

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

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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 or Velcade. Velcade 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 upregulated the proapoptotic protein Bik. However, increasing concentrations of diltiazem in combination with lactacystin, or Velcade, induced apoptosis in a dose-dependent and synergistic manner. The combination of diltiazem and lactacystin also upregulated 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 upregulated GRP78, suggesting also the involvement of ER 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 Ca^{2+} ($[\text{Ca}^{2+}]_c$) is maintained at a lower level inside the cell compared to 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 ER and mitochondria. The endoplasmic reticulum (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, 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 Ca^{2+} channels.

Many therapies for cardiologic diseases are based on the manipulation of voltage-dependent 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, while 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

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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 utilize 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 U.S. for treating relapsed multiple myeloma and mantle cell lymphoma. Velcade 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 proteasome inhibitors, Velcade 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. 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 NF- κ B, 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 pro-apoptotic proteins, thereby allowing these proteins to overwhelm anti-apoptotic proteins (Lomonosova et al., 2009). Furthermore, proteasome inhibitors induce accumulation of misfolded proteins to induce the unfolded protein response (UPR) [e.g., (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.

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We have previously shown that blocking the mitochondrial $\text{Na}^{2+}/\text{Ca}^{2+}$ exchanger significantly enhanced the apoptotic effects of TNF 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 Ca^{2+} channel blocker with a proteasome inhibitor. We determined whether alterations in $[\text{Ca}^{2+}]_c$ affected prostate cancer cell survival and apoptosis induced by proteasome inhibitors. Diltiazem is known to decrease the basal $[\text{Ca}^{2+}]_c$, resulting in reduced Ca^{2+} 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 prostate cancer cell line that is resistant to the apoptotic effects of proteasome inhibitors, DU145, to lactacystin or Velcade. In addition to clinical relevance, these results provide a model to investigate the role of ER calcium concentration in proteasome inhibitor-induced apoptosis.

Methods

Cell culture and experimental design

Prostate cancer cell lines, DU145 and LNCaP (American Type Cell Culture, Manassas, VA) were maintained in RPMI 1640 (Hyclone, Logan, Utah) containing 10% fetal bovine serum (Hyclone), 0.5% 10,000 U/mL-μg/mL penicillin-streptomycin, and 0.1% 250 μg/mL fungizone. Cells were seeded and incubated for 20-24 h to reach a confluency of about 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-yl]ethanoate; Calbiochem, San Diego, CA] for 15 min prior to addition of the proteasome inhibitors, lactacystin [3*S*-hydroxy-2*R*-(1-hydroxy-2-methylpropyl)-4*R*-methyl-5-oxo-2-pyrrolidinecarboxylate-*N*-acetyl-L-cysteine; Cayman Chemical Company, Ann Arbor, MI] or Velcade [[(1*R*)-3-methyl-1-({(2*S*)-3-phenyl-2-[(pyrazin-2-ylcarbonyl)amino]propanoyl}amino)butyl]boronic acid; also known as Bortezomib, Millenium, Cambridge, MA] for 24 h. In the drug combination experiments, 10 μM lactacystin or 40 μM Velcade were used in DU145 cells or 2.5 μM lactacystin in LNCaP cells. For experiments to study the mitochondrial permeability transition pore, 30 μM cyclosporin A [(*E*)-14,17,26,32-tetrabutyl-5-ethyl-8-(1-hydroxy-2-methylhex-4-enyl) 1,3,9,12,15,18,20,23,27-nonamethyl-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-undecaone; Calbiochem, San Diego, CA] 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 [(3*S*,3*aR*,4*S*,6*S*,6*aR*,7*S*,8*S*,9*bS*)-6-(acetyloxy)-4-(butyryloxy)-3,3*a*-dihydroxy-3,6,9-trimethyl-8-{[(2*Z*)-2-methylbut-2-enoyl]oxy}-2-oxo-2,3,3*a*,4,5,6,6*a*,7,8,9*b*-

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decahydroazuleno[4,5-*b*]furan-7-yl octanoate; Calbiochem] or ionomycin [(4*R*, 6*S*, 8*S*, 10*Z*, 12*R*, 14*R*, 16*E*, 18*R*, 19*R*, 20*S*, 21*S*)-19, 21- dihydroxy-22- {(2*S*, 2'*R*, 5*S*, 5'*S*)-5'-[(1*R*)-1-hydroxyethyl]-2, 5'-dimethyloctahydro-2, 2'-bifuran-5-yl}-4, 6, 8, 12, 14, 18, 20-heptamethyl-11-oxido- 9-oxodocosa-10, 16-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 (Cedar-Sinai Medical Center, Los Angeles, CA). These cells were maintained in T-medium (Invitrogen, Carlsbad, CA) containing 5% fetal bovine serum (Hyclone) and 1% 10,000 U/mL-μg/mL penicillin–streptomycin. The human microvascular endothelial cells from adult dermis (HMVECad, Life Technologies, Grand Island, NY) were maintained in EBM-2 medium (Lonza, Walkersville, MD) containing endothelial, epidermal and fibroblast growth factors, heparin, ascorbic acid and gentamycin-amphotericin (Cambex, 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 (1X PBS, 1% Triton X100, 0.5% sodium deoxycholate, 0.1% SDS, 1 mM EDTA, 0.5 μg/μl leupeptin, 1 μg/μl pepstatin, 1 μg/μl phenylmethyl sulfonyl 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 (BD Biosciences Clontech, Palo Alto, CA), Bak, Bik, Bok, PUMA, Bcl-xL (Cell Signaling Technology, Danvers, MA) and GRP78

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(Santa Cruz Biotechnology, Santa Cruz, CA). Positive signals were developed using ECL-Plus (Amersham, Piscataway, NJ) and the signal was captured on a digital imager (Alpha Innotech 8900, San Leandro, CA) and/or exposed to ECL Hyperfilm (Amersham). The blots were stripped and re-probed with β -actin or GAPDH antibody for use as loading controls.

Measurement of apoptosis

Apoptosis was measured using the Peviva M30 Apoptosense kit (Diapharma, West Chester, OH). This ELISA 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 utilized 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 pre-coated 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 Spectra MAX 340 microplate reader (Molecular Devices Corporation, Sunnyvale, CA). Standard solution supplied by the manufacturer was utilized 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 (DAPI; Molecular Probes, Carlsbad, CA) after harvesting by a brief trypsinization. After incubation with stains on ice in the dark for 30 minutes, binding buffer (400 μ L) was added to each sample followed by flow cytometric

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analysis within 30 minutes. The cells were analyzed on a Becton Dickinson LSRII digital analyzer flow cytometer (Applied Biosystems, Carlsbad, CA).

Transfection of prostate cancer cells

DU145 cells were transiently transfected with Bak SMART pool siRNA (Dharmacon, Inc. Chicago, IL) using DMRIE-C (Invitrogen, Carlsbad, CA) transfection reagent. The siRNA pool sense sequences used were CGACAUCAACCGACGCUAUUU, UAUGAGUACUUCACCAAGAUU, GACGGCAGCUCGCCAUCAUUU and AAUCAUGACUCCCAAGGGUUU. Transfected cells were incubated for 8 hr, the medium was replaced with fresh medium, and the cells incubated for an additional 36 hr prior to harvesting and seeding in dishes for treatment. For the introduction of Mito-red into cells for mitochondrial morphology experiments, DU145 cells were resuspended in electroporation buffer {120 mM KCl, 0.15 mM CaCl₂, 10 mM K₂HPO₄/KH₂PO₄, 25 mM HEPES [(4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid], 2 mM EGTA [ethylene glycol-bis(2-aminoethylether)-*N,N,N',N'*-tetraacetic acid], 5 mM MgCl₂, 50 mM glutathione and 2 mM ATP at pH 7.6} at a density of 10⁷ cells/mL. The construct pDsRed2-Mito (Mito-red), a red fluorescent label targeted for expression in mitochondria (Clontech Laboratories, Inc., Mountain View, CA), was added to 4 million cells in 0.4 mL buffer. The mixture of cells and Mito-red DNA were 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 PoratorTM 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 hr. Stably transfected cells were selected

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from single colonies and maintained in the same medium containing G418 antibiotic (Invitrogen Corporation Carlsbad, CA).

Measurement of fragmented mitochondria

To measure fragmented mitochondria, Mito-red stably transfected DU145 cells were seeded on cover slips, treated as described above and examined on a fluorescent 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 5 fields (with more than 100 cells) per slide were counted in 4-5 experiments.

Immunoprecipitation of Bak

The DU145 cells were treated as above and harvested in RIPA 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 PAGE and processed for western analysis. The membranes were probed with Bcl-xL antibody (Cell Signaling Technology, Inc.) and re-probed with a different Bak antibody (Cell Signaling Technology, Inc.).

Measurement of cytosolic calcium

DU145 cells were cultured on glass cover slips and incubated in the dark with PBS containing 1% BSA and 4 μ M Fura 2-AM {acetoxymethyl 2-[5-[bis[(acetoxymethoxy-oxo-methyl)methyl]amino]-4-[2-[2-[bis[(acetoxymethoxy-oxo-methyl)methyl]amino]-5-methyl-phenoxy]ethoxy]benzofuran-2-yl]oxazole-5-carboxylate; Molecular Probes} for 40 min. Cells

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were washed with PBS and incubated in Hank's Balanced Salt Solution for 10 min to allow cleavage of the AM ester. For measurement of calcium, glass cover slips were assembled on a cell cultivation system and placed on a temperature-controlled (37°C) Zeiss fluorescent microscope stage connected to a PTI ImageMaster monochromator (Photon Technology Internations, Birmingham, NJ). The intensity of fluorescence was measured at 340/380 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 1,000g 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 re-probed with β -actin antibody (as a loading control).

Statistical analysis

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All values are presented as means \pm SEM; an ANOVA 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 $[Ca^{2+}]_c$ 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^+/Ca^{2+} exchanger inhibitor, 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 $[Ca^{2+}]_c$ was responsible for apoptosis in prostate cancer cells, DU145 cells were treated with ionomycin and thapsigargin. Ionomycin significantly increased $[Ca^{2+}]_c$ but did not induce apoptosis, in contrast to thapsigargin, which increased $[Ca^{2+}]_c$ to a lesser extent, but induced significant apoptosis (Fig. 1a and b). In addition, decreasing extracellular calcium using EGTA, an extracellular calcium chelator, did not induce noticeable apoptosis up to 300 μ M, while decreasing $[Ca^{2+}]_c$ using BAPTA-AM [1,2-Bis(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid tetrakis(acetoxymethyl ester)], an intracellular calcium chelator, induced apoptosis significantly at a dose of only 30 μ M (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 Velcade.

Preventing calcium influx enhanced the apoptotic effect of the proteasome inhibitors lactacystin and Velcade

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 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 μ M (Fig 2a). However, in combination with a low dose of lactacystin (2.5 μ 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, co-treatment with 10 μ M lactacystin induced about 10-fold apoptosis compared to 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. Like 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).

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Apoptosis induced by diltiazem and lactacystin is mediated through mitochondria

Previous studies have suggested that $[Ca^{2+}]_c$ 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. As 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

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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 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 the lower panel (Fig.4b). 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 (Petronilli et al., 2001; Schild et al., 2001). 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

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transition pore formation involves hexokinase, the voltage-dependent anion channel, adenine nucleotide translocase and cyclophilin D, a protein inhibited by cyclosporin A. 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. Surprisingly, cyclosporin A, a permeability transition pore inhibitor, 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 pro-apoptotic members of the Bcl2 family of proteins that have been shown to play critical roles in mitochondria-mediated apoptosis. Bax and Bak are involved in mitochondrial membrane permeabilization and release of cytochrome c into the cytoplasm (Autret and Martin, 2009). As DU145 cells do not express pro-apoptotic 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, upper and lower panels). When treated with lactacystin or diltiazem alone, Bak continued to remain sequestered by the anti-apoptotic Bcl-xL and thus was inactive as far as its pro-apoptotic 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 pro-apoptotic activity. To confirm the role of Bak in diltiazem- and lactacystin-induced apoptosis, cells were transfected with either

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scrambled or Bak-specific siRNA. Western analysis indicated that transfection of 50nM 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 pro-apoptotic protein Bak is involved in the induction of apoptosis by combined diltiazem and lactocystin. 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.

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 (tBid). tBid 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 induce 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 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

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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 upregulation 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 downregulated 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. (Nikrad et al., 2005).

Endoplasmic reticulum stress may be involved in lactacystin/diltiazem-induced apoptosis

There is growing evidence that endoplasmic reticulum (ER) stress, as well as ER-mitochondrial cross-talk, are 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), while individually diltiazem and lactacystin did not affect GRP78.

Diltiazem enhanced the apoptotic effect of the proteasome inhibitor Velcade

Bortezomib (trade name Velcade) 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

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investigate whether diltiazem could also enhance the apoptotic effect of Velcade. At doses that did not induce apoptosis with either agent alone, the combination of diltiazem and Velcade 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 important roles in cell survival or in cell death, depending on the extent of the alteration in calcium metabolism and the organelle/compartments 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 utilized to treat some of these conditions. However, the relationship between cancer prevalence and calcium levels remains unclear (Cornwell et al., 1987; Olsen et al., 1997), and agents that affect calcium mobilization are being investigated for their effectiveness in cancer therapy (Roderick and Cook, 2008). Diltiazem is a voltage-dependent calcium channel blocker that inhibits calcium influx, thereby relaxing the smooth muscle of the blood vessels and reducing blood pressure. Diltiazem has been used for decades in cardiologic diseases, and thus, based on its ability to enhance apoptosis in combination with proteasome inhibitors (Fig. 2), could be a drug of choice in cancer treatment, since the population at greatest risk for prostate cancer is also prone to cardiovascular disease and hypertension (Yoshinaga et al., 2006).

Proteasome inhibitors are gaining importance as possible anti-cancer agents. Velcade is an approved therapy for patients with hematologic malignancies and is also in clinical trials as a therapeutic to treat solid tumors. Indeed, bortezomib (Velcade) has been tested in phase I trials in androgen-independent prostate cancer patients with advanced solid tumors. Antitumor activity was found in these patients 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 treatment 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

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these proteins and allowing them to overwhelm anti-apoptotic 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-independent prostate cancer cells (Fig. 2). Importantly, 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 post-mitochondrial caspases 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, more effectively decreases cell viability and immunoglobulin secretion than either agent alone (Meister et al., 2010).

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To understand the mechanisms leading to apoptosis, the possible roles of the pro-apoptotic members of the Bcl2 were investigated. As the prostate cancer cells DU145 do not express the pro-apoptotic protein Bax, the role of the other pro-apoptotic 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 anti-apoptotic protein Bcl-xL (Fig. 6). It is well known that binding of Bak by anti-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 endoplasmic reticulum (ER) stress, as well as ER-mitochondria crosstalk, 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 (Fribley 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 upregulated the ER stress marker GRP78 when added separately, but GRP78 levels were significantly upregulated 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

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apoptosis induced by thapsigargin, and BAPTA-AM in our (Fig. 1a, 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, cardiovascular diseases, for which diltiazem is a common therapy, often exhibit a high incidence 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 therapeutics. 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., on other cell types that are not cancerous. Thus, in this imminent era of 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 prostate cancer patients who also need calcium channel blockers for the treatment of their cardiovascular disease.

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Authorship Contributions

Participated in research design: Kaddour-Djebbar, Choudhary, Shirley, Bollag, Kumar

Conducted experiments: Kaddour-Djebbar, Choudhary, Lakshmikanthan, Shirley,

El Gaish, Al-Husein, Zhong, Davis

Performed data analysis: Kaddour-Djebbar, Al-Shabrawey, Bollag

Contributed new reagents: Dong

Contributed to the writing of the manuscript: Kaddour-Djebbar, Bollag, Kumar

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Footnotes

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Legends for Figures

Fig. 1 Alterations of cytosolic calcium concentration ($[Ca^{2+}]_c$) induced apoptosis in prostate cancer cells. **a** DU145 prostate cancer cells were treated for 24 hr with 1 or 10 μ M ionomycin (Iono), a calcium ionophore, or 1 or 10 μ M thapsigargin (Thaps), a sarco/endoplasmic reticulum Ca^{2+} -ATPase inhibitor. Apoptosis was measured using the M30 Apoptosense kit as described in Materials and Methods and expressed as M30 antigen units/ μ g protein compared to the control. **b** $[Ca^{2+}]_c$ was measured by Fura-2 fluorescence as described in Materials and Methods. During the course of measurement, cells were treated with ionomycin or thapsigargin (indicated by the arrow head). **c** Intracellular or extracellular calcium ions were chelated with increasing concentrations of BAPTA-AM (dark bars) or EGTA (grey bars), respectively. Apoptosis was measured using the M30 Apoptosense kit. The values represent the means \pm SEM of 3 experiments (* P <0.05, *** P <0.001 versus the control).

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 μ M) of diltiazem (DZ) in the presence and absence of 2.5 μ M lactacystin (Lact) for 24 h. Apoptosis was measured using the M30 Apoptosense kit. The values represent the means \pm SEM of 3 experiments (*** P <0.001 versus the control). **b** DU145 cells were treated as in Fig. 2a, except that a higher concentration of lactacystin (10 μ M) was used. **c** DU145 cells were treated with or without lactacystin (10 μ M), diltiazem (400 μ M) or the combination for 24 hr, harvested and stained with Annexin V and DAPI as described in 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 upper panel. The lower panel shows cumulative

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values from multiple experiments for the percentage of apoptotic cells from flow cytometric analyses. Values represent the means \pm SEM from 3 separate experiments; * $p < 0.05$ versus the control, + $p < 0.05$, ++ $p < 0.01$ versus the combination of lactacystin and diltiazem. **d** Shown are representative phase contrast microscopic images (20X) illustrating the cell confluence observed after a 24 hr treatment with vehicle (control, C), 10 μ M lactacystin (L), 400 μ M diltiazem (D) or the combination (DL). The inset illustrates the number of cells at time 0 (prior to treatment). **e** HMVECad normal human microvascular endothelial cells derived from adult dermis were treated with 10 μ M lactacystin, 400 μ M diltiazem and the combination and apoptosis was monitored using the M30 Apoptosense kit. The values represent the means \pm SEM of 3 experiments performed in at least duplicate.

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 μ M) and/or diltiazem (DZ, 400 μ M) for 24 hr. 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 fluorescence as described in Materials and Methods. During the measurement, diltiazem (400 μ M) was added at the arrow. **b** In the upper

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panel, DU145 cells were transfected with the mitochondrial marker Mito-Red, and were treated with vehicle (C), 400 μ M diltiazem (D), 10 μ M lactacystin (L) or the combination (DL) for 24 h. Mitochondria were visualized with a Carl Zeiss Axioskop fluorescent microscope. The lower panel shows the percentage of cells exhibiting fragmented mitochondria, counted using the criteria described in Materials and Methods. The data are shown as the means \pm SEM of duplicate samples from three experiments (*** P <0.001 versus the control or lactacystin alone).

Fig. 5 The mitochondrial permeability transition pore was not involved in lactacystin/diltiazem-induced apoptosis. DU145 cells were pretreated with vehicle or cyclosporin A (30 μ M) for 30 min, then treated for 24 hr, with or without lactacystin (10 μ M) and/or diltiazem (400 μ M). Apoptosis was measured using the M30 aptosense kit and is expressed as M30 antigen units/ μ g protein compared to control. Values represent the means \pm SEM of 3 experiments (*** P <0.001 versus the control or either agent alone; NS not significant).

Fig. 6 A pro-apoptotic member of the Bcl2 family, Bak mediated the apoptotic effects of lactacystin and diltiazem. **a** DU145 cells were treated as in Fig. 5, Bak was immunoprecipitated and immunoprecipitates were processed for western analysis for the levels of Bak and Bcl-xL. The upper panel shows a representative experiment. In the lower panel is shown cumulative data for multiple experiments quantified using an Alpha Innnotech 8900, and the values are expressed as the ratio of Bcl-xL to Bak. Values represent the means \pm SEM of 3 experiments (*** p <0.001 versus the control or either agent alone). **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

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loading control. **c.** DU145 cells were transfected with 50 nM scrambled or Bak-specific siRNA and treated with vehicle or diltiazem (400 μ M) in the absence or presence of lactacystin (10 μ M) as indicated. Apoptosis was measured using the M30 Apoptosense kit and is expressed as M30 antigen units/ μ g protein versus the control. The values represent the means \pm SEM of at least three experiments. (***) $P < 0.001$ as indicated).

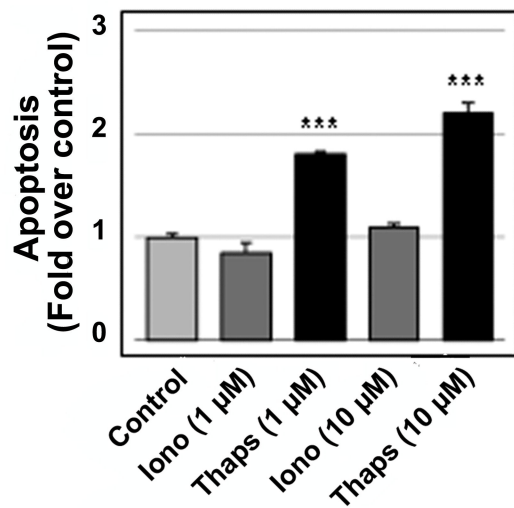
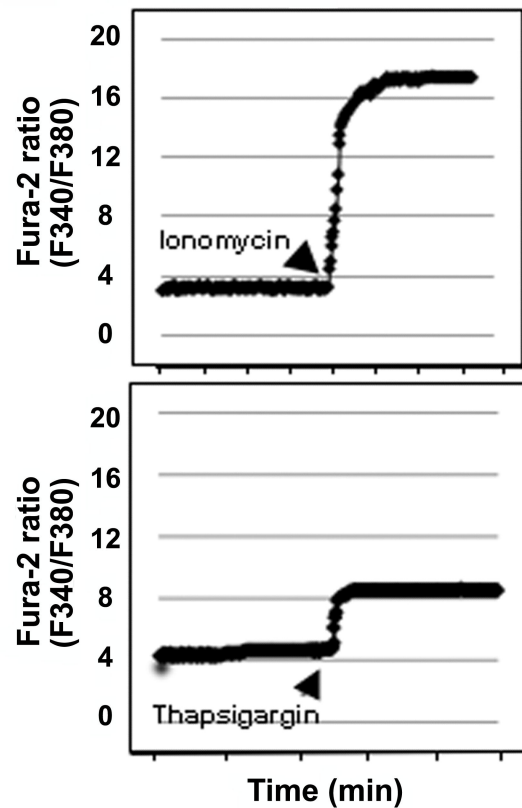
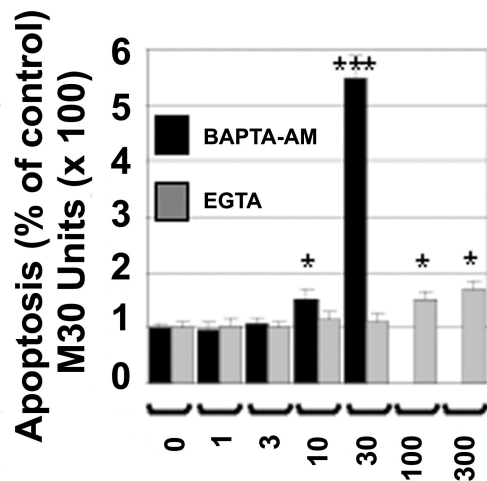
Fig. 7 Bik, rather than Bid, is the likely upstream activator of Bak-mediated apoptosis in cells treated with diltiazem and lactacystin. **a** DU145 cells were treated with vehicle or diltiazem (400 μ M) in the presence or absence of lactacystin (10 μ M) for 24 hr, and cell lysates were processed for western blotting to examine Bid and caspase-8 cleavage. **b** DU145 cells were treated with vehicle or diltiazem (400 μ M) in the presence and absence of lactacystin (10 μ M) for 24 h, and cell lysates were processed for western blotting to examine Bak, Bik, Bok, and PUMA levels. GAPDH served as the loading control. Blots are representative of a minimum of three experiments.

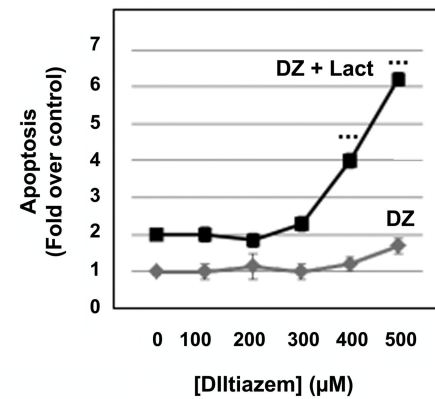
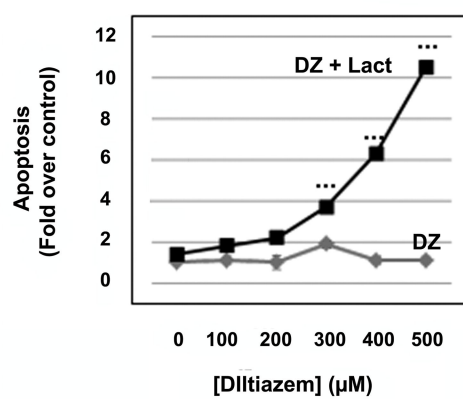
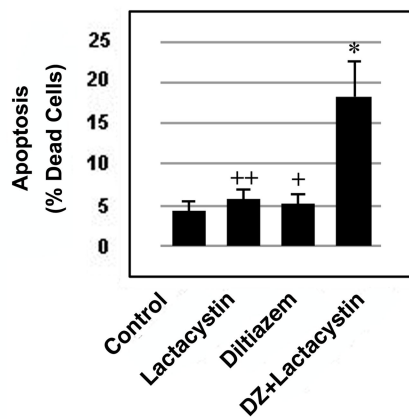
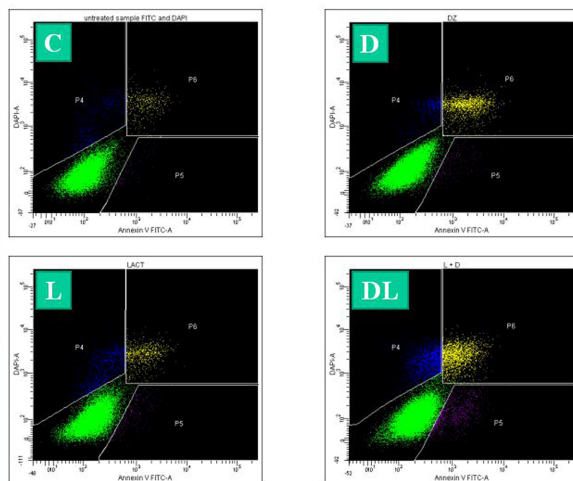
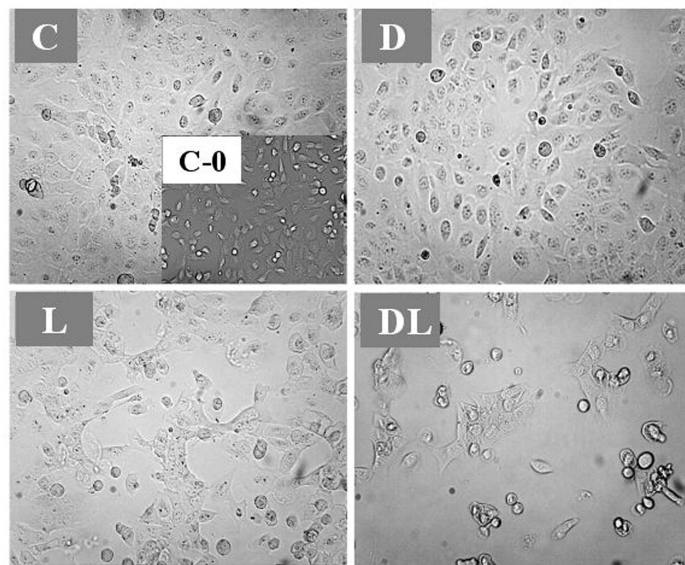
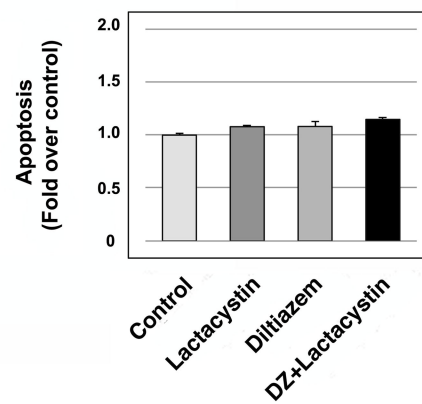
Fig. 8 Endoplasmic reticulum stress may be involved in apoptosis induced by diltiazem and lactacystin. DU145 cells were treated with vehicle or diltiazem (400 μ M) in the presence and absence of lactacystin (10 μ M). Cell lysates were processed for western blotting to examine the ER stress marker Grp78/BiP. The blots were stripped and reprobed for β -actin, which served as a loading control. Blots are representative of a minimum of three experiments.

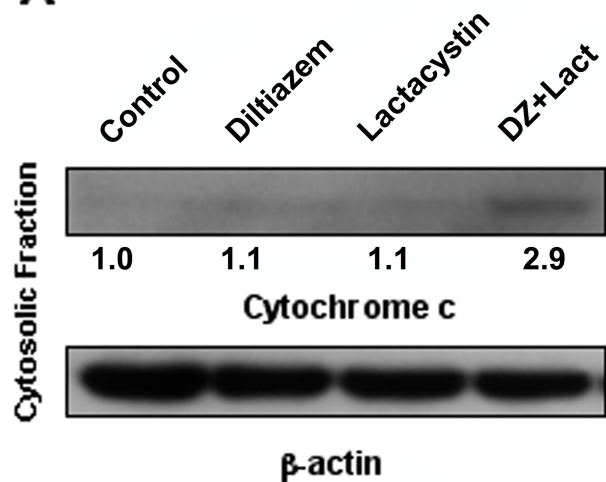
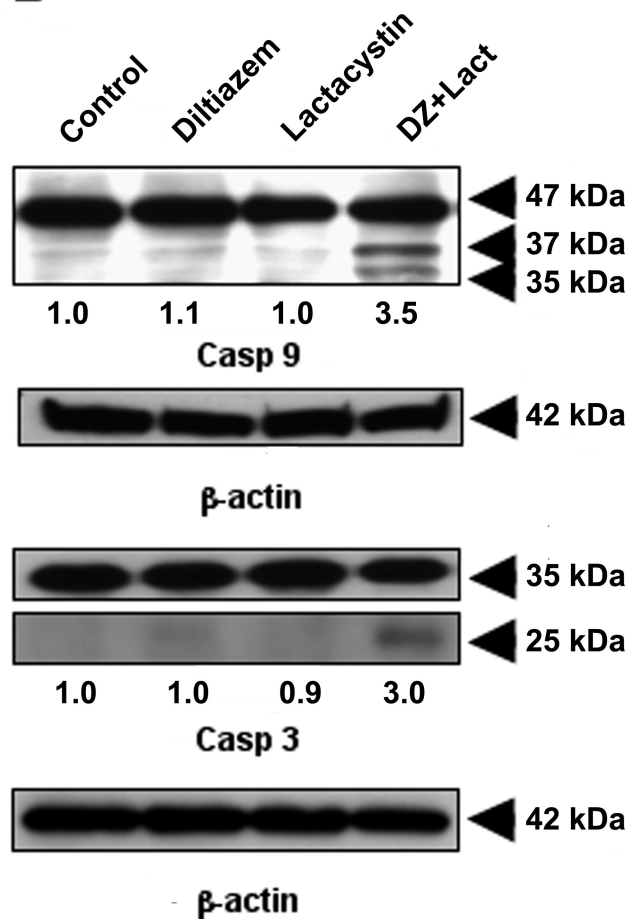
Fig. 9 Diltiazem enhanced the apoptotic effect of Velcade. DU145 cells were treated with vehicle or the proteasome inhibitor, Velcade (40 μ M) alone or in combination with diltiazem

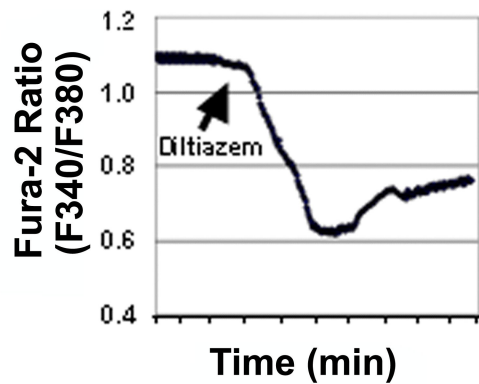
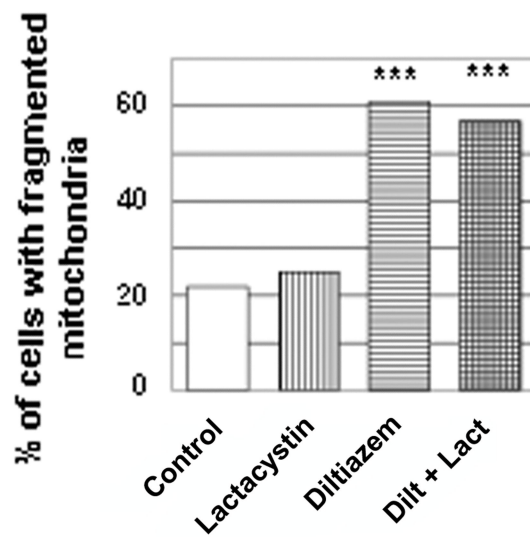
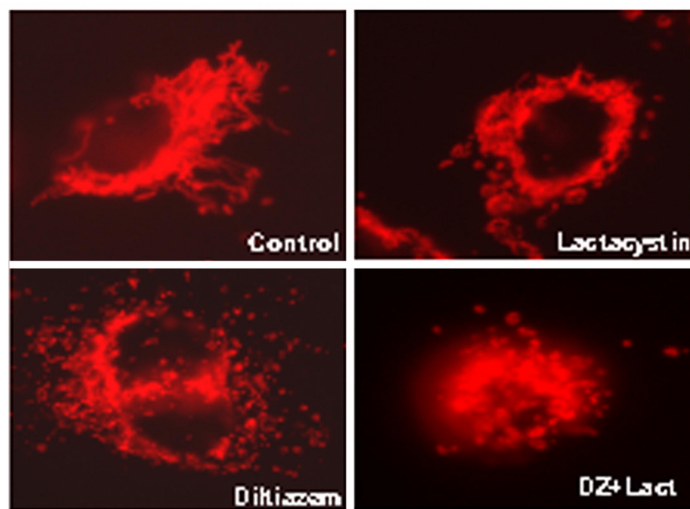
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(400 μ M). Apoptosis was monitored using the M30 Apoptosense kit. The data illustrate the fold increase in apoptosis as compared to control levels and represent the means \pm SEM of duplicate samples. The experiment was repeated once with similar results.

A**B****C****Figure 1**

A**LNCaP****B****DU145****C****D****E****Figure 2**

A**B****Figure 3**

A**B****Figure 4**

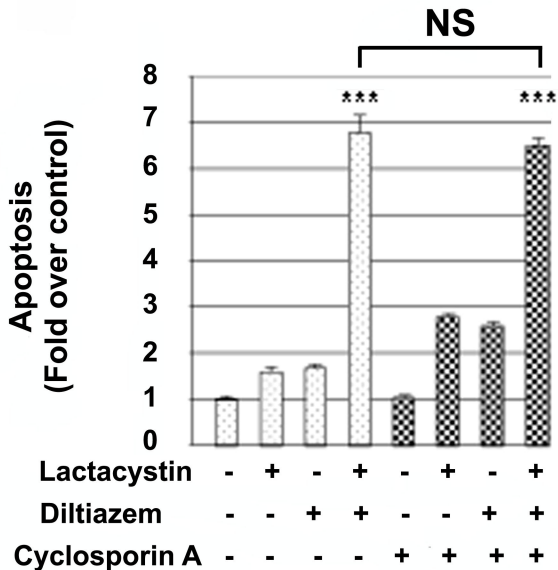
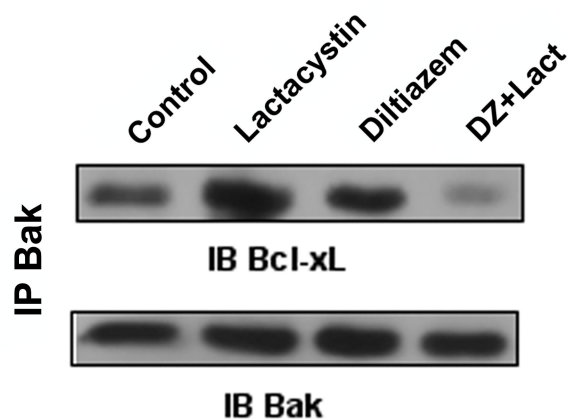
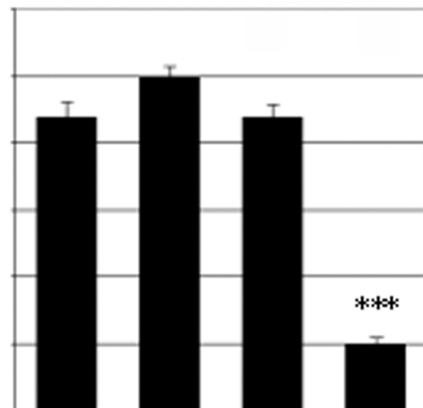
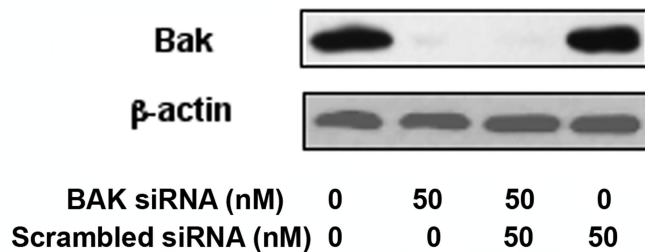
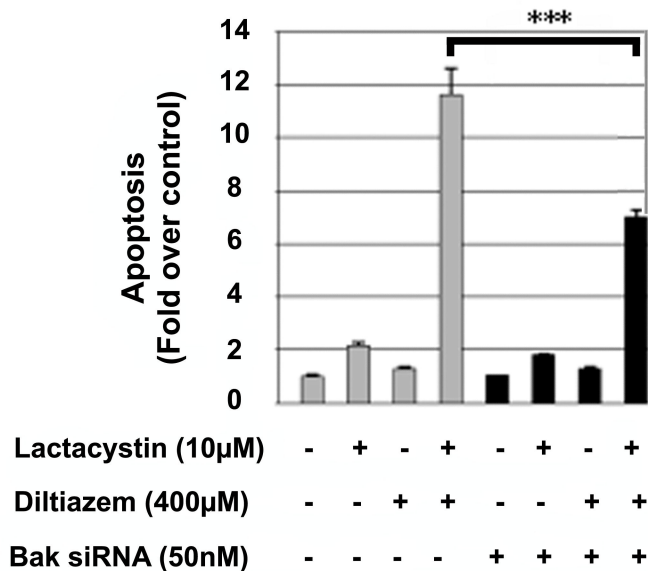
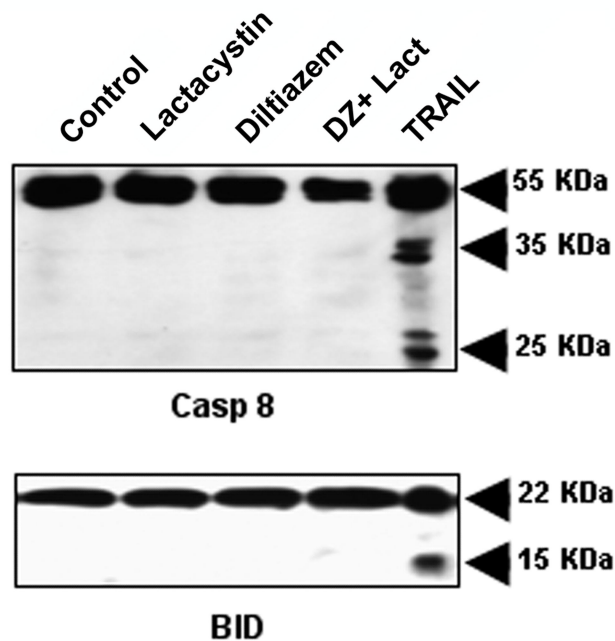
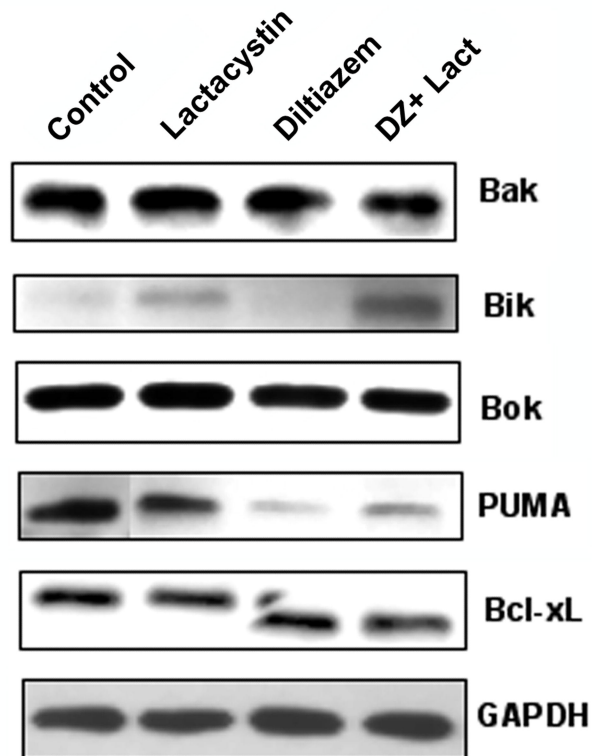


Figure 5

A**Bcl-xL/Bak Ratio****B****C****Figure 6**

A**B****Figure 7**

Control

Lactacystin

Diltiazem

DZ+Lact



Grp78/BiP



β -actin

Figure 8

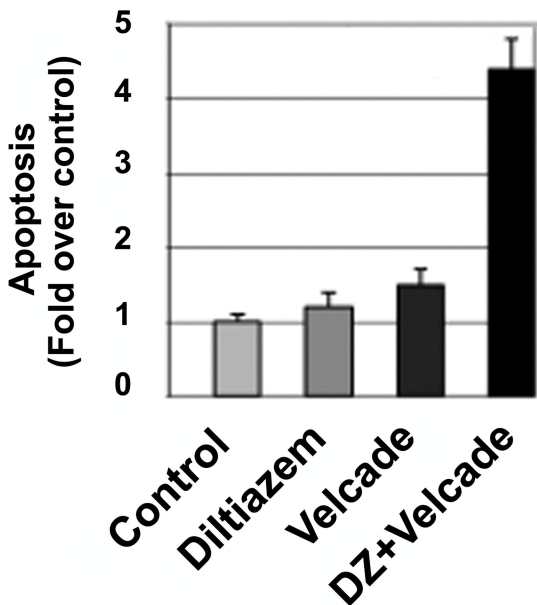


Figure 9