sideward scatter (SSC-H) analysis of the stat. phase promastigotes. (f) AnxA5-Fluos binding of the population A in e (white histogram) and population B in e (black histogram).

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Bcl-2, Bcl-xL, Bcl-w, Mcl-1, and A1, share four regions of homology that contain three major subgroups. Antiapoptotic members, including caspase activation and cell demolition (4). The Bcl-2 protein family d dirial release of apoptogenic molecules (e.g., cytochrome c) for cell killing (3, 4). The BH3-only proteins (Bad, Bik, Bid, Bim, Hrk, Bmf, Noxa, and Puma) have only the 9- to 16-aa BH3 region and are regulated by a range of transcriptional and posttranslational mechanisms (5). Experiments with gene-targeted mice have shown that different BH3-only proteins are required for apoptosis induction in response to distinct developmental cues and cytotoxic stimuli (6).

Previous work has shown that overexpression of Bcl-2 or Bcl-xL (7) and, to a lesser extent, RNAi-mediated reduction in Bcl-2 (8, 9), inhibits imatinib-induced killing of Bcr/Abl+ leukemic cells. We demonstrate here that imatinib activates not only Bim, but also Bad and Bmf, and we show that Bim plus Bad account for most, perhaps all, imatinib-induced killing of Bcr/Abl+ leukemic cells. Remarkably, resistance caused by Bcl-2 overexpression or loss of Bim (plus Bad) could be overcome by cotreatment with the BH3 mimetic ABT-737. These results demonstrate that Bim and Bad account for most, perhaps all, imatinib-induced killing of Bcr/Abl+ leukemic cells and suggest previously undescribed drug combination strategies for cancer therapy.

Cell killing is a critical pharmacological activity of imatinib to eradicate Bcr/Abl+ leukemias. We found that imatinib kills Bcr/Abl+ leukemic cells by triggering the Bcl-2-regulated apoptotic pathway. Imatinib activated several proapoptotic BH3-only proteins: bim and bmf transcription was increased, and both Bim and Bad were activated posttranslationally. Studies using RNAi and cells from gene-targeted mice revealed that Bim plays a major role in imatinib-induced apoptosis of Bcr/Abl+ leukemic cells and that the combined loss of Bim and Bad abrogates this killing. Loss of Bmf or Puma had no effect. Resistance to imatinib caused by Bcl-2 overexpression or loss of Bim (plus Bad) could be overcome by cotreatment with the BH3 mimetic ABT-737. These results demonstrate that Bim and Bad account for most, perhaps all, imatinib-induced killing of Bcr/Abl+ leukemic cells and suggest previously undescribed drug combination strategies for cancer therapy.

Results

Imatinib Activates the Bcl-2-Regulated Apoptotic Pathway in Bcr/Abl+ Leukemic Cell Lines. The Bcr/Abl+ human leukemia lines K562 and BV173 were chosen for initial studies on the effects of imatinib. Imatinib inhibited the proliferation of both cell lines, as reflected by decreased metabolic activity (MTS assay) (Fig. 6a, which is published as supporting information on the PNAS web site). Whereas most K562 cells had undergone apoptosis within 48 h, the BV173 cells were more resistant, as measured by staining with vital dyes and annexin V (Fig. 6b; see Fig. 7, which is published as supporting information on the PNAS web site). Western blot analysis showed that Bcl-2 levels dropped significantly in imatinib-treated K562 cells but remained stable, or even increased slightly, in BV173 cells. The level of Bcl-1xL was significantly higher in BV173 cells than in K562 cells, and imatinib treatment caused no obvious change in Bcl-1xL, McI-1, or Bcl-w levels in either cell line (Fig. 6c). In contrast, a previous report (10) indicated that imatinib causes a reduction in Bcl-1xL in K562 cells, and we speculate that this difference is explained by clonal variation amongst distinct K562 isolates. Re-
cells. When K562 cells were pretreated with the pan-caspase inhibitor Z-VAD-fmk, imatinib still elicited a marked increase in BimEL and BimL (Fig. 8, which is published as supporting information on the PNAS web site).

Semi quantitative RT-PCR (Fig. 1c) and quantitative PCR analysis revealed that imatinib treatment increased bim mRNA levels by ~3-fold in K562 cells within 3 h. We also noted that BimEL protein from imatinib-treated K562 cells migrated more rapidly in SDS/PAGE than BimEL from untreated cells (Fig. 1a), suggesting a loss of phosphorylation, which is known to increase Bim’s proapoptotic activity in cytokine-deprived cells (11–13). Two-dimensional gel electrophoresis and Western blotting demonstrated that imatinib treatment caused the disappearance of the most negatively charged BimEL proteins (arrows) in K562 cells, indicating the accumulation of hypophosphorylated forms of Bim (Fig. 1d). This reduction in Bim phosphorylation is probably a consequence of loss of ERK activity caused by Bcr/Abl blockade (Fig. 9, which is published as supporting information on the PNAS web site). These results show that imatinib increases Bim levels in a caspase-independent manner by up-regulating mRNA levels and by posttranslational modification.

**Imatinib Increases Bim Expression in Bcr/Abl+ Leukemia Cell Lines.** To examine the role of Bim in imatinib-induced cell killing, we first generated multiple subclones of K562 and BV173 cells in which Bim levels were suppressed to various extents by stable expression of an RNAi construct (Fig. 2a). Expression of a control RNAi construct had no effect on cell proliferation, Bcr/Abl expression, or the response to imatinib (Fig. 10, which is published as supporting information on the PNAS web site). In contrast, Bim knockdown reduced the susceptibility to imatinib-induced killing, and the extent of Bim suppression correlated with the extent of protection from imatinib-induced cell killing (Fig. 2b and c).

This observation and the finding that many Bim knockdown cells eventually died after exposure to imatinib (Fig. 2b) indicated that the residual amount of Bim in these cells might be sufficient for cell killing. Alternatively, additional proapoptotic factors might contribute to imatinib-induced cell killing. We found that Bim levels were increased after imatinib treatment, even in K562/shBim#18 and BV173/shBim#4 clones, although basal levels were almost undetectable (Fig. 3a). In addition, we observed that Bad became dephosphorylated and that Bmf was up-regulated in response to imatinib (Fig. 3a), and this up-regulation correlated with the extent of protection from imatinib-induced cell killing (Fig. 2d). Bcl-2 overexpression promoted even greater retention of clonogenic potential (Fig. 2d).

Imatinib Increases Bim Expression in Bcr/Abl+ Leukemia Cell Lines. To further explore how imatinib activates apoptosis, we examined the effect of imatinib on the expression of BH3-only proteins, distant proapoptotic relatives of Bcl-2 (6). Treatment with imatinib induced a rapid and sustained increase in the levels of BimEL and, to a lesser extent, also BimL in K562 (Fig. 1a) and BV173 (Fig. 1b) cells. When K562 cells were pretreated with the pan-caspase
(embryonic day 14.5) were transformed with a bcr-abl retrovirus and clonal lines expressing bcr-abl mRNA derived for tests of sensitivity to imatinib. At least three independent clones from each genetic background were assessed; each had a morphology (Fig. 11, which is published as supporting information on the PNAS web site) and surface marker expression that was characteristic of myeloid progenitors (e.g., Sca-1, c-Kit, and Sca-2). Imatinib inhibited proapoptotic effects of etoposide and dexamethasone (14, 15), remained as sensitive to imatinib as wt./H20841 transformed cells (Fig. 12, which is published as supporting information on the PNAS web site).

Consistent with the notion that Bim and Bad are activated independently by imatinib, we found similar induction of Bim in wt and bad−/− bcr-abl transformed myeloid progenitors, and similar loss of phosphorylation of Bad was seen in wt and bcr-abl transformed cells (Fig. 12, which is published as supporting information on the PNAS web site). In addition, imatinib caused Bmf up-regulation in lines of all genotypes (although induction in bim−/− lines was variable) but had no impact on the levels of puma mRNA or Bcl-2, Bcl-xL, and Bax protein (Fig. 13).

Because many of the bcr-abl transformed bim−/− and bad−/− cells eventually died after treatment with imatinib (Fig. 4b), we hypothesized that these two BH3-only proteins might have overlapping function. Strikingly, >90% of the bcr-abl-transformed bim−/− bad−/− fetal liver cells remained viable even after 7 days of exposure to 3 μM imatinib, a degree of resistance that was only recapitulated by Bcl-2 overexpression (Fig. 4b). These cells, however, did undergo growth arrest (Fig. 4e), demonstrating that the drug was able to inhibit its target in these cells. These results demonstrate that Bim plus Bad account for most, perhaps all, imatinib-induced killing of bcr-abl transformed cells.

Resistance to Imatinib Caused by Loss of Bim and Bad or Bcl-2 Overexpression Can Be Overcome by Cotreatment with the BH3 Mimetic ABT-737. De novo or acquired resistance to chemotherapeutic drugs is a significant problem in the treatment of CML and other cancers (1, 2). Up-regulation of prosurvival Bcl-2-like proteins or loss of their proapoptotic relatives has been shown to influence responses to cancer therapy (3, 4). Recently, it has been reported that a BH3-mimetic compound, ABT-737, which binds to Bcl-2, Bcl-xL, and Bcl-w, can kill certain tumor cells when used alone or in combination with chemotherapeutic drugs (16). Because the loss of Bim and/or Bad, or Bcl-2 overexpression rendered
Bcr/Abl+ leukemic cells resistant to imatinib, we wondered whether ABT-737 could resensitize them. We therefore treated parental K562 cells and subclones overexpressing Bcl-2 or those with suppressed levels of Bim for 48 h with either drug alone or in combination and then measured their survival. By itself, ABT-737 had relatively little effect on any of the K562 sublines (maximum 25% cell killing). Significantly, however, its addition greatly enhanced imatinib-induced cell death; 5 μM ABT-737 increased killing by imatinib (1 μM; open squares) of both Bcl-2 overexpressing and Bim-deficient K562 cells from 10–20% to ~70% (Fig. 5a).

Even more remarkably, although bcr-abl-transformed bim−/−bad−/− myeloid progenitors cells were refractory to imatinib (1.5 progenitors transformed with bcr-abl (three independent lines from three separate mice for each genotype) were treated with vehicle (DMSO; solid line) or 1.5 (dashed line) or 3 μM imatinib (dotted line), and cell growth (a) was scored after 1, 2, 4, and 7 days. Data represent means ± SD of three independent experiments. (b) Cell viability was assessed after 7 days of treatment with 1.5 or 3 μM imatinib by staining with trypan blue dye and counting in a hemocytometer. Results represent means ± SD of three independent experiments indicating percentage of viable cells normalized to percentage of viable cells in control (DMSO)-treated cells.
μM, open triangles; 3 μM, closed triangles), cotreatment with as little as 2.5 μM ABT-737 resulted in >90% killing of these cells (Fig. 5b). This synergy between imatinib and ABT-737 appeared to be specific for Bcr/Abl+ cells, because normal human hepatocytes treated in culture with 3 μM imatinib plus 5 μM ABT-737 retained >90% viability (Fig. 14, which is published as supporting information on the PNAS web site). These results demonstrate that cotreatment with the BH3 mimetic ABT-737 can overcome resistance of Bcr/Abl-transformed cells to imatinib caused by loss of Bim and Bad or Bcl-2 overexpression.

Discussion

Imatinib has shown remarkable clinical benefit for treatment of Bcr/Abl+ leukemia, especially CML patients in early chronic phase. However, it has become clear that rare clones with mutations that confer resistance to imatinib (e.g., mutations in bcr-abl that prevent imatinib binding) can survive, and this resistance can lead to relapse and limits the effects for patients with advanced disease (1). Because the inhibition of cell proliferation by the blockade of Bcr/Abl with imatinib is not sufficient for eradicating Bcr/Abl+ leukemic clones, a better understanding of the mechanisms by which imatinib kills cells and how this killing can be augmented may lead to improved therapeutic strategies.

Although our study confirmed that Bcl-2 or Bcl-xL overexpression (7) or RNAi-mediated reduction of Bim (8, 9) inhibits imatinib-induced apoptosis in K562 cells, we found that it is the combination of Bim and Bad that accounts for the killing activity of imatinib. Imatinib caused a marked reduction in Bcl-2 in the highly sensitive K562 cells, whereas Bcl-2 was maintained at high levels in the more-resistant BV173 cells. Because Bcl-2 overexpression affords protection, it appears likely that Bcl-2 reduction contributes to the high sensitivity of K562 cells to imatinib. The imatinib-induced reduction in Bcl-2 in K562 cells may be a consequence of Stat5 inhibition, a transcription factor activated by Bcr/Abl and known to promote bcl-2 transcription (17).

In agreement with published data (8, 9), we found that imatinib caused up-regulation of Bim protein levels in two Bcr/Abl+ human leukemic cell lines and in bcr-abl-transformed mouse myeloid progenitor lines. Transcriptional up-regulation of Bim was reported to be mediated by FOXO3A (9), and it appears likely that this transcription factor is activated by imatinib because of shutdown of PI3K/Akt signaling, a Bcr/Abl-stimulated pathway known to suppress FOXO3A activity (18). Our 2D gel electrophoresis analyses indicate that posttranslational activation of Bim also contributes to imatinib-induced cell killing. Most likely, imatinib causes loss of Bim phosphorylation by diminishing the activity of Erk, a Bcr/Abl-activated kinase that is known to inhibit Bim’s proapoptotic activity by phosphorylating Bim, thereby targeting it for ubiquitination and proteasomal degradation (11–13).

Reduction of Bim expression by using stable expression of an RNAi vector promoted not only short-term survival, as reported in refs. 8 and 9, but even enhanced clonogenic survival of imatinib-treated K562 cells. Many of the Bim knockdown cells, however, died after prolonged exposure to imatinib, raising the question whether Bim is the only BH3-only protein that initiates apoptosis. Because the levels of Bim in many of the imatinib-treated Bim knockdown clones still were lower than the basal levels of Bim found in untreated parental K562 and BV173 cells, it appeared more likely that Bim cooperates with other proapoptotic proteins. Indeed, in K562 cells and bcr-abl transformed murine myeloid progenitor lines, imatinib caused increased expression of Bmf (19) and substantial dephosphorylation of Bad, a process known to enhance its proapoptotic activity (20). Bad dephosphorylation is likely to be a consequence of a shutdown of the PI3K/Akt and/or Ras/Raf-1/Mek/Erk pathways (both Bcr/Abl targets), which are known to inhibit the proapoptotic activity of Bad (20).

To systematically examine the role of BH3-only proteins in imatinib-induced cell killing, we generated bcr-abl-transformed myeloid progenitor lines from fetal liver of mice lacking Bim, Bad, or both. Loss of Bim provided substantial but incomplete protection against imatinib-induced cell death. These observations indicate that the death of the human leukemic cell lines was not due to merely residual levels of Bim. Loss of Bad also provided partial protection against imatinib and, remarkably, all bcr-abl transformed bim−/− bad−/− cell lines were highly resistant to imatinib-induced cell killing. These results are consistent with the notion that Bcr/Abl inhibits apoptosis in Bcr/Abl+ leukemic cells by keeping both Bim and Bad in check. The finding that Bim loss affords greater protection against imatinib than Bad deficiency can be explained by the fact that Bim binds all prosurvival Bcl-2 family members with high affinity, whereas Bad interacts only with a subset (21). Bmf was induced in imatinib-treated bcr-abl-transformed bim−/− and bcl-2−/− cells, but bcr-abl transformed murine myeloid progenitors lacking Bmf or Puma normally were sensitive to imatinib. Given the critical role of Bim in imatinib-induced apoptosis of bcr-abl-transformed leukemic cells in culture, it will be interesting to examine whether imatinib induces Bim in primary CML in vivo and whether imatinib-resistant CML cells with unmutated bcr-abl have abnormalities in their bim genes or in genes encoding upstream regulators of Bim, such as FOXO3A.

How to augment the therapeutic effect of imatinib or other Bcr/Abl kinase inhibitors is a topic of great interest (1). With respect to studies on combination therapies, inhibitors of the Ras/Mek/Erk or the PI3K/Akt/mTOR pathways have been reported to act synergistically with imatinib (22). Our results may explain these synergistic effects, because these inhibitors are expected to enhance activation of Bim and Bad by transcriptional and/or posttranslational mechanisms, such as FOXO3A-mediated induction of bim mRNA synthesis, and loss of Bim and Bad phosphorylation due to inactivation of Erk and Akt kinases.

Our studies put forward an attractive strategy for additional improvements in the treatment of CML: the use of BH3 mimetic compounds to inhibit the action of antiapoptotic Bcl-2 family members, thereby leading to Bax/Bak activation (16). Our data show that the BH3 mimetic ABT-737 enhances imatinib-induced killing of bcr-abl+ human and mouse leukemic cells and even can overcome resistance to imatinib caused by Bcl-2 overexpression or loss of Bim and/or Bad. This approach may be relevant particularly for imatinib-resistant CML with unmutated bcr-abl, which undergoes proliferation arrest but not death in response to treatment (1), particularly if changes in Bcl-2 family are shown to play a role. Perhaps, combined imatinib/ABT-737 treatment may also be advantageous for primary treatment of imatinib-sensitive CML to enhance eradication of the neoplastic clone, because some authors have hypothesized that imatinib acts predominantly by inhibiting proliferation of leukemic cells (2). ABT-737-induced cell killing therefore may help prevent emergence of (bcr-abl) mutated subclones, thereby producing longer remission or even cure. Finally, our studies suggest that combined treatment with a cell death-inducing BH3 mimetic plus a specific inhibitor of the product of an oncogene causative of transformation in a particular tumor (i.e., Bcr/Abl in CML or B-Raf in melanoma) may be a more generally applicable strategy for treatment of other types of cancer.

Materials and Methods

Cell Lines, Expression Constructs, and Cell Transfection. Cells were maintained as suspension cultures in RPMI medium 1640 with 10% or 15% heat-inactivated FCS and 2 mM L-glutamine. Expression constructs for human Bcl-2, Bcl-xL, and a dominant-interfering mutant of FADD/MORT1, all containing an N-terminal FLAG epitope tag and the puromycin resistance gene were described in ref. 23. A Gene Pulser (Bio-Rad Laboratories, Hercules, CA) was used for electroporation, and transfected cells were selected with 5 μg/ml puromycin. Cells were single-cell cloned by limiting dilution. FLAG-tagged proteins were detected by cytoplasmic immunofluorescence staining with anti-FLAG antibody (M2; Sigma, St. Louis, Missouri).
MO) and flow cytometric analysis in a FACScan (Becton Dickinson, Franklin Lakes, NJ).

Generation of the anti-Bim short hairpin RNA construct cloned into pSUPER vector with the neomycin-resistant gene was described in ref. 24.

Mice and Retroviral Infection of Fetal Liver-Derived Hemopoietic Cells.

All experiments with mice were performed according to the guidelines of the Melbourne Health Research Directorate Animal Ethics Committee. The bim−/− (25), bad−/− (26), puma−/− (14), and vav-bcl-2 transgenic (27) mice have all been described, but not bmf−/− mice (A.V. and A.S., unpublished work). These animals either were generated on a C57BL/6 background or had been backcrossed with C57BL/6 mice for more than eight generations. The bim−/−bad−/− mice were produced by intercrossing bim−/− and bad−/− mice. Fetal liver cells were harvested from 14.5-day-old embryos and pre cultured for 24 h in DMEM containing 20% FCS, 2 mM 1-glutamate, 50 μM 2-mercaptoethanol, and cytokines (50 ng/ml stem cell factor/50 ng/ml thrombopoietin/500 ng/ml fetal liver kinase-ligand/100 units/ml IL-6; all gifts of W. Alexander, The Walter and Eliza Hall Institute of Medical Research). The pMPZen.bcr-abl expression vector has been described in ref. 28, and was transfected into packaging Phoenix cells by using FuGENE6 (Roche, Indianapolis, IN). Pre cultured fetal liver cells were infected by co-cultivation on Retronectin (Takara, Kusatsu, Japan)-coated plates in virus-containing medium supplemented with cytokines (see above). Infected cells expressing Bcr/Abl were selected by growth in the absence of cytokines.

Western Blotting. Western blotting was performed with antibodies against Abl (clone 8E9; BD Pharmpingen, Franklin Lakes, NJ), human bcl-2 (Bel-2-100), mouse Bcl-2 (clone 3F11), Bcl-w (clone 13F9; Alexis, Lausen, Switzerland), Bcl-x, (Transduction Laboratory, Lexington, KY), Bim (clone 3C5, Alexis; or polyclonal Ab from Stressgen, Victoria, BC, Canada), Bad (Stressgen), phospho-Bad (Ser112), phospho-Bad (Ser136) (both from Cell Signaling Technology, Beverly, MA), Bax (Upstate Biotechnology, Lake Placid, NY), mouse Bmf (clone 12E10, Alexis), human Bmf (polyclonal Ab; Alexis), Heat Shock Protein 70 (Hsp70) (Nov; a gift from R. Anderson, Peter MacCallum Cancer Institute, Melbourne, Australia), Mcl-1 (Dako, Glostrup, Denmark) and β-actin (Sigma) were used. Detection was performed with HRP-conjugated secondary Abs (specific to rat, mouse, hamster, or rabbit IgG) and enhanced chemiluminescence (Amersham Biosciences).

Two-Dimensional Gel Electrophoresis. Two-dimensional protein electrophoresis was performed by using the IPGphor isoelectric focusing (IEF) system (Amersham Biosciences, Piscataway, NJ). Protein lysates were loaded onto IEF gels, rehydrated at 20°C for 12 h, and subjected to IEF for at least 12,000 volt × h. After equilibration with SDS/PAGE buffer for 10 min at room temperature, the IEF gel was subjected to SDS/PAGE. Transfer, immunoblotting, and visualization were performed as described above.

RT-PCR. Total RNA was extracted by using the Microto-Midi Total RNA Extraction Kit (Invitrogen, San Diego, CA), and 40 ng/μl total RNA (in a volume of 25 μl) was subjected to RT. One microliter of the resulting cDNA was subjected to PCR by using primers for bim, bcr/abl, bmf, or puma or β-actin as a control for the quality and abundance of RNA (sequences will be provided upon request).

Cell Death Assays. Cell death was assessed either by trypan blue staining and cell counting in a hemocytometer or by staining with propidium iodide (PI) with or without staining with annexin V-FITC and flow cytometric analysis. The MTS assay was performed with the cell proliferation assay kit (MBL, Nagoya, Japan) according to the manufacturer’s instructions. For clonogenic survival assays, K562 cells were seeded at 2.0 × 104 cells per ml and treated with 1.5 or 3 μM imatinib (Novartis Pharma, Basel, Switzerland) for 24 or 48 h. Cells then were washed three times in complete medium to remove imatinib and before plating on 0.5% soft agar containing 20% FCS and 30% DMEM. Clonogenic potency was examined by counting colony numbers after 8 days of culture.

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