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

Ismail Kaddour-Djebbar, Vivek Choudhary, Vijayabaskar Lakshmikanthan,1 Robert Shirley, Manal El Gaish, Mohamed Al-Shabrawey, Belal Al-Husein, Roger Zhong, Michael Davis, Zheng Dong, Wendy B. Bollag, and M. Vijay Kumar2

Charlie Norwood VA Medical Center, Augusta, Georgia (I.K.-D., V.C., V.L., R.S., M.E.G., B.A.-H., R.Z., M.D., Z.D., W.B.B., M.V.K.); Department of Surgery (Urology), (I.K.-D., V.C., V.L., R.S., M.V.K.); Departments of Physiology (I.K.-D., V.C., W.B.B.), Oral Biology (M.A.-S., W.B.B.), and Cell Biology and Anatomy, Georgia Health Sciences University, Augusta, Georgia (Z.D., W.B.B.); and College of Pharmacy, University of Georgia, Augusta, Georgia (B.A.-H.)

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ABSTRACT

Diltiazem is a calcium channel blocker used to treat cardiovascular ailments. In addition, reports suggest that diltiazem induces cell death, which could make it a drug of choice for the treatment of cancer associated with hypertension. The goal of this research was to determine whether diltiazem is capable of inducing apoptosis in prostate cancer cells, either alone or in combination with the proteasome inhibitors, lactacystin and bortezomib (Velcade). Bortezomib is approved for the treatment of multiple myeloma; unfortunately, it has side effects that limit its utility. Presumably these side effects could be decreased by reducing its dose in combination with another drug. We have previously shown that lactacystin induces apoptosis in LNCaP cells; here, we show that this effect was enhanced by diltiazem. Furthermore, in proteasome inhibitor-resistant DU145 cells, diltiazem alone did not induce apoptosis but decreased cytosolic calcium levels and induced mitochondrial fission; likewise, lactacystin did not induce apoptosis but up-regulated the proapoptotic protein Bik. However, increasing concentrations of diltiazem in combination with lactacystin or bortezomib induced apoptosis in a dose-dependent and synergistic manner. The combination of diltiazem and lactacystin also up-regulated the levels of Bik and released Bak from Bcl-XL, indicating the involvement of the Bcl2 family pathway in this apoptosis. In addition, the drug combination up-regulated GRP78, suggesting also the involvement of endoplasmic reticulum stress in the apoptotic response. Thus, our results demonstrate a potential therapeutic advantage of combining a frequently used calcium channel blocker with proteasome inhibitors in the treatment of prostate cancer.

Introduction

The concentration of cytosolic $\text{Ca}^{2+}$ ($[\text{Ca}^{2+}]_c$) is maintained at a lower level inside the cell compared with that in the extracellular space. This gradient of $\text{Ca}^{2+}$ 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, removing and buffering excess calcium in the cytosol (Ferreiro et al., 2008). Finely tuned changes in $[\text{Ca}^{2+}]_c$ modulate a variety of intracellular functions ranging from muscle contraction to secretion. Ionic calcium also plays a major role in the complex interplay that leads to cell death (Tagliarino et al., 2001; Roderick and Cook, 2008). Indeed, $\text{Ca}^{2+}$ signals in the nucleus, ER, and mitochondria have been shown to affect checkpoints of the cell death process, thus modulating the sensitivity of cells to various challenges (Gill et al., 1996). $[\text{Ca}^{2+}]_c$ can also be modulated by calcium influx through voltage-dependent $\text{Ca}^{2+}$ channels.

Many therapies for cardiological diseases are based on the manipulation of voltage-dependent $\text{Ca}^{2+}$ channels. Thus, calcium channel blockers are used by millions of patients to treat hypertension, angina, and heart rhythm abnormalities. These drugs prevent calcium influx into cells and maintain $[\text{Ca}^{2+}]_c$ at low levels, thereby allowing relaxation of vascular...
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 proteasome 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/trials). Similar to other proteasome inhibitors, bortezomib acts by blocking the 26S proteasome. In normal cells, the proteasome regulates protein expression and function by degrading ubiquitylated proteins and destroying abnormal or misfolded proteins. Proteasome 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-κB, a protein that is constitutively activated in some cancer cells (Goldberg and Rock, 2002). Another possible mechanism by which proteasome 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, proteasome inhibitors induce accumulation of misfolded proteins to induce the unfolded protein response (Meister 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.

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-[(2R)-2-methylaminoethyl]-5-(4-methoxyphenyl)-3-oxo-6-thia-2-azabicyclo[5.4.0]undecane-7,9,11-trien-4-yllethanolate; Calbiochem, San Diego, CA] for 15 min before addition of the proteasome inhibitors. Lactacystin [3S-hydroxy-2R-(1-hydroxy-2-methylpropyl)-4R-5-(4-methoxyphenyl)-3-oxo-2-pyrrolidinonecarboxylate-N-acetyl-L-cysteine; Cayman Chemical Company, Ann Arbor, MI] or bortezomib [(1R)-3-methyl-1-[(1S)-3-phenyl-2-[(pyrazin-2-ylcarbonyl)aminol]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-tetrabutyl-5-ethyl-8-(1-hydroxy-2-methylhexyl)-4-enyl], 1,3,9,12,15,18,20,23,27-nonanemethyl-11,29-dipropyl-1,3,6,9,12,15,18,21,24,27,30-undecaazacyclodotriacontan-2,4,7,10,13,16,19,22,25,28,31-undecacone; Calbiochem) was added 30 min before diltiazem. Increasing concentrations of diltiazem were used for dose-response experiments and a submaximal concentration, 400 μM, was chosen for further experiments.

The DU145 cells were treated with thapsigargin [(2S,3αR,4S,6S,7S,9βS)-3,3-di(hydroxyacetoyl)-4-(butyryloxy)-1,3,6,9-tetramethyl-8-[(2S,2R)-2-methylbutyl-2-enoyl]oxy]-2-oxo-2,3,4,5,6,7,8,9,10-decaphenylazulen-4-(5H)-furan-7-y]octanolate; Calbiochem) or ionomycin [(4R,6S,8S,10Z,12R,14R,16F,18R,19R,20S,21S)-19-21-dihydroxy-22-[(2S,2'R,5R,5'S)-5'-((1R)-1-hydroxyethyl)-2,2'-dimethyloctahydro-2,2'-bifuran-5-yl]-4,6,8,12,14,18,20-heptamethan-11-oxido-9-oxodocosan-10,12-dienoate; Sigma-Aldrich, St. Louis, MO) for 24 h. The cell line p69, an SV40 large T antigen-transformed prostate epithelial cell line with poor tumorigenicity (Bae et al., 1994), was a kind gift from Dr. Lelund Chung (Cedars Sinai Medical Center, Los Angeles, CA). These cells were maintained in T medium (In vitrogen, Carlsbad, CA) containing 5% fetal bovine serum (HyClone) and 1% 10,000 U/ml penicillin-1 μg/ml streptomycin. The human microvascular endothelial cells from adult dermis (HMVEC-d; In vitrogen) were maintained in EBM-2 medium (Lonza Walkersville, Inc., Walkersville, MD) containing endothelial, epidermal, and fibroblast growth factors, heparin, ascorbic acid, and gentamicin-ampicillin (Cambrex, East Rutherford, NJ).

Protein Extraction and Western Blotting. At the end of treatment, floating and attached cells were harvested, washed with phosphate-buffered saline (PBS) and resuspended in lysis buffer (1× PBS, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 1 mM EDTA, 0.5 μg/ml leupeptin, 1 μg/ml pepstatin, 1 μg/ml phenylmethylsulfonyl fluoride, and 1 μg/ml aprotinin). Cells were incubated on ice for 30 min and centrifuged at 10,000g at 4°C for 10 min. The supernatants were collected, and the protein concentration was estimated using Bio-Rad protein reagent (Bio-Rad Laboratories, Hercules, CA). Cell extracts were used in the M30 apoptosis assay (as described below) or separated on Tris-glycine gels and probed for caspases-3, -8, and -9 (Clontech, Mountain View, CA), Bak, Bik, Bok, PUMA, Bel-XL (Cell Signaling Technology, Danvers, MA), and GRP78 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). Positive signals were developed using ECL Plus (GE Healthcare, Fairfield, CT), and the signal was captured on a digital imager (8900; Alpha Innotech, San Leandro, CA) and/or exposed to ECL Hyperfilm (GE Healthcare). The blots were stripped and reprobed with β-actin or glyceraldehyde-3-phosphate dehydrogenase antibody for use as loading controls.

Measurement of Apoptosis. Apoptosis was measured using the Peviva M30-Apoptosense kit (Diapharma, West Chester, OH). This enzyme-linked immunosorbent assay kit uses a specific antibody recognizing a cytokeratin 18 neoepitope that is generated by the
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 (Cummings 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 LSR II 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 transiently transfected with Bak SMART pool siRNA (Dharmacon, Inc., Chicago, IL) using DMRIE-C (Invitrogen) transfection reagent. The siRNA pool sense sequences used were CGACAUCAACCGAGC-CAUAUUU, UAUGAUCAUCACAAAGAUU, GACGGCAGCUCGC-siRNA pool sense sequences used were CGACAUCAACCGAGC-CAUAUUU, UAUGAUCAUCACAAAGAUU, GACGGCAGCUCGC-CAUAUUU, and AAUACGACUCCCAAGGUU. Transfected cells were incubated for 4 h, the medium was replaced with fresh medium, and the cells were incubated for an additional 36 h before harvesting and seeding in dishes for treatment. For the introduction of Mito Red into cells for mitochondrial morphological experiments, DU145 cells were resuspended in electroporation buffer (120 mM KCl, 0.15 mM CaCl2, 10 mM K2HPO4/KH2PO4, 25 mM HEPES, 2 mM EGTA, 5 mM MgCl2, 50 mM glutathione, and 2 mM ATP at pH 7.6) at a density of 106 cells/ml. The construct pDeRed2-Mito (Mito Red), a red fluorescent label targeted for expression in mitochondria (Clontech), was added to 4 million cells in 0.4 ml of buffer. The mixture of cells and Mito Red DNA was incubated for 5 min at room temperature, transferred to 4-mm cuvettes (BTX Inc., San Diego, CA) and subjected to electroporation pulses (3 pulses of 300 V for a period of 5 ms each) using the Electro Square Porator ECM 830 (BTX Inc.). The cells were incubated with the DNA on ice for another 5 min before plating in dishes and incubation for 24 to 48 h. Stably transfected cells were selected from single colonies and maintained in the same medium containing G418 antibiotic (Invitrogen).

Measurement of Fragmented Mitochondria. To measure fragmented mitochondria, Mito Red stably transfected DU145 cells were seeded on coverslips, treated as described above, and examined on a fluorescein microscope (Zeiss Axioskop; Carl Zeiss Inc., Thornwood, NY). Cells in which less than 50% of the mitochondria were elongated were scored as positive for fragmented mitochondria. At least five fields (with more than 100 cells) per slide were counted in four to five experiments.

Immunoprecipitation of Bak. The DU145 cells were treated as above and harvested in radioligand immunoprecipitation assay buffer (Millipore Corporation, Billerica, MA). Equal amounts of protein lysates were incubated overnight with Bak antibody (Santa Cruz Biotechnology, Inc.) and protein-A/G agarose beads. The beads were washed three times, and equal amounts of proteins were separated by SDS-polyacrylamide gel electrophoresis and processed for Western analysis. The membranes were probed with Bcl-xL antibody (Cell Signaling Technology) and reprobed with a different Bak antibody (Cell Signaling Technology).

Measurement of Cytosolic Calcium. DU145 cells were cultured on glass cover slips and incubated in the dark with PBS containing 1% bovine serum albumin and 4 μM Pura 2-AM (ace-toxymethyl 2-[5-[bis[acetoxyethylthio-oxo-methyl]methyl]lamin]-4-[2-[5-[bis[acetoxyethylthio-oxo-methyl]methyl]lamin]-5-methyl-phenoxyl ethoxy]benzofuran-2-yl]oxazole-5-carboxylate; Invitrogen) for 40 min. Cells were washed with PBS and incubated in Hanks’ balanced salt solution for 10 min to allow cleavage of the AM ester. For measurement of calcium, glass coverslips were assembled on a cell cultivation system and placed on a temperature-controlled (37°C) Zeiss fluorescent microscope stage connected to a PTL ImageMaster monochromator (Photon Technology International, Birmingham, NJ). The intensity of fluorescence was measured at 540/380 nm excitation and 510 nm emission wavelengths to measure cytosolic calcium before and after treatments.

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 again 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 reprobed with β-actin antibody (as a loading control).

Statistical Analysis. All values are presented as ± 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 [Ca 2+]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, using 1,2-bis(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid tetrakis(acetoxyethyl 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\textsuperscript{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 μM (Fig. 2A). However, in combination with a low dose of lactacystin (2.5 μM), 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 μM 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\textsuperscript{2+}], alterations can induce mitochondrial damage and apoptosis (Jambriya 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 pro-caspase-9, formation of the apoptosome complex, and activation of pro-caspase-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 pro-caspase-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

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**Fig. 2.** The combination of diltiazem and lactacystin induced significant apoptosis of prostate cancer cells. A, LNCaP prostate cancer cells were treated with increasing concentrations (100–500 μM) of diltiazem (DZ) in the presence and absence of 2.5 μM lactacystin (Lact) for 24 h. Apoptosis was measured using the M30-Apoptosense kit. The values represent the means ± S.E.M. of three experiments. **B**, DU145 cells were treated as in A, except that a higher concentration of lactacystin (10 μM) was used. C, DU145 cells were treated with or without lactacystin (10 μM), diltiazem (400 μM), or the combination for 24 h, harvested, and stained with annexin V and 4′,6-diamidino-2-phenylindole as described under Materials and Methods. Cells were then analyzed by flow cytometry. Representative data are shown for control (C), lactacystin (L), diltiazem (D), or the combination (DL) in the top. The bottom shows cumulative values from multiple experiments for the percentage of apoptotic cells from flow cytometric analyses. Values represent the means ± S.E.M. from three separate experiments. **D**, shown are representative phase-contrast microscopic images (20×), illustrating the cell confluence observed after a 24-h treatment with vehicle (control, C), 10 μM lactacystin (L), 400 μM diltiazem (D), or the combination (DL). The inset illustrates the number of cells at time 0 (before treatment). E, HMVECad normal human microvascular endothelial cells derived from adult dermis were treated with 10 μM lactacystin, 400 μM diltiazem, and the combination, and apoptosis was monitored using the M30-Apoptosense kit. The values represent the means ± S.E.M. of three experiments performed in at least duplicate.
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 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.

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Calcium Involvement in Prostate Cancer Cell Apoptosis

Fig. 3. Lactacystin in combination with diltiazem induced cytochrome c release and activated caspases-9 and -3. A, DU145 cells were treated with lactacystin (Lact, 10 μM) and/or diltiazem (DZ, 400 μM) for 24 h. Cytosolic fractions were processed for Western analysis for cytochrome c. B, DU145 cells were treated as above, and cell extracts were processed for Western analysis of caspase-9 (Casp 9) and caspase-3 (Casp 3) activation by cleavage. β-Actin was used as a loading control. Shown under each blot are the densitometric values of the relevant bands (normalized to the loading control) relative to the average (normalized) control value. The experiment was repeated with similar results.

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. *** 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. 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.

Fig. 6A. 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 presence of Bak. The top shows a representative experiment. In the bottom, cumulative data for multiple experiments quantified using an Alpha Innotech S900 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. B, DU145 cells were transfected with 50 nM scrambled or Bak-specific siRNA and treated with vehicle or diltiazem (400 μM) or lactacystin (10 μM) as indicated. 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 at least three experiments. ***, P < 0.001 versus the control or either agent alone; NS, not significant.
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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 procaspase-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 proapoptotic 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. 7A). 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 proapoptotic 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 proapoptotic 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, proapoptotic 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., 2005). 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.

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 neurologological 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 tin, as are many cancer cells, which seem to be adept at therapeutic agents. In addition, DU145 prostate cancer cells doses of proteasome inhibitors, in combination with other side effects could presumably be reduced by using lower side effects. The hypothesis for the present work is that these promote cancer cell death, proteasome inhibitors often show proteins and allowing them to overwhelm antiapoptotic pro-

are involved in cell death, thereby extending the life of these Proteasome inhibitors block the degradation of proteins that combination with other agents is warranted for the treat-

at tolerated doses of bortezomib. However, it was concluded that further investigation of this proteasome inhibitor in combination with other agents is warranted for the treat-

ment of advanced prostate cancer (Papandreou et al., 2004). Proteasome inhibitors block the degradation of proteins that are involved in cell death, thereby extending the life of these proteins and allowing them to overwhelm antiapoptotic proteins, consequently promoting apoptosis (Papandreou and Logothetis, 2004). Unfortunately, at the doses required to promote cancer cell death, proteasome inhibitors often show side effects. The hypothesis for the present work is that these side effects could presumably be reduced by using lower doses of proteasome inhibitors, in combination with other therapeutic agents. In addition, DU145 prostate cancer cells are relatively resistant to apoptotic agents such as lactacystin, as are many cancer cells, which seem to be adept at resisting various chemotherapeutic approaches to destroy them, and combining drugs may be a necessary step in cancer therapy. Therefore, the therapeutic advantages of combining the proteasome inhibitor, lactacystin, and a calcium channel blocker, diltiazem, were examined. These results showed that altering the levels of intracellular calcium using a variety of methods resulted in apoptosis of prostate cancer cells. The combination of lactacystin and diltiazem induced apoptosis in both androgen-responsive and androgen-indepen-
dent prostate cancer cells (Fig. 2). Note that, although the androgen-independent prostate cancer cells (DU145) showed resistance to lactacystin, diltiazem was able to sensitize these cells to the proteasome inhibitor, leading to apoptosis (Fig. 2B). Furthermore, increased cytochrome c efflux from the mitochondria and activation of postmitochondrial caspasases such as caspase-9 and caspase-3 (Fig. 3) suggested the involvement of mitochondria in the observed apoptosis. Similar results have been reported by others in that bortezomib sensitizes cancer cells to the apoptotic action of TRAIL through the intrinsic pathway (Nikrad et al., 2005). Likewise, in myeloma cells, the combination of bortezomib and verapamil, another calcium channel blocker, decreases cell viability and immunoglobulin secretion more effectively than either agent alone (Meister et al., 2010).

To understand the mechanisms leading to apoptosis, the possible roles of the proapoptotic members of the Bcl2 family were investigated. Because the DU145 prostate cancer cells do not express the proapoptotic protein Bax, the role of an-
other proapoptotic protein, Bak, in the apoptosis induced by lactacystin and diltiazem was determined. Western analysis showed that treatment with lactacystin and diltiazem did not alter the levels of Bak (Fig. 7). However, the combination treatment significantly reduced the interaction between Bak and the antia apoptotic protein Bcl-xl (Fig. 6). It is well known that binding of Bak by antia apoptotic 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 cross-talk, in apoptotic events (Li et al., 2006; Puthalakath et al., 2007; 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 (Pribley 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 cardio-
vascular diseases, for which diltiazem is a common therapy,
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Stebbins DS, Hook JB, Thomas AP, and White MK (1999) Cellular activation by Ca2+ 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 imminent 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, Choudary, Shirley, Bollag, and Kumar.

**Conducted experiments:** Kaddour-Djebbar, Choudary, Lakshmikanth, 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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Address correspondence to: Dr. Ismail Kaddour-Djebbar, Charlie Norwood VA Medical Center, One Freedom Way, Augusta, GA 30904. E-mail: ismail.kaddour-djebbar@va.gov