Diltiazem Enhances the Apoptotic Effects of Proteasome Inhibitors to Induce Prostate Cancer Cell Death

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Introduction

The concentration of cytosolic Ca\(^{2+}\) ([Ca\(^{2+}\)\(_c\)]\) is maintained at a lower level inside the cell compared with that in the extracellular space. This gradient of Ca\(^{2+}\) is tightly controlled by interactions with binding proteins and movement across the plasma membrane, in addition to transport into and out of key organelles such as the endoplasmic reticulum (ER) and mitochondria. The ER plays the role of a calcium reservoir, whereas mitochondria are a calcium sink, controlling the entry into and out of key organelles such as the endoplasmic reticulum (ER) and mitochondria. The ER plays the role of a calcium reservoir, whereas mitochondria are a calcium sink, remov-
smooth muscle and leading to decreased blood pressure. Less clear is the effect of calcium channel blockers in cancer, particularly prostate cancer. Thus, whereas some investigators report that the use of calcium channel blockers is inversely associated with prostate cancer (Debes et al., 2004), others have failed to find such a relationship (Rodriguez et al., 2009). Thus, it is of interest to investigate the potential effectiveness of these drugs in prostate cancer therapy, especially because both cardiovascular problems and prostate cancer are common in elderly men (Yoshinaga et al., 2006). Therefore, it may be justifiable to use calcium channel blockers along with cytotoxic drugs to treat prostate cancer in patients with associated hypertension.

Bortezomib (PS-341; marketed as Velcade) is the first proteasomal inhibitor to be approved in the United States for treating relapsed multiple myeloma and mantle cell lymphoma. Bortezomib is also in phase I and II clinical trials for the treatment of other cancers, including Kaposi sarcoma, metastatic breast cancer, melanoma, renal cell carcinoma, brain tumors and prostate cancer, often in combination with other therapeutic agents (http://www.cancer.gov/clinicaltrials). Similar to other proteasomal inhibitors, bortezomib acts by blocking the 26S proteasome. In normal cells, the proteasome regulates protein expression and function by degrading ubiquitinated proteins and destroying abnormal or misfolded proteins. Proteasomal inhibitors are expected to block proliferation and induce apoptosis by stabilizing several key tumor suppressors and cell cycle inhibitors, and also by blocking the activation of nuclear factor-kB, a protein that is constitutively activated in some cancer cells (Goldberg and Rook, 2002). Another possible mechanism by which proteasomal inhibitors may induce apoptosis in cancer cells is by preventing the degradation of proapoptotic proteins, thereby allowing these proteins to overwhelm antiapoptotic proteins (Lomonosova et al., 2009). Furthermore, proteasomal inhibitors induce accumulation of misfolded proteins to induce the unfolded protein response (Meister et al., 2010). Under physiological conditions, the ER chaperone GRP78 (BiP) binds at least three ER transmembrane proteins (IRE1, ATF6, and PERK) (Egger et al., 2007). Upon ER stress, GRP78 associates with misfolded proteins and releases the ER transmembrane proteins, leading by different mechanisms to apoptosis, if the stress is sustained.

We have previously shown that blocking the mitochondrial Na+/Ca2+ exchanger significantly enhanced the apoptotic effects of tumor necrosis factor α-related apoptosis-inducing ligand (TRAIL) in prostate cancer cells (Kaddour-Djebbar et al., 2006). In the present study, we examined the advantages of combining a clinically approved Ca2+ channel blocker with a proteasome inhibitor. We determined whether alterations in [Ca2+]i affected prostate cancer cell survival and apoptosis induced by proteasome inhibitors. Diltiazem is known to decrease the basal [Ca2+]i, resulting in reduced Ca2+ supply to the ER, which creates ER stress (Ghosh et al., 1991; Paschen et al., 2003). We showed that diltiazem enhanced the apoptotic effect of the proteasome inhibitor lactacystin in LNCaP cells and sensitized a proteasome inhibitor-resistant prostate cancer cell line (DU145) to the apoptotic effects of lactacystin or bortezomib. In addition to clinical relevance, these results provide a model to investigate the role of ER calcium concentration in proteasome inhibitor-induced apoptosis.

Materials and Methods

Cell Culture and Experimental Design. Prostate cancer cell lines DU145 and LNCaP (American Type Culture Collection, Manassas, VA) were maintained in RPMI 1640 (HyClone, Logan, Utah) containing 10% fetal bovine serum (HyClone), 0.5% 10,000 U/ml penicillin-1 μg/ml streptomycin, and 0.1% 250 μg/ml amphotericin B (Fungizone). Cells were seeded and incubated for 20 to 24 h to reach a confluence of approximately 70%. They were then treated with fresh medium containing vehicle or diltiazem [cis-][2-(2-dimethylaminoethyl)-5-(4-methoxyphenyl)-3-oxo-6-thia-2-azabicyclo[5.4.0]undeca-7,9,11-trien-4-ylthethane; Calbiochem, San Diego, CA] for 15 min before addition of the proteasome inhibitors. Lactacystin [3S-hydroxy-2R-1-hydroxy-2-methylpropyl]R-4R-methyl-5-oxo-2- pyrrolidinolinecarboxylate-N-acetyl-L-cysteine; Cayman Chemical Company, Ann Arbor, MI] or bortezomib [iR3-3-methyl-1-[iR3-3-phenyl-2-[(pyrazin-2-ylcarbonyl)amino]propanoyl]amino] butylboronic acid; Velcade; Millenium, Cambridge, MA] for 24 h. In the drug combination experiments, 10 μM lactacystin or 40 μM bortezomib was used in DU145 cells or 2.5 μM lactacystin was used in LNCaP cells. For experiments to study the mitochondrial permeability transition pore, 30 μM cyclosporine [E]-14,17,26,32-tetrahydro-3,5-ethyl-8-1-hydroxy-2-methylhexyl-4-enyl-1,3,9,12,15,18,20,23,27-nonanamethyl-11,29-dipropyl-1,3,6,9,12,15,18,21,24,27,30-undecacazyclodotriacontan-2,4,7,9,11,14,16,19,22,25,28,31-undecanone; Calbiochem] was added 30 min before diltiazem. Increasing concentrations of diltiazem were used for dose-response experiments and a submaximal concentration, 400 nM, was chosen for further experiments. The DU145 cells were treated with thapsigargin [iS3,3aR,4S,65,67,68,69,7S-acetoxy-2,3,4a-decahydroazuleno[4,5-a][1,3,6,9,12,15,18,21,24,27,30-undecacazyclodotriacontan-2,4,7,9,11,14,16,19,22,25,28,31-undecanone; Calbiochem] with a concentration of 8 μM for 1 h to induce ER stress.

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action of caspases activated in response to apoptosis. This highly sensitive assay is specific for cell death due to apoptosis and does not measure necrotic cells. We (Shirley et al., 2005; Kaddour-Djebbar et al., 2006; Lakshmikanthan et al., 2006) and other laboratories (Cumnings et al., 2008; Greystoke et al., 2008) have successfully used the Apoptosense kit to specifically measure apoptosis. Upon completion of the experiments, cells were harvested, and total proteins were extracted as described above. Protein extracts were added to 96-well plates precoated with mouse monoclonal M30 antibody, horseradish peroxidase tracer solution was added to the wells, and the reactions were incubated for 4 h. Color was developed by adding tetramethyl benzidine solution and the optical density was measured at 450 nm on a SpectraMax 340 microplate reader (Molecular Devices, Sunnyvale, CA). Standard solution supplied by the manufacturer was used for generating standard curves.

To quantify apoptosis using flow cytometry, cells were stained with annexin V (Beckman Coulter, Miami, FL) and 4′-6-diamidino-2-phenylindole (Invitrogen) after harvesting by a brief trypsinization. After incubation with stains on ice in the dark for 30 min, binding buffer (400 μl) was added to each sample followed by flow cytometric analysis within 30 min. The cells were analyzed on a BD LSRII digital analyzer flow cytometer (Applied Biosystems, Foster City, CA).

Transfection of Prostate Cancer Cells. DU145 cells were transiently transfected with Bak SMART pool siRNA (Dharmacon, Inc., Chicago, IL) using DMRIE-C (Invitrogen) transfection reagent. The siRNA pool sense sequences used were CGCACAUCAACCGACGC-UAUUU, UAUGAGAUACUUCACCAAGAU, GACGGCAGCUCGC-GACGGCAGC-UUUU, and AAUUCACUGCCAGGGUUU. Transfected cells were incubated for 5 h, the medium was replaced with fresh binding buffer (400 μl) was added to each sample followed by flow cytometric analysis within 30 min. The cells were analyzed on a BD LSRII digital analyzer flow cytometer (Applied Biosystems, Foster City, CA).

Isolation of Cytosolic Fractions. Cells were washed and incubated in ice-cold buffer containing 20 mM HEPES-KOH, pH 7.2, 10 mM KCl, 1.6 mM MgCl2, 1 mM EDTA, 1 mM EGTA, and 250 mM sucrose and protease inhibitor cocktail (Boehringer Ingelheim Pharmaceuticals, Inc., Ridgefield, CT). Cells were homogenized (200 strokes) on ice with a Dounce homogenizer and were centrifuged at 1000g for 15 min to collect the nuclear fraction. The supernatant was then centrifuged at 16,000g for 20 min to obtain the mitochondrial fraction, which was washed and resuspended in mitochondrial extraction buffer (10 mM Tris-HCl, pH 7.8, and 0.1% Triton X-100). The supernatant was subjected to high-speed centrifugation (100,000g) for 60 min, and the supernatant was collected as the cytosolic fraction. Cytosolic fractions were analyzed by Western blotting for cytochrome c as described above. The membranes were stripped and repробed with β-actin antibody (as a loading control).

Statistical Analysis. All values are presented as means ± S.E.M.; an analysis of variance single factor test was performed to assess significant differences between the groups. Significant differences were defined at P < 0.05.

Results
Specific Alterations in Cytosolic Calcium Concentrations Induced Apoptosis in Prostate Cancer Cells. Although alterations in calcium levels have been correlated with cancer incidence, the importance of this association is not clear. We have demonstrated that increasing [Ca2+]c did not necessarily result in apoptosis. For example, treatment of prostate cancer cells with ionomycin, a strong calcium ionophore, did not enhance the apoptotic effect of the mitochondrial Na+/Ca2+ exchanger inhibitor, 7-chloro-5-(2-chlorophenyl)-1,5-dihydro-4,1-benzothiazepin-2(3H)-one (CGP-37157), whereas thapsigargin, a less efficacious [Ca2+]c-elevating agent, enhanced the apoptosis induced by CGP-37157 (Kaddour-Djebbar et al., 2006). To determine whether [Ca2+]c was responsible for apoptosis in prostate cancer cells, DU145 cells were treated with ionomycin and thapsigargin. Ionomycin significantly increased [Ca2+]c but did not induce apoptosis, in contrast to thapsigargin, which increased [Ca2+]c to a lesser extent but induced significant apoptosis (Fig. 1, A and B). In addition, decreasing extracellular calcium using EGTA, an extracellular calcium chelator, did not induce noticeable apoptosis up to 300 μM, whereas decreasing [Ca2+]c by 1,2-bis(2-aminophenoxy)ethane-N,N,N′,N′-tetraacetic acid tetrakis(acetoxymethyl) ester (BAPTA-AM), an intracellular calcium chelator, induced apoptosis significantly at a dose of only 30 μM (Fig. 1C). Together, these results suggest that changes in cytosolic calcium alone may not be sufficient to trigger apoptosis. Instead, it seems that alterations in calcium levels within organelles, such as the ER and mitochondria (e.g., ER calcium depletion or mitochondrial calcium overload), may be more important determinants of apoptosis. Therefore, the goal of this report was to further...
examine the effect of altering Ca\(^{2+}\) metabolism with a clinically approved drug on the response to the proteasome inhibitors, lactacystin and bortezomib.

**Preventing Calcium Influx Enhanced the Apoptotic Effect of the Proteasome Inhibitors Lactacystin and Bortezomib.** Calcium channel blockers are widely prescribed for hypertension and other cardiovascular ailments. Proteasome inhibitors are a new class of drugs approved for treatment of multiple myeloma and mantle cell lymphoma and are in clinical trials for other cancers. We tested the effect of a combination of calcium channel blockers and proteasome inhibitors on prostate cancer cells. Our prior results showed that the proteasome inhibitor, lactacystin, induced apoptosis in LNCaP cells in a dose-dependent manner (Shirley et al., 2005). In the present work, we found that diltiazem did not induce apoptosis in LNCaP cells even at 500 \(\mu\)M (Fig. 2A). However, in combination with a low dose of lactacystin (2.5 \(\mu\)M), diltiazem increased the apoptotic effect of lactacystin in a dose-dependent manner. Treatment with lactacystin alone resulted in a 2-fold increase in apoptosis, which was enhanced a further 3-fold when combined with diltiazem (Fig. 2A). DU145 cells, which are resistant to lactacystin, were sensitized when combined with diltiazem (Fig. 2B); whereas diltiazem alone did not induce noticeable apoptosis, cotreatment with 10 \(\mu\)M lactacystin induced approximately 10-fold greater apoptosis compared with individual treatments (Fig. 2B). The significant increase in apoptosis by the combination of lactacystin and diltiazem was confirmed using annexin V staining (flow cytometry) (Fig. 2C) and observation of cell numbers using phase-contrast microscopy (Fig. 2D), indicating the efficacy of the combined drugs. As with LNCaP cells, the SV40 large T antigen-immortalized prostate epithelial cell line p69 exhibited sensitivity to lactacystin alone (data not shown). However, normal microvascular endothelial cells derived from adult dermis demonstrated no apoptotic response to either lactacystin or diltiazem alone or to the combination (Fig. 2E).

**Apoptosis Induced by Diltiazem and Lactacystin Is Mediated through Mitochondria.** Previous studies have suggested that [Ca\(^{2+}\)], alterations can induce mitochondrial damage and apoptosis (Jambrina et al., 2003; Mattson and Chan, 2003; Sareen et al., 2007). Therefore, we examined mitochondrial involvement in the apoptotic effects of diltiazem and lactacystin. Because efflux of cytochrome c from the mitochondria is a known marker for the involvement of the mitochondria in apoptosis, cytosolic cytochrome c was monitored in DU145 cells. An increase in the level of cytochrome c was observed in the cytoplasm of cells treated with both diltiazem and lactacystin (Fig. 3A). In the cytoplasm, cytochrome c interacts with Apaf-1, resulting in the recruitment of procaspase-9, formation of the apoptosome complex, and activation of procaspase-9 by cleavage. Activation of caspase-9 in diltiazem- and lactacystin-treated cells was confirmed by the presence of the 37- and 35-kDa cleaved products of caspase-9 (Fig. 3B). In mitochondrial-mediated apoptosis, activated caspase-9 is responsible for the cleavage/activation of procaspase-3, as seen by the appearance of the 25-kDa cleaved fragment of caspase-3 (Fig. 3B), confirming the apoptosis induced by the drug combination.

**Diltiazem Decreased Cytosolic Calcium Levels and Altered Mitochondrial Morphology in Prostate Cancer Cells.** We and others have shown that alterations in cytosolic and mitochondrial calcium levels result in changes in mitochondrial morphology (Hom et al., 2007; Kaddour-Djebbar et
al., 2010), including mitochondrial fragmentation or fission. Our previous studies have also suggested a potential link between mitochondrial fission and apoptosis (Choudhary et al., 2011); however, these results also indicate that this link is not absolute. In the current work we find that inhibition of calcium influx by diltiazem decreased the basal levels of cytosolic calcium (Fig. 4A). To determine the effect of these changes in cytosolic calcium on mitochondria, DU145 cells...
were transfected with a Mito Red construct that is targeted for expression in mitochondria, and the number of cells undergoing mitochondrial fission was monitored. As expected in control untreated cells, the mitochondria were elongated filamentous structures (Fig. 4B), whereas diltiazem treatment led to mitochondria that were punctate, pinhead-like structures typical of fragmented mitochondria. Lactacystin by itself did not significantly alter the structure of mitochondria. Mitochondrial morphology in diltiazem- and lactacystin-treated cells was similar to that of diltiazem alone-treated cells, suggesting that the diltiazem-induced alterations in mitochondrial shape were not affected by lactacystin. The number of cells with fragmented mitochondria was determined for each treatment and is shown in Fig. 4B (bottom). With diltiazem treatment, 61% of cells contained fragmented mitochondria, whereas the combination of diltiazem and lactacystin induced mitochondrial fission in 58% of cells (not significantly different from diltiazem alone), again suggesting that diltiazem-induced mitochondrial changes were not affected by lactacystin. The percentage of cells with fragmented mitochondria was similar in both control and lactacystin-treated cells and was less than 25%.

**Involvement of Bak, Rather than the Mitochondrial Permeability Transition Pore, in Diltiazem/Lactacystin-Induced Apoptosis.** The mitochondrial permeability transition pore facilitates the efflux of cytochrome c from the mitochondria to the cytoplasm. The permeability transition pore is a multiprotein complex joining the inner and the outer mitochondrial membranes, and its function is regulated by different agents including calcium. Permeability transition pore formation involves hexokinase, the voltage-dependent anion channel, adenine nucleotide translocase, and cyclophilin D, a protein inhibited by cyclosporine. Based on our finding that the combination of diltiazem and lactacystin induced cytochrome c release (Fig. 3A), the importance of this pore in the apoptosis induced by the combined treatment was examined. Cyclosporine, a permeability transition pore inhibitor, surprisingly did not mitigate the apoptotic effect of combined diltiazem and lactacystin (Fig. 5). This result suggests that the permeability transition pore is not involved in the apoptotic response to the combination treatment, although mitochondria were clearly involved.

Bax and Bak are proapoptotic members of the Bcl2 family of proteins that have been shown to play critical roles in

**Fig. 4.** Diltiazem decreased cytosolic calcium levels and induced mitochondrial fission. A, cytosolic calcium was measured using Fura 2-AM fluorescence as described under Materials and Methods. During the measurement, diltiazem (400 μM) was added at the arrow. B, top, DU145 cells were transfected with the mitochondrial marker Mito Red and were treated with vehicle, 400 μM diltiazem (Dilt), 10 μM lactacystin (Lact), or the combination for 24 h. Mitochondria were visualized with a Carl Zeiss Axioskop fluorescent microscope. The bottom shows the percentage of cells exhibiting fragmented mitochondria, counted using the criteria described under Materials and Methods. The data are shown as the means ± S.E.M. of duplicate samples from three experiments. **III**, *P* < 0.001 versus the control or lactacystin alone.
mitochondria-mediated apoptosis. Bax and Bak are involved in mitochondrial membrane permeabilization and release of cytochrome c into the cytoplasm (Autret and Martin, 2009). Because DU145 cells do not express proapoptotic Bax (Kaddour-Djebbar et al., 2010), Bax cannot have a role in apoptosis induced by diltiazem and lactacystin in these cells. Therefore, we investigated the role of Bak in the induction of apoptosis by diltiazem and lactacystin. Bak is a mitochondrial protein capable of forming homo- or heterodimers but is inactive when sequestered by antiapoptotic proteins such as Bcl-xL. Immunoprecipitation and Western blotting for Bcl-xL showed that in control cells, Bak is sequestered by Bcl-xL (Fig. 6A, top and bottom). When treated with lactacystin or diltiazem alone, Bak continued to remain sequestered by the antiapoptotic Bcl-xL and thus was inactive as far as its proapoptotic function was concerned. When cells were treated with both drugs, the levels of Bcl-xL immunoprecipitated with Bak decreased, suggesting reduced binding between Bak and Bcl-xL. Bak released from Bcl-xL would then be available to exert its proapoptotic activity. To confirm the role of Bak in diltiazem- and lactacystin-induced apoptosis, cells were transfected with either scrambled or Bak-specific siRNA. Western analysis indicated that transfection of 50 nM Bak siRNA reduced the expression of Bak (Fig. 6B). Measurement of apoptosis in these cells showed that siRNA-mediated abrogation of Bak expression significantly decreased apoptosis in cells treated with the combination of diltiazem and lactacystin (Fig. 6C). These results demonstrate that the proapoptotic protein Bak is involved in the induction of apoptosis by combined diltiazem and lactacystin. As suggested earlier (Scorrano and Korsmeyer, 2003), in the absence of a role for the permeability transition pore (Fig. 5), Bak may mediate the formation of a mitochondrial pore that is responsible for the release of cytochrome c to the cytoplasm, leading to apoptosis involving mitochondria.

Fig. 5. The mitochondrial permeability transition pore was not involved in lactacystin/diltiazem-induced apoptosis. DU145 cells were pretreated with vehicle or cyclosporine (30 μM) for 30 min and then were treated for 24 h with or without lactacystin (30 μM) and/or diltiazem (400 μM). Apoptosis was measured using the M30-Apoptosense kit and is expressed as M30 antigen units per microgram of protein compared with control. Values represent the means ± S.E.M. of three experiments. ***, $P < 0.001$ versus the control or either agent alone; NS, not significant.

Fig. 6. A proapoptotic member of the Bcl2 family, Bak, mediated the apoptotic effects of lactacystin and diltiazem (DZ). A, DU145 cells were treated as in the legend to Fig. 5. Bak was immunoprecipitated (IP), and immunoprecipitates were processed for Western analysis for the levels of Bak and Bcl-xL. The top shows a representative experiment. In the bottom, cumulative data for multiple experiments quantified using an Alpha Innotech 8900 are shown, and the values are expressed as the ratio of Bcl-xL to Bak. Values represent the means ± S.E.M. of three experiments. ***, $P < 0.001$ versus the control or either agent alone. IB, immunoblot. B, to inhibit the expression of Bak, cells were transfected with 50 nM Bak-specific siRNA or an equivalent amount of scrambled siRNA. Protein extracts were analyzed by Western blotting for the presence of Bak. β-Actin was used as a loading control. C, DU145 cells were transfected with 50 nM scrambled or Bak-specific siRNA and treated with vehicle or diltiazem (400 μM) in the absence or presence of lactacystin (10 μM) as indicated. Apoptosis was measured using the M30-Apoptosense kit and is expressed as M30 antigen units per microgram of protein versus the control. The values represent the means ± S.E.M. of at least three experiments. ***, $P < 0.001$ as indicated.
Bik, Rather than Bid, May Be the Upstream Activator of Bak in Diltiazem/Lactacystin-Induced Apoptosis. Next, the mechanism by which Bak is activated was investigated. One mechanism by which Bak is activated is by the conversion (and activation) of pro-caspase-8 to caspase-8, which then in turn cleaves Bid to truncated Bid. Truncated Bid is transported into the mitochondria to result in the activation of pro-apoptotic members of the Bcl2 pathway, such as Bak. Western blot analysis showed that treatment with diltiazem and lactacystin individually or in combination neither activated caspase-8 nor induced the formation of truncated Bid (Fig. 7B). Cells treated with TRAIL as a positive control exhibited both activation of caspase-8 and truncation of Bid (Kaddour-Djebbar et al., 2006; Lakshmikanthan et al., 2006). To determine whether other members of the Bcl2 family were affected by diltiazem and lactacystin, the expression of the pro-apoptotic proteins Bak, Bik, Bok, and PUMA was examined. No obvious differences in the levels of Bak and Bok were noted (Fig. 7B). The lack of difference in the levels of Bak indicates that the pro-apoptotic effect of Bak does not require increased expression of Bak; instead Bak is activated when it is released from Bcl-xl in response to treatment (Fig. 6A). Western blots showed up-regulation of Bik by lactacystin alone, and this effect was significantly enhanced when diltiazem was added (Fig. 7B). On the other hand, pro-apoptotic PUMA is down-regulated by diltiazem alone and in combination with lactacystin (Fig. 7). The levels of Bcl-xl were not altered under these treatment conditions (Fig. 7B), in agreement with Nikrad et al. (2005).

Endoplasmic Reticulum Stress May Be Involved in Lactacystin/Diltiazem-Induced Apoptosis. There is growing evidence that ER stress, as well as ER-mitochondrial cross-talk, is involved in apoptotic events (Li et al., 2006; Puthalakath et al., 2007; Madeo and Kroemer, 2009). Thapsigargin, an ER Ca\(^{2+}\) pool-depleting agent has been shown to induce ER stress (Fribley et al., 2004), and proteasome inhibitors have also been suggested to deplete the ER Ca\(^{2+}\) pool (Landowski et al., 2005). In the current research, to determine whether ER stress is involved in apoptosis under our treatment conditions, the levels of the ER stress marker GRP78 were examined. Western analysis showed that GRP78 levels significantly increased in cells treated with both diltiazem and lactacystin (Fig. 8), whereas individually diltiazem and lactacystin did not affect GRP78.

Diltiazem Enhanced the Apoptotic Effect of the Proteasome Inhibitor Bortezomib. Bortezomib is a proteasome inhibitor that has been approved for the treatment of multiple myeloma and mantle cell lymphoma and is in phase I and II clinical trials for the treatment of other cancers including prostate cancer. Therefore, it was of interest to investigate whether diltiazem could also enhance the apoptotic effect of bortezomib. At doses that did not induce apoptosis with either agent alone, the combination of diltiazem and bortezomib enhanced apoptosis (Fig. 9). These results have a potentially high clinical relevance, in that these two drugs are approved for different ailments that often occur simultaneously (Yoshinaga et al., 2006) and therefore could be indicated in prostate cancer patients with hypertension.

Discussion

Calcium is a second messenger that plays an important role in cell survival or in cell death, depending on the extent of the alteration in calcium metabolism and the organelle/compartment that is targeted (Mussche et al., 2000). Alterations in calcium mobilization and metabolism in disease conditions such as cardiovascular and neurological ailments are well established. Therefore, therapies based on manipulation of calcium have been used to treat some of these conditions.
therapy. Therefore, the therapeutic advantages of combining them, and combining drugs may be a necessary step in cancer resisting various chemotherapeutic approaches to destroy side effects could presumably be reduced by using lower amounts of bortezomib. However, it was concluded that combination treatment significantly reduced the interaction between Bak and the antiapoptotic protein Bcl-xl (Fig. 6). It is well known that binding of Bak by antiapoptotic proteins such as Bcl-xL or Bcl2 prevents Bak activation. Thus, although treatment with lactacystin and diltiazem did not alter the levels of Bak, it certainly enabled the activation of Bak. Furthermore, the involvement of Bak in apoptosis induced by lactacystin and diltiazem was confirmed by knocking down Bak levels using specific siRNA (Fig. 6).

There is growing evidence for the involvement of ER stress, as well as ER-mitochondria crosstalk, in apoptotic events (Li et al., 2006; Puthalakath et al., 2005; Bakhshi et al., 2008; Madeo and Kroemer, 2009). Proteasome inhibitors deplete calcium levels in the ER (Landowski et al., 2005), resulting in ER stress, which is known to lead to apoptosis (Pribyle et al., 2004). Here, we show that diltiazem treatment decreased intracellular calcium levels (Fig. 4A), a condition that would decrease the calcium supply to the ER, resulting in ER stress. Indeed, thapsigargin, an agent that depletes ER calcium has been shown to induce ER stress (Strayer et al., 1999). Under our treatment conditions, neither lactacystin nor diltiazem up-regulated the ER stress marker GRP78 when added separately, but GRP78 levels were significantly up-regulated with the combination treatment (Fig. 8). The resultant ER stress could be another explanation for the observed apoptotic synergy, as well as for the demonstrated apoptosis induced by thapsigargin and BAPTA-AM in our (Fig. 1, A and C) and other laboratories (Strayer et al., 1999; Soboloff and Berger, 2002).

These results have potential clinical relevance, in that these two drugs are approved for different ailments that often occur simultaneously. Indeed, the incidence of cardiovascular diseases, for which diltiazem is a common therapy,
is often high in the same population predisposed to or diagnosed with prostate cancer. This study demonstrates the advantage of using the clinically approved calcium channel blocker diltiazem in combination with proteasome inhibitors, especially on DU145 prostate cancer cells that are otherwise resistant to the apoptotic effects of these proteasome inhibitors, which are under consideration as novel cancer therapy agents. Moreover, the combination was not harmful in normal human cells (Fig. 2E), such that neither lactacystin nor diltiazem alone nor the combination induced significant apoptosis in human microvascular endothelial cells derived from adult dermis (HMVECad). This result suggests that the combination treatment may have fewer “side effects,” i.e., effects on other cell types that are not cancerous. Thus, in this era of imminently personalized medicine, our results suggest the possible benefit of using calcium channel blockers as new tools for cancer therapy. In particular, the approach of combining calcium channel blockers with other agents such as proteasome inhibitors might be particularly suitable for at least some patients with prostate cancer who also need calcium channel blockers for the treatment of their cardiovascular disease.

Authorship Contributions
Participated in research design: Kaddour-Djebbar, Choudhary, Shirley, Bollag, and Kumar.
Conducted experiments: Kaddour-Djebbar, Choudhary, Lakshmi-kanthan, Shirley, El Gaish, Al-Husein, Zhong, and Davis.
Contributed new reagents or analytic tools: Dong.
Performed data analysis: Kaddour-Djebbar, Al-Shabrawey, and Bollag.
Wrote or contributed to the writing of the manuscript: Kaddour-Djebbar, Bollag, and Kumar.

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