The Bcl-2 Homology Domain 3 (BH3) Mimetic ABT-737 Reveals the Dynamic Regulation of Bad, a Proapoptotic Protein of the Bcl-2 Family, by Bcl-xL[S]

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ABSTRACT

The proteins of the B-cell lymphoma 2 (Bcl-2) family are important regulators of apoptosis under normal and pathological conditions. Chemical compounds that block the antiapoptotic proteins of this family have been introduced, such as 4-[4-[(4′-Chloro[1,1′-biphenyl]-2-yl)methyl]-1-piperazinyl]-N-[[4-[[(1R)-3-(dimethylamino)-1-[(phenylthio)methyl]propyl]amino]-3-nitrophenyl]sulfonyl]benzamide (ABT-737), a BH3-mimetic that neutralizes Bcl-2 and Bcl-xL. In this study, we used ABT-737 to explore the dynamic regulation of Bcl-2 proteins in living cells of different origins. Using ABT-737 as well as RNA interference or the application of growth factors, we examined the impact of the functional availability of the antiapoptotic proteins Bcl-2 and Bcl-2-extra large (Bcl-xL) on the Bcl-2 network. We report that ABT-737 increases the expression of Bcl-2-associated death promoter (Bad), a proapoptotic partner of the proteins Bcl-2 and Bcl-xL. Our observations indicate that Bad overexpression induced by ABT-737 results from the control of its normally rapid protein turnover, leading to the stabilization of this protein. We demonstrate the relevance of Bad post-translational regulation by Bcl-xL to the physiological setting using RNA interference against Bcl-xL as well as the application of epidermal growth factor, a growth factor that promotes the dissociation of Bad from Bcl-xL. Our results highlight a new facet of the mode of action of the antiapoptotic proteins Bcl-2 and Bcl-xL consisting of the regulation of the stability of the protein Bad. Finally, our results shed light on the mode of action of ABT-737, currently the best characterized inhibitor of the antiapoptotic proteins of the Bcl-2 family, and bear important implications regarding its use as an anticancer drug.

Introduction

Proteins of the B-cell lymphoma 2 (Bcl-2) family are pivotal regulators of apoptosis that share one or more domains of sequence homology called the Bcl-2-homology (BH1–BH4) domains (for review, see Youle and Strasser, 2008; Chipuk et al., 2010). Bcl-2 proteins are functionally classified into different subsets, depending on their pro- or antiapoptotic effect, and the presence of a unique BH3 or multiple BH1 to BH4 homology domains. Cell sensitivity to apoptosis depends on the mutual interactions and cross-regulations between the pro- and antiapoptotic proteins. The proapoptotic proteins of the "BH3-only" subset, including BH3-interacting domain death agonist (Bid), Bcl-2-interacting mediator of cell death (Bim), p53 up-regulated modulator of apoptosis (Puma), or Bcl-2-associated death promoter (Bad), are up-

ABBREVIATIONS: Bcl-2, B-cell lymphoma 2; BH, Bcl-2-homology domain; Bim, Bcl-2-interacting mediator of cell death; Bad, Bcl-2-associated death promoter; Bax, Bcl-2–associated X protein; Bak, Bcl-2 homologous antagonist/killer; Mcl-1, myeloid-cell leukemia sequence 1; Bcl-xL, B-cell lymphoma-extra large; ABT-737, 4-[4-[(4′-Chloro[1,1′-biphenyl]-2-yl)methyl]-1-piperazinyl]-N-[[4-[[(1R)-3-(dimethylamino)-1-[(phenylthio)methyl]propyl]amino]-3-nitrophenyl]sulfonyl]benzamide; HSMC, human smooth muscle cell; HUVEC, human umbilical vein endothelial cell; EGF, epidermal growth factor; MG132, N-benzoyloxycarbonyl (Z)-Leu-Leu-leucinal; PAGE, polyacrylamide gel electrophoresis; PCR, polymerase chain reaction; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]propanesulfonate; HA14-1, 2-amino-6-bromo-α-cyano-3-(ethoxycarbonyl)-4H-1-benzopyran-4-acetic acid ethyl ester; siRNA, small interfering RNA.

stream regulators that control the multidomain proteins of the Bcl-2 family, whereas their multidomain counterparts, such as Bax, Bcl-2–associated X protein (Bax) and Bcl-2 homologous antagonist/killer (Bak), are considered to be core components of the mitochondrial apoptotic machinery (Youle and Strasser, 2008; Chipuk et al., 2010). Under normal conditions, the proteins of the BH3-only subset and the proapoptotic multidomain proteins of the Bcl-2 family are functionally neutralized by the antiapoptotic proteins of the Bcl-2 family, such as Bcl-2, B-cl-extra large (Bcl-xL), or myeloid cell leukemia 1 (Mcl-1). The neutralizing effect of the proapoptotic proteins is induced by the specific interaction of the BH3 domain of these proteins with a hydrophobic pocket present on the surface of the antiapoptotic Bcl-2 proteins (Youle and Strasser, 2008; Chipuk et al., 2010). Recently, compounds with a BH3 mimetic activity have been developed, with the aim of sensitizing cells to apoptosis, principally in the context of cancer treatment (Certo et al., 2006; van Delft et al., 2006; Letai, 2008). The main BH3 mimetic developed to date is the compound 4-[4-[(4′-Chloro[1,1′-biphenyl]-2-yl)methyl]-1-piperazinyl]-N-[[4-[[(1R)-3-(dimethylamino)-1-[(phenylthio)methyl]propyl]amino]-3-nitrophenyl]sulfonyl]benzamide (ABT-737), developed by Abbott Laboratories (Abbott Park, IL), which inhibits the antiapoptotic proteins Bcl-2 and Bcl-xL with a reactivity that mimics the BH3-only protein Bad (Oltersdorf et al., 2005; Lee et al., 2007).

The interaction between the BH3-only protein Bad and the antiapoptotic proteins Bcl-2 and Bcl-xL regulates cell sensitivity to apoptosis (Yang et al., 1995; Kelekar et al., 1997; Petros et al., 2000; Letai et al., 2002; Chen et al., 2005; Kuwana et al., 2005; Kim et al., 2006; Hinds et al., 2007). As is the case for other BH3-only proteins of the Bcl-2 family, Bad operates as a sentinel and an upstream regulator of Bcl-2 proteins in response to specific death stimuli: Bad is under negative regulation by trophic stimuli, such as those provided by growth factors present in the cell culture medium (for review, see Danial, 2008). Serine phosphorylations of Bad play an important role in the regulation of this protein: unphosphorylated Bad is able to neutralize Bcl-2 and Bcl-xL, as seen upon cellular deprivation of prosurvival signals (Danial, 2008). Bad phosphorylation is a complex process that involves multiple protagonists and occurs on distinct positions of the molecule in a tied fashion (Wang et al., 1996; Zha et al., 1996; Scheid et al., 1999; Datta et al., 2000; She et al., 2005). It has been proposed, on the basis of the convergence of several kinase signaling pathways, that Bad operates as a point of integration of the activity of prosurvival kinase pathways (She et al., 2005).

Although the general principles of the regulation of apoptosis by the interaction between Bcl-2/Bcl-xL and the proapoptotic proteins such as Bad are well accepted, the dynamic aspects of this regulation inside living cells remain poorly known. The recent introduction of inhibitory compounds, such as ABT-737, so far the most specific inhibitor of the proteins Bcl-2 and Bcl-xL, offers an interesting possibility to study this aspect of the Bcl-2 protein network (van Delft et al., 2006; Lee et al., 2007; Vogler et al., 2009). Based on our previous observation that ABT-737 increases the expression of the protein Bad in liver cancer cells (Galmiche et al., 2010), we decided to use ABT-737 to examine the functional consequences of the neutralization of Bcl-2 and Bcl-xL

on the dynamic regulation of the Bcl-2 network. Here, we report that the neutralization of Bcl-xL profoundly affects several aspects of the regulation of Bad. Our findings highlight the dynamic participation of Bad in the Bcl-2 protein network and reveal an aspect of the regulation of this protein that has until now received little attention (i.e., the regulation of this protein's stability). Our results bear important implications regarding the mode of action of ABT-737 and the use of BH3-mimetics in oncology.

Materials and Methods

Cell Culture. All cell lines were maintained at 37°C in a humidified atmosphere with 5% CO_2. Huh7, BxPC3, and HeLa cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum (Jacques Boy, Reims, France), 2 mM glutamine, and penicillin/streptomycin. Human smooth muscle cells (HSMCs) derived from aortic tissue were cultured in medium 231 supplemented with smooth muscle growth supplement (all from Invitrogen, Cergy Pontoise, France). Human umbilical vein endothelial cells (HUVECs) were purchased and cultured in endothelial basal medium 2 supplemented with endothelial growth medium 2 (Lonza France Sàrl, Levallois-Perret, France).

Reagents. ABT-737 and its inactive enantiomer were kindly provided by Abbott Laboratories (Oltersdorf et al., 2005). Recombinant human epidermal growth factor (EGF), HA14-1, actinomycin D, and cycloheximide were purchased from Sigma (Lyon, France). Sorafenib was a kind gift from Bayer Healthcare (Wayne, NJ). N-Benzoyloxy-carbonyl (Z)-Leu-Leu-leucinal (MG132), Ac-Leu-Leu-norleucinal, and epoxomicin were purchased from Calbiochem (San Diego, CA). Antibodies raised against Bad, Bad phosphorylated on Ser75 (Ser112 of murine Bad), Bad phosphorylated on Ser99 (Ser136 of murine Bad), Bad phosphorylated on Ser118 (Ser155 of murine Bad), Bcl-xL, Bax, Bak, Bid, or Bim were from Cell Signaling Technology (Danvers, MA). Antibodies directed against Mcl-1, Bcl-xL (7B2.5), Bad (5E6), 14-3-3 proteins, and Hsp60 were from Santa Cruz Biotechnology (Heidelberg, Germany). Antibodies against β-actin and α-tubulin were from Sigma (St. Louis, MO). Secondary antibodies coupled with horseradish-peroxidase were purchased from GE Healthcare (Saclay, France).

Western Blot. Complete cell extracts were prepared in radioimmunoprecipitation assay buffer. Their protein concentration was determined with the BCA kit (Thermo Fisher Pierce, Brebriere, France). The proteins were precipitated, loaded on SDS-PAGE and transferred to nitrocellulose membranes using standard procedures. The enhanced chemiluminescence reaction was used for revelation on Hyperfilm (GE Healthcare). Immunoblots were scanned and quantified using the software ImageJ (http://rsbweb.nih.gov/ij/).

Quantitative PCR. Total RNA was extracted using RNeasy Mini Kit (QIAGEN, Courtaboeuf, France) and reverse-transcribed using High Capacity cDNA Reverse Transcription kit and random hexamer (Applied Biosystems, Courtaboeuf, France). Amplification was performed with the TaqMan Universal PCR master Mix on an ABI 7900HT Sequence Detection System (Applied Biosystems) using primers and probe sets for Bad and human β-actin (TaqMan Gene Expression Assay; Applied Biosystems).

Cellular Fractionation. Mitochondria and cytoplasmic fractions were prepared as previously reported (Galmiche et al., 2008). In brief, 5×10^6 Huh7 cells were homogenized in a buffer containing 250 mM sucrose, 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, and 20 mM HEPES-KOH, pH 7.4, together with protease and phosphatase inhibitors. Mitochondria were recovered in the heavy membrane fraction, after centrifugation of the postnuclear supernatant at 13,000g for 15 min. Equal amounts of proteins from the different fractions were applied on SDS-PAGE.

Immunoprecipitation. Huh7 cells were seeded in 10-cm diameter dishes and treated as indicated in the legend to Fig. 3. After one

rinsing in ice-cold PBS, extracts were prepared in a buffer containing 150 mM NaCl, 5 mM EDTA, 1% CHAPS, and 20 mM HEPES-KOH, pH 7.4, with protease inhibitors and a 10-min centrifugation step at 15,000g. For each condition, 1 mg of protein was incubated with 1 μg of rabbit anti-Bcl-xL antibody (Cell Signaling Technology) for 4 h at 4°C. The extracts were then incubated for 2 h with protein A immobilized on Sepharose beads (Sigma). The beads were washed three times and diluted in Laemmli sample buffer before their loading on SDS-PAGE. The immunoprecipitates were analyzed by immunoblotting using mouse monoclonal antibodies raised against Bcl-xL and Bad.

RNA Interference. Silencer select validated siRNAs directed against Bcl-xL (GGAACUCUAUGGGAACAAUtt, GCUGGAGU-CAGUUUAGUGAtt), Mcl-1 (CCAGUAUACUUCUUAGAAAtt), Bad (GGAGGAUGAGUGACGAGUUtt, GCAACGCAGAUGCGGCAAAtt), and Silencer negative controls #1 and #2 were purchased from Applied Biosystems. Transfections were performed using the siPORT-*NeoFX* reagent (Applied Biosystems) and Opti-MEM transfection medium (Invitrogen) according to the manufacturer's instructions.

Cytotoxicity Assessment. Cells grown on glass coverslips were processed as described previously (Galmiche et al., 2010). Nuclei were stained with 4,6-diamidino-2-phenylindole, and cells were observed with a microscope equipped for fluorescence microscopy (Eclipse TE2000U; Nikon, Tokyo, Japan).

Statistical Analyses. Data are presented as means ± S.D. Student's t test was used, and $p < 0.05$ was considered the threshold for significance.

Results

ABT-737 Increases Bad Expression Levels in a Panel of Human Cell Lines. To examine the effect of Bcl-2/Bcl-xL neutralization, we applied ABT-737 on a panel of different cell lines and measured the expression levels of the members of the Bcl-2 protein family by immunoblotting (Fig. 1). We found that ABT-737 induces a massive increase in the expression of Bad in all the cell lines examined (Fig. 1A; Supplemental Fig. 1). This increase in the expression of Bad occurred progressively over the first 3 h after exposure to ABT-737. It was a specific event, because the protein levels of

most of the other members of the Bcl-2 family remained stable at that time point (Fig. 1A). The expression of BIM, a protein of the BH3-only subset, was also slightly increased after cell exposure to ABT-737, although the effect was much smaller than with Bad (Fig. 1A). The effect of ABT-737 was noticed irrespective of the tissue of origin or the transformed status of the cells that were examined (Fig. 1B): the expression levels of Bad were increased in cells originating from the liver (Huh7), cervix epithelium (HeLa), pancreas (BXPC3), endothelium (HUVEC), or in HSMCs originating from the vascular wall. Although ABT-737 increased the expression levels of Bad at micromolar concentrations, its inactive enantiomer produced no effect (Fig. 1C). We concluded that ABT-737 is a potent inducer of Bad protein expression in several types of cultured cells.

ABT-737 Stabilizes the Bad Protein. To explore the mechanisms that lead to this increase in the expression of Bad, we measured Bad mRNA levels in Huh7 cells exposed to ABT-737 or its inactive enantiomer for 3 h (Fig. 1D). It is noteworthy that Bad mRNA levels remained stable in ABT-737-treated cells (Fig. 1D). To directly address the role of transcription in the regulation of Bad, we applied actinomycin D, a blocker of RNA polymerase, at a concentration of 2 μg/ml, previously reported by us to induce a complete block of transcription in eukaryotic cells (Rolando et al., 2010). Actinomycin D did not prevent the induction of Bad in Huh7 exposed to ABT-737 (Supplemental Fig. 2), further suggesting that transcription does not play an important role in the increase in the expression of Bad upon exposure to ABT-737. We therefore envisioned the possibility that ABT-737 might regulate the expression of Bad at the post-transcriptional level and, in particular, through an effect on the stability of this protein. To measure the stability of Bad, we used the antibiotic cycloheximide that blocks protein translation, and we analyzed the pace at which the expression levels of Bad decayed. Huh7 cells were treated for 1 h with ABT-737 or its inactive enantiomer after a preincubation with 50 μM cyclo-
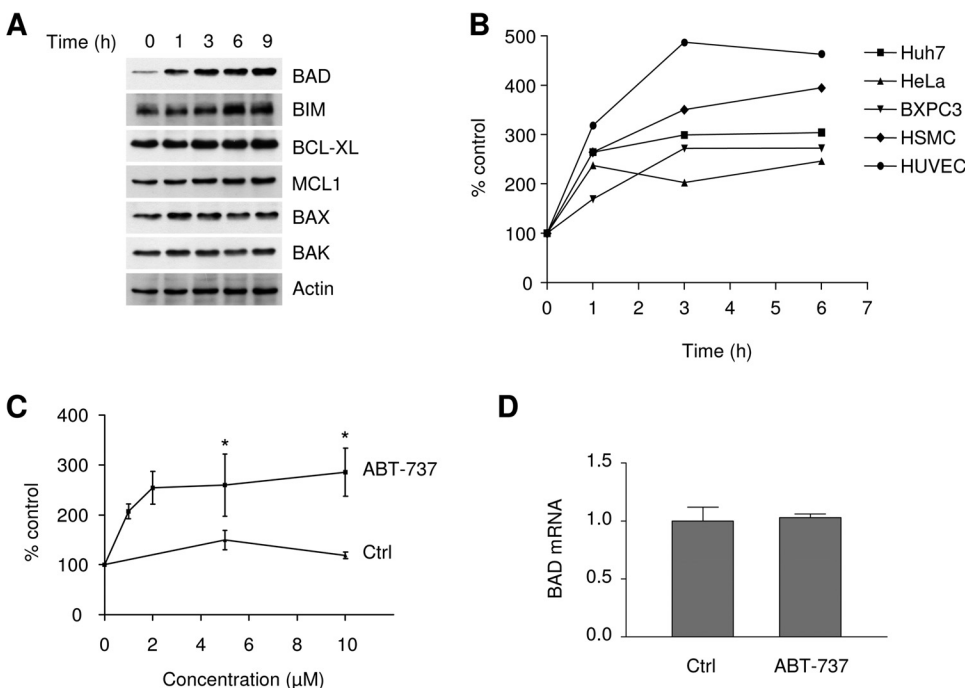
Fig. 1. ABT-737 increases the expression of Bad protein. A, time-course induction of Bad in Huh7 cells treated with ABT-737 (10 μM for 0, 1, 3, 6, and 9 h). B, quantification of the expression of Bad protein in Huh7, HeLa, and BXPC3 cells, HUVECs, and HSMCs exposed to 10 μM ABT-737 at 0, 1, 3, 6 h. The values are expressed as percentage of initial content and are taken from a single representative experiment. C, concentration dependence. Graded concentrations of ABT-737 or its inactive enantiomer were applied on Huh7 cells for 3 h, and the amount of Bad was analyzed by immunoblotting. The values are average of three independent experiments. D, Huh7 cells exposed to 10 μM ABT-737 for 3 h were analyzed for their content of Bad mRNA. Results are normalized with regard to β-Actin mRNA.

heximide, and the corresponding protein extracts were analyzed for their content of Bad or the other proteins of the Bcl-2 family (Fig. 2). We found a striking change in the stability of the protein Bad upon ABT-737 exposure: although the Bad protein presents a rapid turnover in control cells, with a half-life estimated at approximately 1 h, this turnover was clearly abolished upon cell exposure to ABT-737 (Fig. 2). This stabilization of Bad was a specific effect, because we noted no effect of ABT-737 on the turnover of the other proteins of the Bcl-2 family that were tested (Fig. 2). It is noteworthy that ABT-737 stabilized Bad at a dose of 1 μM (data not shown), consistent with its effect on Bad expression as shown in Fig. 1C. We concluded that ABT-737 specifically prevents the rapid turn-over of Bad and stabilizes this protein.

ABT-737 Promotes the Cytosolic Accumulation of Bad. Based on a previous report in which it was found that heterodimers formed between Bad and Bcl-xL had a high affinity for mitochondrial membranes (Jeong et al., 2004), we decided to further examine the impact of ABT-737 on the subcellular distribution of Bad and its association with Bcl-xL. Complete extracts, cytosolic fractions, and heavy membrane fractions enriched in mitochondria were prepared from Huh7 cells exposed to ABT-737 and analyzed by immunoblot-

ting for their content of Bad (Fig. 3A). The cytosolic fraction was considerably enriched in Bad, whereas a slight decrease in the mitochondrial content of this protein was apparent after ABT-737 treatment (Fig. 3A). We concluded that ABT-737 reduces the affinity of Bad for mitochondria in living cells. The interaction of Bad with membrane organelles had previously been reported to be controlled by phosphorylation (Danial, 2008). We decided to examine the phosphorylation status of Bad using antibodies raised against the three positions documented to play the main role in its regulation (i.e., Ser75, Ser99, and Ser118) of the human molecule (Danial, 2008). We detected a different pattern of phosphorylation of Bad in each of the cell lines that were exposed to ABT-737; phosphorylated forms of Bad remained undetectable in Huh7 cells. Bad was phosphorylated at high levels on Ser75 in BXPC3 cells and at intermediate levels in HeLa cells, HUVECs, or HSMCs (Supplemental Fig. 3). Bad phosphorylation on Ser118 was not detected under our experimental conditions. We concluded that ABT-737 changes the subcellular localization of Bad, whereas its effect on the phosphorylation of Bad is context-specific and depends on the various cell lines or experimental conditions that were applied here. Next, we tested the possibility that a change in the association of Bad with proteins of the 14-3-3 family might account for the cytosolic accumulation of Bad upon ABT-737 treatment. We performed an immunoprecipitation of 14-3-3 proteins to examine the amount of Bad associated with this family of cytosolic proteins (Supplemental Fig. 4). We found no evidence of an association between these proteins in Huh7 cells that had been exposed to ABT-737, whereas the kinase c-Raf, used here as a positive control, remained stably associated with 14-3-3 proteins (Supplemental Fig. 4). Finally, we
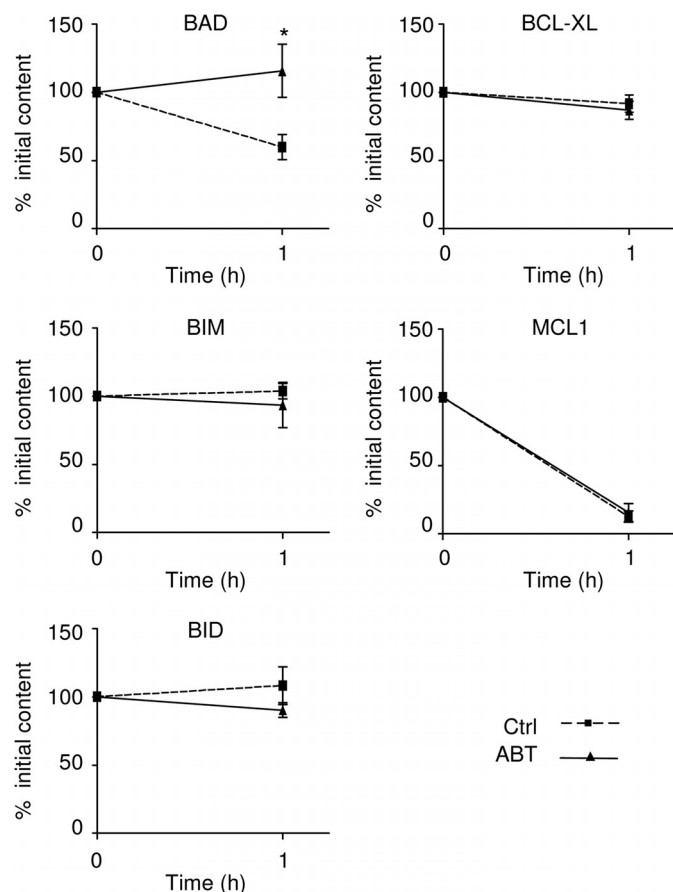

Fig. 2. The increase in expression of Bad by ABT-737 proceeds through the stabilization of the Bad protein. Huh7 cells were pretreated for 30 min with 50 μM cycloheximide and treated with ABT-737 or its inactive enantiomer (both applied at 10 μM) for 1 h. Total protein extracts were analyzed for their content of the indicated Bcl-2 proteins by immunoblotting, and the amount of protein was calculated as a ratio to their initial content. Data are presented as mean $\pm$ S.D. of three independent experiments. ∗, $p < 0.05$ compared with control.
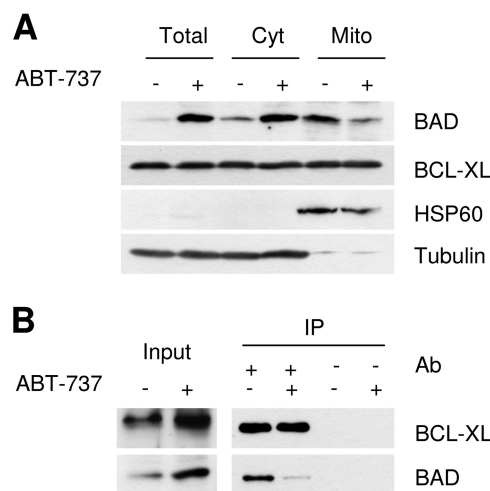

Fig. 3. ABT-737 promotes the cytosolic accumulation of Bad. A, Huh7 exposed to ABT-737 or its inactive enantiomer (10 μM) for 3 h were fractionated as indicated under *Materials and Methods*, and equal protein amounts of the indicated fractions were analyzed by immunoblotting to determine their content of the indicated proteins. The mitochondrial chaperone HSP60 and tubulin are used as markers of the mitochondrial (mito) and cytosolic (cyt) fractions, respectively. This experiment is representative of three experiments giving comparable results. B, analysis of the complexes formed between Bcl-xL and Bad by immunoprecipitation. Huh7 cells treated with ABT-737 for 3 h (10 μM) were lysed, and Bcl-xL was immunoprecipitated. The indicated markers were analyzed by immunoblotting. The input corresponds to 5% of the extract used for each condition of immunoprecipitation, and the experiment includes a set of control conditions performed in the absence of the immunoprecipitating antibody.

examined the effect of ABT-737 on the association of Bad with Bcl-xL (Fig. 3B). We performed an immunoprecipitation of Bcl-xL from Huh7 exposed to ABT-737 for 3 h, and the levels of Bad present in the immunoprecipitate were examined by immunoblotting. We found that ABT-737 prevents the interaction between Bcl-xL and Bad, in agreement with the previous biochemical characterization of this drug (Oltersdorf et al., 2005) (Fig. 3B). We concluded that ABT-737 induces the cytosolic accumulation of the molecule Bad in a free, unliganded, form.

Bcl-xL Regulates the Expression of Bad. To check whether the neutralization of Bcl-xL accounts for the effect of ABT-737 on Bad, we combined several approaches. First, we tested another inhibitor of the antiapoptotic proteins of the Bcl-2 family structurally unrelated to ABT-737, the compound 2-amino-6-bromo-α-cyano-3-(ethoxycarbonyl)-4H-1-benzopyran-4-acetic acid ethyl ester (HA14-1; Wang et al., 2000). HA14-1 also induced an overexpression of Bad at the protein level, albeit with somewhat lower potency than ABT-737 (Supplemental Fig. 5). More importantly, we performed RNA interference experiments directed against antiapoptotic proteins of the Bcl-2 family in Huh7 cells. Although ABT-737 can target Bcl-xL and Bcl-2, we detected no expression of Bcl-2 in Huh7 cells, and we consequently decided to explore the role of Bcl-xL in the regulation of Bad. We compared the effect of two independent siRNA targeting sequences of Bcl-xL with control siRNAs directed against irrelevant sequences or against MCL1, another prosurvival member of the Bcl-2 family that is not targeted by ABT-737, on Bad expression levels (Oltersdorf et al., 2005) (Fig. 4A). Strikingly, the two siRNAs targeting Bcl-xL were found to specifically increase Bad expression and therefore independently reproduced the effect of ABT-737 (Fig. 4A). To check the possibility that transcriptional mechanisms might account for the variations in the

expression of Bad, we measured Bad mRNA levels with the use of quantitative PCR. We found no increase in Bad mRNA expression levels in cells treated with Bcl-xL siRNA, clearly indicating that a post-transcriptional mechanism explains the increase in Bad protein expression in these cells (Supplemental Fig. 6). A further indication that Bad expression levels are regulated post-translationally was obtained with the application of chemical inhibitors of the proteasome on Huh7 cells. Three nonstructurally related inhibitors of the proteasome (MG132, Ac-Leu-Leu-norleucinal, and epoxomycin) were applied on Huh7 for a short period of time and found to clearly increase Bad expression levels, confirming the importance of the regulation of this protein at the level of the protein turnover (Supplemental Fig. 7). To further examine the possibility that the neutralization of Bcl-xL accounted for the overexpression of Bad, we examined the effect of ABT-737 on cells in which Bcl-xL levels were reduced by RNA interference (Fig. 4B). We noted no additive increase in the expression of Bad after exposure to ABT-737 in cells where Bcl-xL expression was reduced by RNA interference (Fig. 4B). These findings further suggested the specificity of the effects of ABT-737 that we had reported, and we concluded that the neutralization of Bcl-xL most likely accounts for the effect of ABT-737 on the turnover of Bad.

Physiological and Pharmacological Relevance to the Regulation of Bad. To examine the consequences of our findings in a physiologically relevant setting, we turned our attention to EGF, a growth factor that potently activates the Raf/mitogen-activated protein kinase kinase/extracellular signal-regulated kinase kinase cascade and promotes the phosphorylation of Bad on Ser75 (She et al., 2005). It was reported previously that Bad phosphorylation induced by EGF disrupts the complexes formed between Bcl-xL and Bad (Scheid et al., 1999). We therefore exposed Huh7 cells to EGF (15 ng/ml) for 1 h and examined the protein and mRNA levels of Bad by immunoblotting and quantitative PCR, respectively (Fig. 5). We noticed that EGF induced a clear increase in Bad protein levels without altering its mRNA levels (Fig. 5, A and B). The increase in Bad expression observed after EGF exposure was dependent on the activation of intracellular kinase cascades because it was blocked by sorafenib, an inhibitor of the RAF kinase family (Fig. 5A). To rule out a possible contribution of a block of translation in these effects of sorafenib, as was observed, for example, with Mcl-1 (Rahmani et al., 2005), we directly measured the stability of the Bad protein using cycloheximide (Fig. 5C). The results clearly showed that Bad phosphorylation, which controls the ability of this protein to associate with Bcl-xL, regulates Bad protein turnover in Huh7 cells (Fig. 5C).

To further demonstrate the relevance of our findings, we decided to examine the role of Bad in the cytotoxic effects of ABT-737 on cancer cells. ABT-737 exerts modest cytotoxic effects when it is applied on cancer cells as a single agent (Oltersdorf et al., 2005), but a potent synergistic proapoptotic effect results from its coapplication with oncogenic kinase inhibitors (Cragg et al., 2008, 2009; Galmiche et al., 2010; Hikita et al., 2010). Considering that Bad operates as a point of integration of the activity of important prosurvival kinases in cancer cells (She et al., 2005), we reasoned that the induction of Bad by BH3 mimetics, such as ABT-737, might partially explain this synergy. To explore the pharmacological relevance of the induction of Bad, we decided to examine the
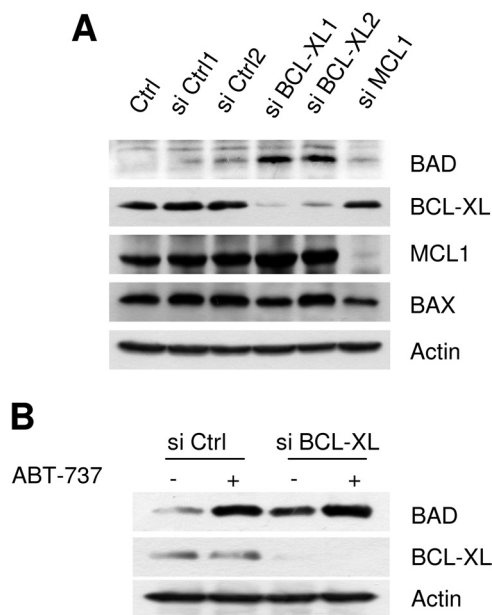
Fig. 4. Endogenous Bcl-xL modulates the expression of Bad. A, RNA interference against Bcl-xL increases Bad protein expression levels. Huh7 cells were transfected with the indicated siRNA and maintained in culture for 3 days. The levels of expression of the indicated proteins were subsequently analyzed by immunoblotting. B, Huh7 cells transfected with a ctrl siRNA or a siRNA directed against Bcl-xL were maintained in culture for 48 h, and ABT-737 (10 μM) was applied as indicated for 3 h.

role of Bad in the cytotoxicity of ABT-737 coappled with sorafenib on hepatocellular carcinoma cells (Galmiche et al., 2010; Hikita et al., 2010). Huh7 cells in culture were transfected with Bad-specific siRNAs, according to a previously reported procedure (Galmiche et al., 2010). After 48 h of exposure to the siRNAs, cells were treated with ABT-737 and sorafenib (both applied at 10 μM and maintained for 3 h), and the percentage of dead cells was evaluated by counting the number of cells with condensed chromatin after a 4,6-diamidino-2-phenylindole staining under a microscope (Fig. 6). In our experimental conditions, we noticed that a 2 -old decrease in the expression of Bad significantly reduced the cytotoxic effect of ABT-737 and sorafenib (Fig. 6). We concluded that Bad plays a role in the cytotoxicity of ABT-737 upon coapplication of this BH3 mimetic with a blocker of oncogenic kinases, such as sorafenib.

Discussion

The proteins of the Bcl-2 family are pivotal regulators of apoptosis and multiple aspects of cell physiology. In the present report, we took advantage of the recently developed inhibitor of the antiapoptotic proteins of the Bcl-2 family, the BH3-mimetic ABT-737, to examine in a dynamic fashion how the antiapoptotic proteins Bcl-2 and Bcl-xL regulate one of their proapoptotic binding partners, Bad. Bad is an impor-

tant member of the BH3-only subset of proapoptotic Bcl-2 proteins that plays a pivotal role as an apoptosis sensitizer and a regulator of metabolism in the liver and pancreas (Danial, 2008). We report the dramatic consequences of the application of ABT-737 on the expression and regulation of Bad. Our results bear important consequences regarding the cellular mode of action of ABT-737 as well as the regulation of Bad.

Our observations extend the current knowledge on the mode of action of ABT-737 in the cell. In addition to the direct and well characterized inhibition of Bcl-xL by ABT-737 (Oltersdorf et al., 2005; Lee et al., 2007), our observations suggest not only that ABT-737 is able to neutralize the antiapoptotic proteins Bcl-2 and Bcl-xL but also that it could exert indirect effects on proteins of the Bcl-2 family. Whether the induction of Bad might contribute to the neutralization of Bcl-2 and Bcl-xL upon cellular exposure to ABT-737 is currently unclear, because we did not detect an increase in complex formation between these proteins after treatment with ABT-737. Nevertheless, our findings bear important implications concerning the therapeutic use of the pharmacological inhibitors of the antiapoptotic Bcl-2 proteins. It has recently been noted that these agents produce potent synergistic proapoptotic effects when coappled with oncogenic kinase inhibitors on cancer cells (Cragg et al., 2008, 2009; Galmiche et al., 2010; Hikita et al., 2010). The results that we present in this
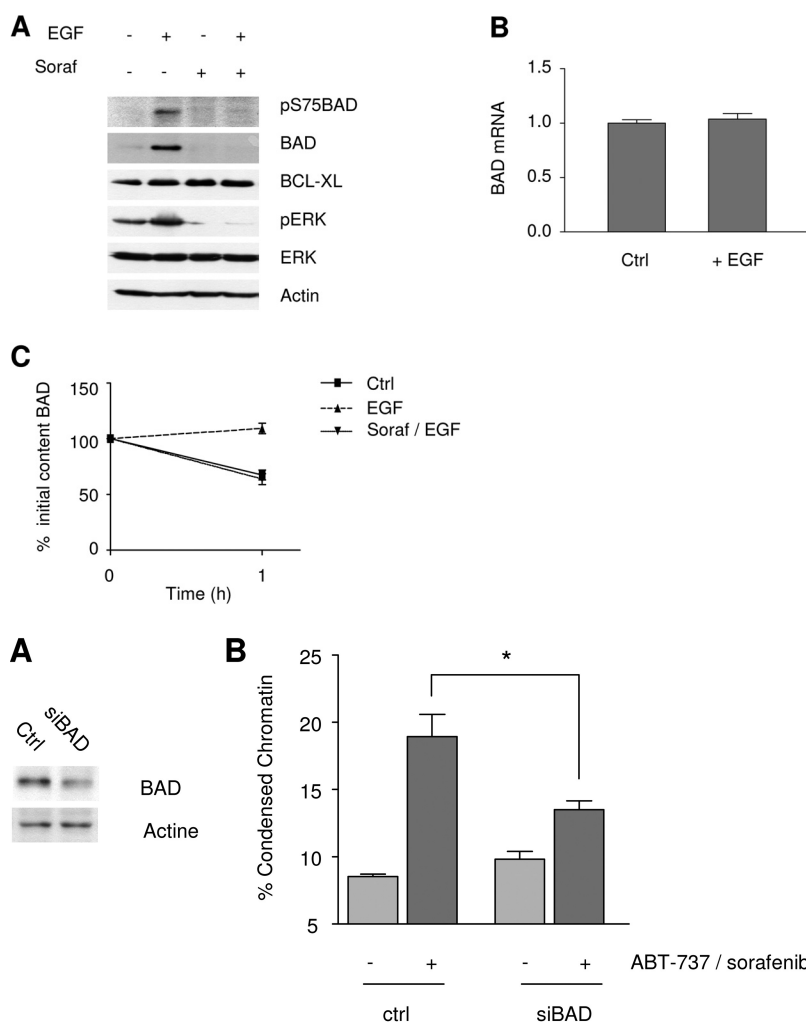

Fig. 5. Post-transcriptional regulation of Bad after EGF treatment. A, Huh7 cells were serum-starved for 3 h and exposed to EGF (15 ng/ml) for 1 h, either alone or in the presence of the kinase inhibitor sorafenib (10 μM). The levels of expression of the indicated proteins were subsequently analyzed by immunoblotting. Results are from a representative experiment, performed three times with comparable results. B, mRNA levels of Bad were analyzed in Huh7 cells treated for 1 h with EGF.

Fig. 6. RNA interference targeted against Bad protects Huh7 cells from the cytotoxic effect of the combination of ABT-737 and sorafenib. A, Huh7 cells were transfected with control or Bad-specific siRNAs and maintained in culture for 48 h. Immunoblotting was used to verify the reduction in the expression levels of Bad. B, Huh7 cells treated as control or with Bad-specific siRNAs were exposed for 3 h to ABT-737 (10 μM) and sorafenib (10 μM), and the cytotoxicity was evaluated by condensed chromatin formation under microscopic examination. Results shown are from a representative experiment performed in triplicate, with each condition based on at least 500 cells counted on random fields. *, $P < 0.05$.

report suggest that the induction of Bad, a BH3-only protein that defines a convergence point for several oncogenic kinases that regulate the survival of cancer cells, at least partially explains this synergy.

Our results also provide interesting new insights on the regulation of the protein Bad. Although several studies on Bad have put emphasis on the role of phosphorylation in the regulation of this protein (for review, see Danial, 2008), it has remained unclear whether Bad is constitutively neutralized by phosphorylation in healthy cells, or whether it encounters regulated cycles of association/dissociation with Bcl-2 and Bcl-xL. Our results suggest that Bad is a protein that constantly interacts with the antiapoptotic proteins Bcl-2 and Bcl-xL. Our results also bring useful insights regarding the interaction of Bad with mitochondria. How the affinity of Bad for mitochondrial membranes is regulated is an important question that has been previously explored in vitro using purified components (Jeong et al., 2004; Hekman et al., 2006). In a recent report, the possibility that Bad might directly possess an affinity for membranes was raised based on the lipophilic properties of the carboxyl-terminal part of this protein (Hekman et al., 2006). Our observations showing that Bad accumulates in the cytosol of cells exposed to ABT-737 suggest that the functionality of the antiapoptotic proteins of the Bcl-2 family determines the affinity of Bad for mitochondria and that the direct recognition of these organelles' lipids does not greatly contribute to the interaction between Bad and mitochondria in healthy cells.

Our observations point to an aspect of Bad regulation that until now has received little attention: the control of the expression of Bad through the modulation of this protein's stability (Fueller et al., 2008; Galmiche et al., 2010). Upon Bcl-xL neutralization accomplished through the administration of a BH3 mimetic or upon RNA interference targeted against Bcl-xL, the protein Bad accumulated in a manner that was essentially accounted for by its stabilization. The existence of a regulation of the turnover of Bcl-2 proteins through the association with their binding partners is a property that had already been reported for other members of

the Bcl-2 family, such as the antiapoptotic protein Mcl-1 (Czabotar et al., 2007; Adams and Cooper, 2007) or the proapoptotic BH3-only protein Bim-extra long (Bim-EL; Ewings et al., 2007), but that had not yet been reported for Bad. In Fig. 7, we propose a model summarizing the regulation of Bad with an emphasis on the role of Bcl-xL. Although our results clearly indicate that the interaction of Bad with Bcl-xL promotes its turnover, the molecular mechanisms that control the turn-over of Bad upon its association remain incompletely understood. Future investigations will aim at identifying the possible mechanisms, but two nonmutually exclusive potential explanations can already be envisioned: 1) the association between Bad and Bcl-xL could expose some sequences of Bad involved in this protein's turnover. It is likely to have a strong impact on the folding of Bad, because this protein has been previously reported to be intrinsically unfolded (Hinds et al., 2007). We previously reported the presence of two PEST sequences, known to exert a destabilizing effect on proteins, in Bad (Fueller et al., 2008). The conformation and/or the surface exposure of these sequences might change upon the interaction of Bad with Bcl-xL; 2) alternatively, the association of Bad with Bcl-xL might regulate the turnover of Bad by modulating its subcellular localization or its proximity with the molecular machinery involved in Bad turn-over.

Irrespective of the mechanisms involved, our results suggest that the interaction between Bcl-xL and Bad affects the regulation of Bad beyond the simple mutual neutralization, by controlling the turn-over of this BH3-only protein, an observation that, to our knowledge, had not been previously reported. These findings bear important implications for the control of apoptosis in the context of tumorigenesis. Resistance to apoptosis is a fundamental property of tumor cells, and alterations of the expression of the proteins of the Bcl-2 family frequently contribute to this resistance (Hanahan and Weinberg, 2000; Letai, 2008; Yip and Reed, 2008; Frenzel et al., 2009). Our finding that Bcl-xL regulates Bad protein stability could partially explain why in tumors that overexpress Bcl-xL, such as hepatocellular carcinoma, Bad expression is reduced through post-translational mechanisms (Takehara et al., 2001; Galmiche et al., 2010). Based on the observations that we present in this report, as well as those published in previous studies, we suggest that the control of protein stability is an important facet of Bcl-2 regulation that is controlled via mutual interactions between Bcl-2 proteins. Collectively, our findings illustrate how the use of the novel reagents with a BH3 mimetic mode of action helps the investigation of the dynamic aspects of the regulation of Bcl-2 proteins inside intact cells.
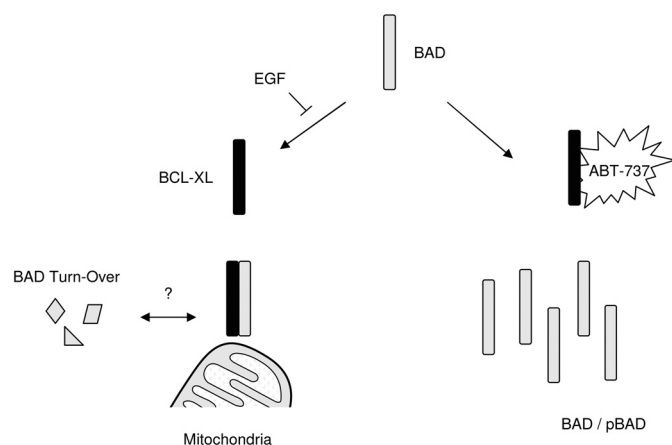

Fig. 7. A model for the regulation of Bad. The model presented here integrates our observations on the control of Bad expression by Bcl-xL association. Upon association with Bcl-xL, Bad tends to preferentially target mitochondrial membranes and encounters a rapid turn-over. When Bcl-xL is inhibited by ABT-737, or when the interaction between Bad and Bcl-xL is prevented by the phosphorylation of Bad (after EGF treatment), the turnover of Bad is prevented and the protein accumulates in the cytosol.

Authorship Contributions

Participated in research design: Ezzoukhry and Galmiche.
Conducted experiments: Ezzoukhry, Louandre, François, and Godin.
Performed data analysis: Ezzoukhry and Galmiche.

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