Cancer Chemother Pharmacol (2008) 61:231–242
DOI 10.1007/s00280-007-0465-0

ORIGINAL ARTICLE

Lysosomotropic acid ceramidase inhibitor induces apoptosis in prostate cancer cells

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Received: 8 November 2006 / Accepted: 4 March 2007 / Published online: 12 April 2007

Abstract
Purpose Alterations in ceramide metabolism have been reported in prostate cancer (PCa), resulting in escape of cancer cells from ceramide-induced apoptosis. Specifically, increased expression of lysosomal acid ceramidase (AC) has been shown in some primary PCa tissues and in several PCa cell lines. To determine if this represents a novel therapeutic target, we designed and synthesized LCL204, a lysosomotropic analog of B13, a previously reported inhibitor of AC
Methods Prostate cancer cell lines were treated with LCL204 for varying times and concentrations. Effects of treatment on cytotoxicity, sphingolipid content, and apoptotic markers were assessed.
Results Treatment of DU145 PCa cells resulted in increased ceramide and decreased sphingosine levels. Interestingly, LCL204 caused degradation of AC in a cathepsin-dependent manner. We also observed rapid destabilization of lysosomes and the release of lysosomal proteases into the cytosol following treatment with LCL204. Combined, these events resulted in mitochondria depolarization and executioner caspase activation, ultimately ending in apoptosis
Conclusions These results provide evidence that treatment with molecules such as LCL204, which restore ceramide levels in PCa cells may serve as a new viable treatment option for PCa.

Keywords Ceramide · Lysosomes · Apoptosis · LCL204 · B13 · Acid ceramidase inhibitors

Abbreviations

PCa	Prostate cancer
HRPC	Hormone-refractory prostate cancer
AC	Acid ceramidase
ASMase	Acid sphingomyelinase
LTR	LysoTracker Red
LMP	Lysosomal membrane permeabilization

Introduction

Prostate cancer (PCa) is the most common non-cutaneous malignancy in men in the United States and the second leading cause of death from cancer. While treatment of localized PCa is frequently effective, many men are unfortunately diagnosed with more advanced disease which, whether local or metastatic, is typically resistant to the current available treatments. Androgen ablation is most often the therapeutic choice, but becomes ineffective as advanced PCa develops androgen independence, known as hormone-refractory prostate cancer (HRPC). HRPC develops, on average, 18 months after beginning androgen ablation therapy and commonly displays resistance to a wide variety of

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chemotherapeutic agents [37]. As a result, HRPC is generally considered incurable, highlighting the need for new treatment options.

Ceramide is the basic building block of the complex sphingolipids and functions as a bioactive lipid in several cellular processes including apoptosis, inflammation, and cell cycle arrest. Cellular ceramide levels are regulated by ceramide-synthesizing enzymes such as acid sphingomyelinase (ASMase) and ceramide-metabolizing enzymes such as acid ceramidase (AC). Ceramide is often produced in response to cellular stress such as hypoxia, nutrient deprivation, genotoxic agents, or immune attack. As these insults are commonly encountered by cells in a growing tumor, successful tumor formation depends on the development of escape mechanisms to surmount this homeostatic control point. Thus, it is not surprising that defects in ceramide signaling and metabolism have been shown to be involved in apoptosis resistance in cancer cells ([20, 21, 23, 36, 38, 40], reviewed in [25]).

One method for cells to escape ceramide-induced apoptosis is to ensure that ceramide produced by the stress response is rapidly removed by ceramide-metabolizing enzymes such as AC (reviewed in [25, 27]). Human AC is synthesized as a 53 kDa polypeptide which is processed into α and β subunits (13 and 40 kDa, respectively) in lysosomes, where it resides and functions to regulate lysosomal ceramide levels [11]. Seelan et al. found the human AC gene to be over-expressed in 42% of PCa specimens analyzed as well as three PCa cell lines [34]. These results suggest that therapeutic strategies aimed at restoring the balance of ceramide in PCa cells may offer a new treatment option for PCa.

Aromatic analogs of ceramide (N-acyl-phenyl-amino-alcohols) have been shown to be potent anti-cancer agents [2, 3]. The AC inhibitor (1R,2R) N-myristoylamino-(4′-nitrophenyl)-propandiol-1,3 (also referred to as B13 or LCL4), had strong anti-cancer activity in the myeloid leukemic cell line HL-60, melanoma, prostate, and colon cancer cells [2, 3, 28, 31, 35]. Here, we introduce a novel analog of B13, LCL204 [(1R,2R) 2-(N-tetradecylamino)-1-(4-NO_2)-phenyl- 1,3-dihydroxy-propane HCl] (Z. M. Szulc et al., Submitted for publication). An independently synthesized compound called AD2646 [(2R,3R) 2-(N-tetradecylamino)-1-(4-NO_2)-phenyl- 1,3-dihydroxy-propane] has been reported to exert cytotoxic effects on the myeloid leukemic cell line HL-60 [8], and to alter sphingolipid metabolism and inhibit AC activity in the leukemic T cell line Jurkat, resulting in cell death [12].

As altered ceramide metabolism associated with up-regulated AC has been implicated in some prostate and head and neck cancers [25], AC inhibitors such as LCL204 are of interest. We report here that LCL204 exerted a rapid effect on lysosomes in PCa cells via elevation of pH and alteration of sphingolipid profiles, which was immediately followed by degradation of both AC and ASMase. These events were proximal to a loss of mitochondria membrane potential ($\Delta\psi_m$) and the activation of executioner caspases, which ultimately culminated in apoptosis. These data illustrate that the use of lysosomal inhibitors of AC may serve as a functional treatment for PCa exhibiting aberrant ceramide metabolism.

Materials and methods

Cell lines

The human PCa cell lines DU145, LNCaP, DuPro, and PC-3 were purchased from ATCC, Manassas, VA, USA, and PPC-1 cells were from Dr. Yi Lu at the University of Tennessee, Memphis, TN, USA. All cells were cultured in RPMI 1640 (Mediatech Inc., Herndon, VA, USA) supplemented with 10% heat-inactivated BGS (Hyclone, Logan, UT, USA). Cells were maintained in 5% CO_2 at 37°C. All experiments were performed in RPMI 1640 supplemented with 2% heat-inactivated BGS.

Reagents

LCL204 [(1R,2R) 2-(N-tetradecylamino)-1-(4-NO_2)-phenyl- 1,3-dihydroxy-propane HCl] was synthesized in the Medical University of SC Lipidomics Core Facility (Charleston, SC, USA) as described [8]. Pepstatin A, leupeptin, aprotinin, phenylmethanesulfonyl fluoride (PMSF), and MG132 were all purchased from Sigma, St. Louis, MO, USA. CA074Me was from Calbiochem, San Diego, CA, USA, while zVAD-fmk was from Biomol, Plymouth Meeting, PA, USA. JC-1 mitochondrial dye and LysoTracker Red (LTR) lysosomal dye were from Molecular Probes, Eugene, OR, USA. Antibodies used for immunoblotting were: mouse monoclonal anti-cytochrome c and anti-AC (Pharmingen, San Diego, CA, USA), rabbit polyclonal anti-actin (Sigma), mouse monoclonal anti-cathepsin B (Oncogene Research Products, San Diego, CA, USA), mouse monoclonal anti-COX IV (Molecular Probes), rabbit polyclonal anti-Bak (Cell Signaling Technology Inc., Beverly, MA, USA), goat anti-rabbit IgG-HRP conjugate (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) and goat anti-mouse IgG-HRP conjugate (Sigma).

MTS cytotoxicity assays for LCL204 treatments

Cell viability was determined using the CellTiter 96 AQ_{ueous} One Solution Cell Proliferation Assay (Promega, Madison, WI, USA). About 1×10^4 cells per well were seeded in 96-well plates overnight. The next day media was removed

and replaced with either 100 µl media with vehicle control or media containing LCL204 at desired concentrations. Assays were carried out as previously described [15]. For experiments using enzyme inhibitors and LCL204, media was removed and replaced with 50 µl media containing vehicle only or indicated inhibitor. Cells were pretreated 1 h at 37°C before adding 50 µl media containing vehicle, inhibitor only, LCL204 only (2× concentration), or a combination. The remainder of the assay was carried out as described above.

Sphingolipid measurement

About 2.1×10^6 cells were seeded in 100 mm plates overnight. The next day, media was removed and replaced with media containing vehicle control or LCL204 (10 µM) for indicated time points. Following treatment, cells were harvested by gentle scraping and immediate centrifugation at 4°C for 5 min at 400×*g*. Cell pellets were then resuspended in ice cold PBS and stored at −80°C. For sphingolipid analysis, mass spectrometry was used as previously described [4].

Caspase 3/7 activity assay

Cells were seeded overnight in clear bottom black 96 well plates (Corning, Acton, MA, USA). The next day, medium was removed and replaced with medium containing vehicle or LCL204 at indicated concentrations. After 24 h treatment, Caspases 3 and 7 activities were measured using Apo-ONE Homogeneous Caspase 3/7 assay according to the manufacturer's instructions (Promega). Fluorescence was measured using a Fluostar dual fluorescence/absorbance plate reader (BMG Laboratories, Durham, NC, USA) with 485 nm excitation and 520 nm emission filter set.

Mitochondria membrane potential measurement

Cells were seeded at a density of 7.49×10^5 cells per plate in 60 mm plates overnight. The next day, media was replaced with media containing vehicle control or LCL204 (5 µM). Cells were lifted using Cell Stripper (Mediatech), washed twice in PBS, and resuspended in 3 ml 1× JC-1 reagent solution (dissolved in medium). Samples were incubated at 37°C for 15 min, washed twice with PBS, and resuspended in 0.5 ml growth medium before analysis by flow cytometry using a Becton-Dickinson FACSCalibur (590/527 nm emission). A minimum of 10,000 events were scored for each sample.

Immunoblot analysis

Cells were seeded in 60 mm plates as described above and treated accordingly. Cells were lifted by gently scraping the plates, washed once with ice cold PBS and then lysed in lysis buffer (PBS, 1% Triton X-100, 10% glycerol) containing protease inhibitors pepstatin A (0.5 µg/ml), leupeptin (0.5 µg/ml), aprotinin (5 µg/ml), and PMSF (100 µg/ml) for 10 min on ice. Insoluble material was removed by centrifugation at 14,000 rpm for 15 min at 4°C. The supernatants were then supplemented with SDS at a final concentration of 2% and stored at −80°C. Protein concentrations were determined using the D_C Protein Assay (Bio-Rad Laboratories, Hercules, CA, USA) according to the manufacturer's instructions. Fifty µg of protein per sample (unless otherwise indicated) were separated on NuPAGE 4-12% Bis–Tris gels (Invitrogen, Carlsbad, CA, USA) and transferred to nitrocellulose membranes (Bio-Rad). Following transfer, membranes were blocked for 1 h at room temperature in Tris-buffered saline (TBS) containing 0.1% Tween-20 and 5% non-fat dry milk and incubated overnight at 4°C with primary antibody at a dilution of 1:2,000 (actin), 1:1,000 (cytochrome c, COX IV, Bak), or 1:400 (cathepsin B). Overnight incubations were performed in 5% milk in TBS-Tween. Following overnight incubation, membranes were washed three times in TBS-Tween and incubated for 1 h at room temperature with secondary antibody in 5% milk TBS-Tween at dilutions of 1:5,000 (goat anti-mouse IgG) or 1:50,000 (goat anti-rabbit). Membranes were then washed again and proteins were visualized with Super Signal HRP substrate (Pierce Biotechnology Inc., Rockford, IL, USA). Results from selected blots were quantitated by densitometry using ImageQuant v1.2 software and normalizing each protein band to the corresponding actin levels.

Lysosomal stability assay

Lysosomal stability was measured using the fluorescent dye LTR. Cells were seeded overnight in 60 mm plates. The next day, medium was removed and replaced with medium containing 200 nM LTR for 30 min at 37°C. LTR was removed and cells were washed once with PBS, then medium containing the treatment was added for the indicated time. After treatment, cells were lifted with trypsin, washed once in PBS, and resuspended in 0.5 ml growth medium. LTR fluorescence was measured using FACS analysis (564–606 nm) as above. A decrease in fluorescence intensity corresponded to an increase in lysosomal pH, and a minimum of 10,000 events were scored for each sample.

Reverse transcriptase PCR

DU145 cells were treated with LCL204 (10 µM) or ethanol control. Cells were collected at indicated time points and total RNA was extracted using RNAqueous-4PCR kit (Ambion Inc., Austin, TX, USA), including the DNase I

treatment step to remove DNA contamination. The levels of AC transcripts were assayed by two-step RT-PCR protocol (Ambion) and Rig/S15 was used as an internal control. The sequences of the primers for amplification of AC were: F—tgtggatagggttcctcactaga, R—ttgtgtatacggtcagcttgttg. All reactions were performed in a programmable thermal cycler (reverse transcription at 55°C for 1 h; PCR at 95°C, 3 min; 95°C, 30 s; 52°C, 1 min and extension at 72°C for 1 min; final extension at 72°C, 10 min). The PCR product was separated on a 2% agarose gel.

Acid sphingomyelinase activity assay

DU145 cells were lysed in 50 mM Tris (pH 7.4) using a probe sonicator. Cellular debris was removed after centrifugation at 3,000×*g* for 10 min. Proteins (50 µg) were adjusted to a total volume of 100 µl and the reaction was started by adding 100 µl of the reaction mixture containing 1 mM EDTA, 250 mM sodium acetate (pH 5.0), 100 µM [*choline-methyl*-^{14}C] sphingomyelin and 0.1% Triton X. After incubation at 37°C for 1 h, the reaction was stopped by adding 1.5 ml of chloroform/methanol (2:1) and then 400 µl of water. Phases were separated by centrifugation at 2,000×*g* for 5 min. Quantitation of the amount of released radioactive phosphocholine was determined by subjecting 400 µl of the upper phase to scintillation counting.

Subcellular fractionation

For cytochrome c immunoblot, cells were harvested at 4, 12, or 24 h after treatment as described previously [16] and proteins (15 µg) were separated by gel electrophoresis and immunoblotted for cytochrome c as above. The protocol for separating cytosolic and heavy membrane fractions is a modified version of that published previously [9]. Briefly, cells were harvested at 0, 0.5, 1, and 2 h after treatment, washed once in PBS, and gently resuspended in isotonic mitochondrial buffer (210 mM mannitol, 70 mM sucrose, 1 mM EDTA, 10 mM HEPES, pH 7.0) supplemented with protease inhibitors. Cells were then transferred to microcentrifuge tubes and homogenized using 40 strokes with a polished (fine grain sandpaper) Teflon pestle. Fractions were separated using differential centrifugation as described in the reference. All fractions were stored at −80°C. Cytosolic and heavy membrane fractions (30 and 15 µg, respectively) were separated on NuPAGE gels and immunoblotted as described above. For cathepsin B activity assays, the same procedure was carried out as above with the exception of protease inhibitors. Enzyme activity per 50 µg lysate was measured using the fluorogenic cathepsin B substrate III (Calbiochem) according to the manufacturer's protocol.

Confocal microscopy

DU145 cells were grown in 4.3 cm^2 chamber slides (Nalge Nunc, Rochester, NY, USA) and transfected with YFPmito using FuGENE6 (Roche, Indianapolis, IN, USA) according to the manufacturer's instructions using 1 µg of total DNA per chamber. About 24 h after transfection cells were treated with 15 µM LCL204. The slides were fixed with 4% paraformaldehyde for 20 min then permeabilized with 0.2% Triton X-100 for 15 min, followed by blocking with 4% bovine serum albumin for 45 min at RT. Cells were probed with rabbit polyclonal 2-14 anti-Bak antibody (1:5,000; BioChem) for 2 h then stained with goat anti-rabbit Alexa Fluor 543 antibodies (1:250; Molecular Probes) for 45 min. After washing, cells were imaged using the 63× objective of an LSM 510 Zeiss confocal microscope.

Results

LCL204 induces cell death and rapid changes in sphingolipid levels in PCa cells

Using an MTS cell viability assay, we found that LCL204 induced concentration-dependent cell death in the micromolar range in five different PCa cell lines cells after 24-h treatment (Fig. 1a). Furthermore, LCL204 was much more toxic at lower concentrations than B13, the parent compound to LCL204, in four different PCa cell lines (Fig. 1b). B13 induces apoptosis in PCa cells and inhibits experimental prostate tumor growth [31]. These anti-tumor activities are thought to result from B13-mediated ceramide elevation [28, 31, 35]. In order to investigate similar biochemical activities of LCL204, sphingosine and ceramide levels were measured following LCL204 treatment. Treatment of both DU145 and PPC-1 cells with LCL204 induced a dose-dependent decrease in sphingosine levels (Fig. 2a, b). Sphingosine results from AC-mediated ceramide hydrolysis, therefore a decline in sphingosine is consistent with inhibition of AC. Additionally, treatment with LCL204 increased total ceramide levels in DU145 cells (Fig. 2c), which is also consistent with AC inhibition. This was detected within the first 2 h after treatment with either 2 or 10 µM LCL204. In PPC-1 cells, however, little change in ceramide levels was seen in response to LCL204 at the time points analyzed (Fig. 2d).

LCL204 induces proteolytic degradation of AC

Following treatment of DU145 cells with LCL204, we detected a dose- and time-dependent decrease in AC expression levels. Increased LCL204 concentration corresponded to decreased AC expression when cells were treated with the compound for 12 h (Fig. 3a). Treatment of DU145 cells

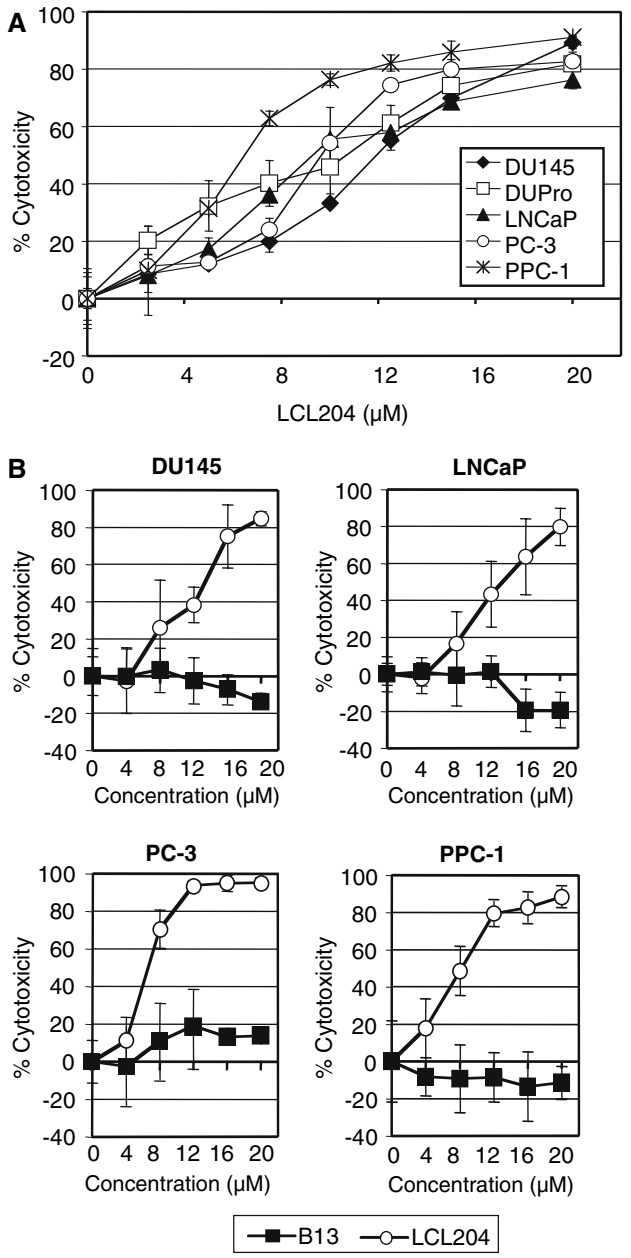

Fig. 1 LCL204 induces cell death in PCa cells. **a** PCa cell lines were treated for 24 h with LCL204. **b** PCa cell lines were treated for 24 h with either LCL204 or B13. Cell death was quantified using MTS cell viability assay. Results are representative of three independent experiments. *Bars*, SD

with 10 μM LCL204 caused decrease of AC expression in a time-dependent manner beginning within the first 2 h of treatment (Fig. 3b). These effects were comparable in all PCa cell lines tested, including PC-3, LNCaP, DuPro, and PPC-1 (data not shown). Using RT-PCR, we determined that this down-regulation was not a transcriptional event as there was no decrease in AC mRNA levels in DU145 cells during treatment with 10 μM LCL204 within the same time frame of protein level decline (Fig. 3c).

Cell death in response to LCL204 involves caspase activity

As caspase involvement has been reported in AD2646-induced cell death in leukemia cell lines [8, 12], we analyzed caspase activity in PCa cell lines in response to LCL204. Pre-treatment with the broad-spectrum caspase inhibitor zVAD-fmk reduced LCL204-induced cytotoxicity at LCL204 concentrations below 20 μM, indicating a role for caspases in LCL204-mediated cell death in PCa cells (Fig. 4a). Using a fluorogenic caspase activity assay, we found LCL204 induced dose-dependent executioner caspase activity in DU145 cells after 24-h treatment (Fig. 4b). Similar results were achieved in PC-3, PPC-1, and DuPro cells (data not shown). Protein degradation is frequently executed through the proteasomal [42] or lysosomal [19] pathways. Alternatively, caspases also serve this function in certain pathways particularly during apoptosis [39]. Therefore, we investigated the effects of LCL204 in the presence of a panel of protease inhibitors (Fig. 4c). Prior to LCL204 treatment, DU145 cells were pretreated for 1 h with vehicle only, zVAD-fmk (pan-caspase inhibitor), MG132 (proteasome inhibitor), CA074Me (cathepsin B inhibitor), or pepstatin A (cathepsin D inhibitor). Treatment with inhibitors alone had no effect on AC protein levels (not shown). Interestingly, more than one protease inhibitor blocked AC degradation. Pretreatment with pepstatin A had no effect on the LCL204-induced AC protein loss, while pretreatment with zVAD-fmk, MG132, or CA074Me all blocked AC degradation, suggesting the involvement of multiple pathways. However, caspase inhibitors such as zVAD-fmk are known to be rather promiscuous in their selectivity [32]. Accordingly, we found both zVAD-fmk and MG132 to have non-specific inhibitory activity against cathepsin B (data not shown). Due to the specificity of CA074Me [6], cathepsin B emerged as a primary candidate for mediating AC degradation. These results led to evaluation of lysosome integrity following LCL204 treatment. AC and ASMase both reside within the lysosomal membrane and are known to closely interact as they have been shown to co-precipitate when secreted into culture medium [13]. Therefore, ASMase activity following LCL204 treatment was analyzed. LCL204 induced a rapid decrease in ASMase activity in DU145 cells beginning as early as 30 min after treatment (Fig. 4d). Furthermore, the loss of activity was almost fully restored in the presence of CA074Me after a 2-h treatment (Fig. 4e), again implicating proteolytic degradation mediated by cathepsin B.

LCL204 induces lysosomal destabilization and membrane permeabilization

The loss of AC and reduction of ASMase activity as well as the suspected involvement of cathepsin B led us to investigate

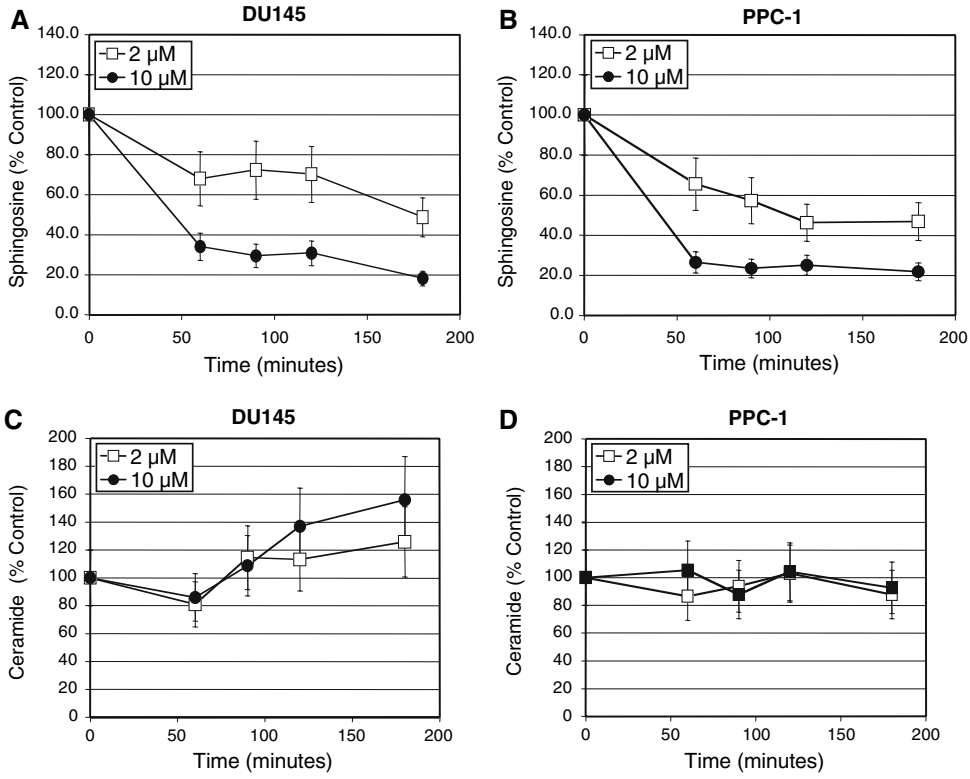

Fig. 2 Sphingolipid profiles are altered in response to LCL204. DU145 or PPC-1 cells were treated for indicated times with LCL204 (2 or 10 μM). After treatment cells were harvested and total sphingosine (**a**, **b**) or total ceramide (**c**, **d**) levels were measured using mass spectrometry and plotted according to percent control. Results are representative of three independent experiments. *Bars*, SD

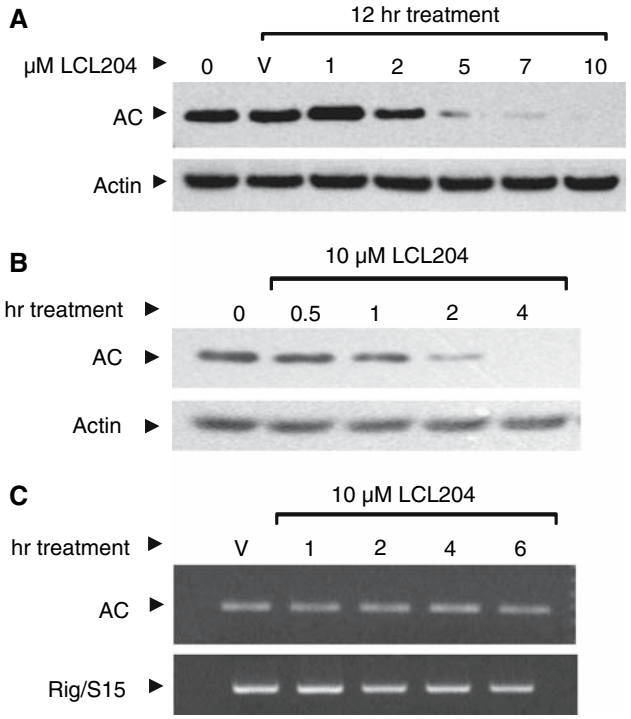

Fig. 3 LCL204 treatment induces degradation of AC. DU145 cells were treated for 12 h with indicated concentrations of LCL204 (**a**) or with 10 μM LCL204 for indicated time (**b**) and cell lysates were analyzed for AC expression. **c** DU145 cells were treated with 10 μM LCL204 for indicated time and AC mRNA levels were determined by RT-PCR. Rig/S15 primers were used as an internal control. *V* vehicle only. Results are representative of three independent experiments

lysosomal stability following LCL204 treatment. Lysosomal destabilization can result in membrane permeabilization and release of lysosomal proteins, including cathepsins B and D, into the cytosol [5, 30]. Using a subcellular fractionation technique, cytosolic proteins were separated from heavy membrane bound proteins in DU145 cells and protein location was analyzed by Western blot (Fig. 5a). There was no contamination of mitochondrial (COX IV) or lysosomal (cathepsin B) proteins from the membrane fraction in the cytosolic fraction. However, after treating DU145 cells with LCL204 the active form of cathepsin B was detected in the cytosolic fraction within 30 min. To confirm these findings, enzymatic activity of cathepsin B in the cytosol following LCL204 treatment was measured (Fig. 5b). As expected, low activity was detected in the cytosol of untreated cells. However, cathepsin B activity increased within 30 min of 10 μM LCL204 treatment before returning to basal levels.

LCL204 carries an N-myristoyl-amino group and represents the secondary lipophilic amines, while the parent compound B13, which is otherwise similar in structure, contains an N-myristoyl-group and represents the lipophilic amides, which are neutral molecules. Lysosomotropic compounds can obtain their properties via an amino group within the polar head of the molecule [17], therefore we investigated lysosomal stability after LCL204 treatment using the acidophilic dye LTR and flow cytometric analysis. The results from these experiments are presented graphically to show

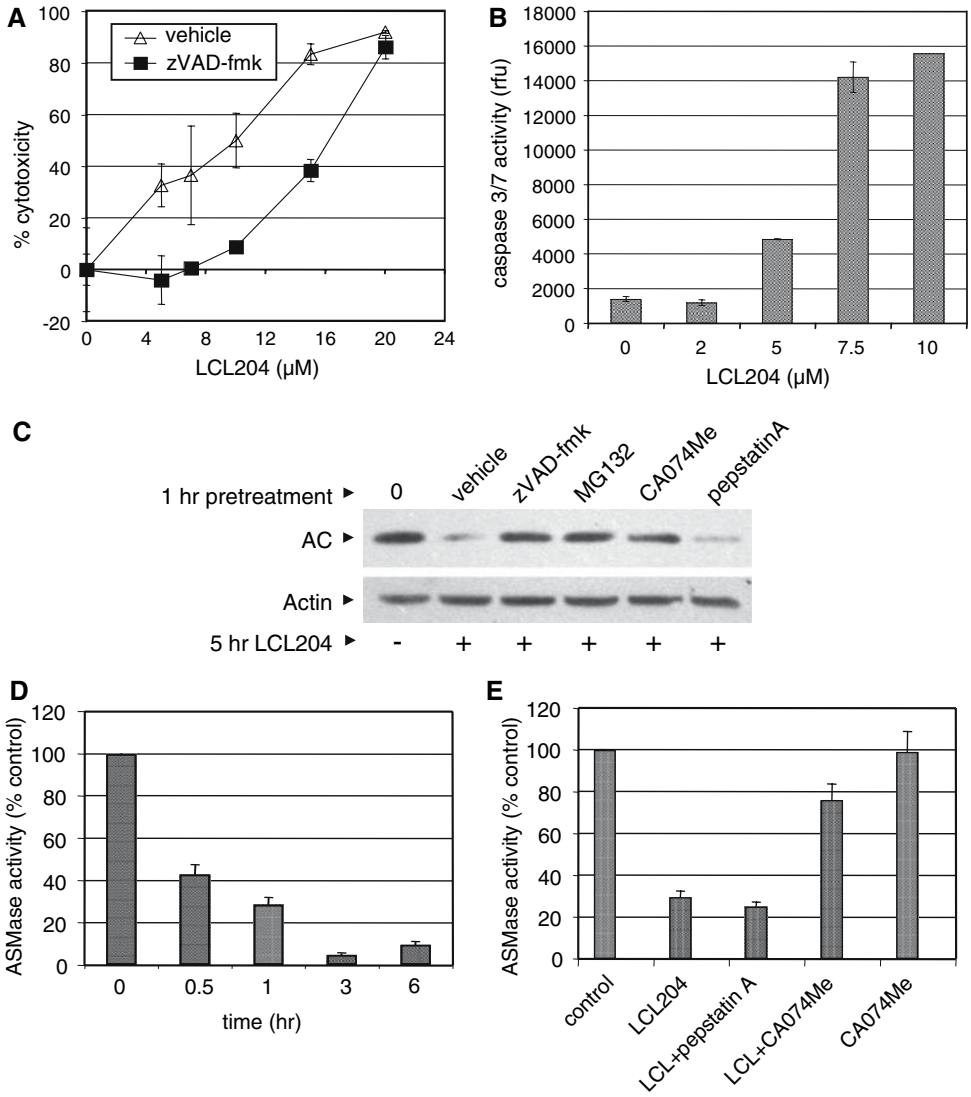

Fig. 4 LCL204-induced cell death involves caspase activity and loss of ASMase activity. **a** DU145 cells were pre-treated for 1 h with vehicle control or zVAD-fmk followed by 24 h treatment with LCL204. Cytotoxicity was measured as in Fig. 1. **b** Caspase 3/7 activities in DU145 cells were measured using a fluorometric activity assay after 24 h treatment with LCL204. **c** DU145 cells were pre-treated for 1 h with vehicle control, zVAD-fmk (50 μM), MG132 (100 nM), CA074Me (10 μM), or pepstatin A (1 μg/ml) followed by LCL204 (10 μM) for 5 h and lysates analyzed by Western Blot for AC expression. **d** ASMase activity was measured in DU145 cells following 10 μM LCL204 treatment for indicated times. **e** ASMase activity was measured in DU145 cells after pre-treatment for 1 h with inhibitors as in **c** followed by addition of 10 μM LCL204 for 2 h. **a**–**c** Results are representative of three independent experiments; **d**–**e** Results are averages from two independent experiments. *Bars*, SD

the mean percentage LTR fluorescence intensity, which is dependent on the acidic pH of lysosomes, therefore a decrease in fluorescence intensity indicates a rise in lysosomal pH [5]. Treatment with 10 mM NH_4Cl served as a positive control. The effect on lysosomal pH was remarkably rapid in DU145 cells, beginning as early as 5 min after treatment with 10 μM LCL204 (Fig. 5c). LCL204 also destabilized lysosomes in a concentration-dependent manner (Fig. 5d). While treating DU145 cells for 1 h with 10 μM LCL204 induced the shift in LTR fluorescence, the same treatment with B13 or C_6-ceramide (30 μM) did not have this effect (Fig. 5e). These data were reproducible in the other PCa cell lines PC-3, PPC-1, and DuPro (not shown).

Treatment with LCL204 induces loss of mitochondrial membrane potential and cytochrome c release

Apoptosis signaling that stems from within the cell typically traverses the intrinsic (type II) apoptotic pathway characterized by loss of mitochondria membrane potential ($\Delta\psi_m$) and the release of cytochrome c from mitochondria into the cytosol [29]. Therefore, we analyzed these parameters in PCa cells in response to LCL204 treatment. We observed a decrease in $\Delta\psi_m$, evident in the shift in fluorescence spectra of the JC-1 dye from 527 to 590 nm, as early as 2 h after treatment with 5 μM LCL204, which continued to decrease over time (Fig. 6a). Cytochrome c was detected in the cytosol 4 h after treatment with 5 or 10 μM LCL204 (Fig. 6b).

LCL204 induces Bak activation

Apoptosis signaling that begins with lysosomal membrane permeabilization (LMP) often involves the mitochondria-dependent intrinsic apoptotic pathway, a process regulated by members of the Bcl-2 family [1, 5, 29]. This led us to investigate the potential roles for the pro-apoptotic Bcl-2 family member Bak in LCL204-induced apoptosis. Bak

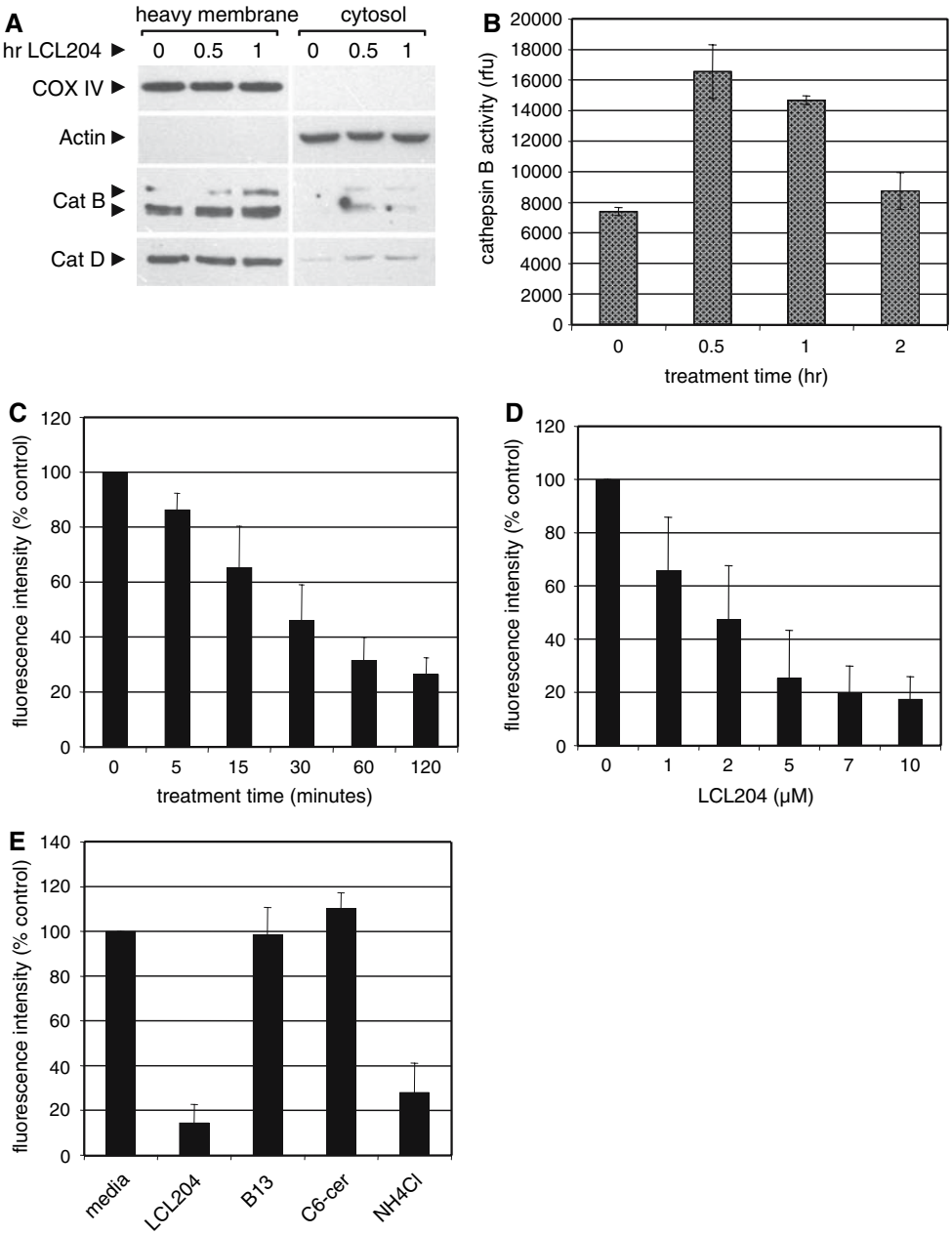

Fig. 5 LCL204 induces lysosomal instability and membrane permeabilization. DU145 cells were treated with 10 μM LCL204 for indicated times and cathepsin B translocation was analyzed by Western blot (**a**) or enzyme activity assay (**b**) using subcellular fractionation. **c**–**e** Lysosomal pH was quantified inversely using LysoTracker Red staining and flow cytometric analysis. Data shown depict the percent change in fluorescence intensity compared to controls and are averages of the results from three independent experiments. **c** DU145 cells treated with 10 μM LCL204 for indicated time points. **d** DU145 cells treated for 1 h with indicated concentrations of LCL204. **e** DU145 cells were treated for 1 h with vehicle control, LCL204 (10 μM), B13 (20 μM), C6-ceramide (30 μM), or NH4Cl (10 mM). Western blot (**a**) is representative of two independent experiments, and Cathepsin activity assay results are averages of three independent experiments. *Bars*, SD

was chosen for these studies instead of Bax, another multi-domain anti-apoptotic protein of the Bcl-2 family, because DU145 cells are null for Bax. Compared to vehicle control, we detected a concentration-dependent up-regulation of Bak protein levels in DU145 cells following a 12-h treatment with increasing concentrations of LCL204 (Fig. 7a). Increased Bak expression was also time-dependent following 10 μM LCL204 treatment (Fig. 7b). Activation of Bak was confirmed using confocal microscopy to visualize formation of Bak mitochondria-associated clusters (Fig. 7c). Formation of Bak foci along the mitochondria is a hallmark of apoptosis [24]. Control cells (left panel) showed even Bak distribution (red) along the mitochondria membrane (green), appearing as yellow in the overlay. However, after 20 h LCL204 treatment we detected formation of Bak foci on the mitochondria membrane, demonstrated by the red Bak clusters interspersed amongst the green-labeled mitochondria membrane marker, YFP-mito (right panel).

Discussion

Alterations in ceramide metabolism pathways are known to contribute to cancer cell resistance to apoptosis and overall malignancy [21, 23, 36, 38, 40]. Therefore, development of strategies to target ceramide pathways may have significant therapeutic potential [18]. In the research presented here we have evaluated the effects of the B13 analog, LCL204, on

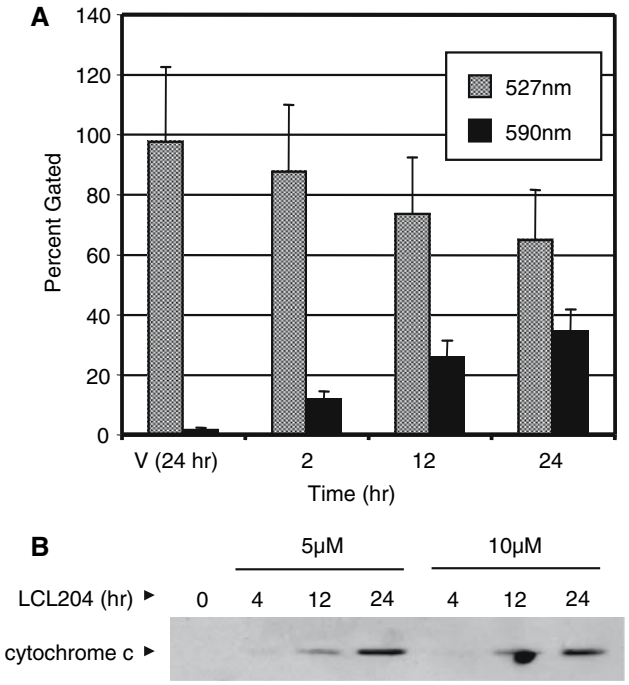

Fig. 6 Mitochondrial membrane potential is decreased and cytochrome c released following LCL204 treatment. **a** Mitochondria membrane potential in DU145 cells was measured after 5 μM LCL204 treatment using JC-1 dye and flow cytometric analysis. A decrease in potential corresponds to a shift in fluorescence from 527 (*gray*) to 590 nm (*black*). **b** Cytochrome c released into the cytosol was measured by Western blot of cytosolic extracts. Results are averages (**a**) or representative (**b**) of two independent experiments. *Bars*, SD

PCa, which has been previously shown to exhibit altered ceramide metabolism [40]. LCL204 had a dose- and time-dependent effect on PCa cell lines with a significant increase in cytotoxicity compared to the parent compound B13, which has been shown previously to elevate ceramide levels in different cancer cell lines [28, 31, 35] (Fig. 1). We observed a decrease in sphingosine levels in PCa cells in response to LCL204 treatment (Fig. 2), suggesting a loss of AC function since AC hydrolyzes ceramide to produce sphingosine. We also observed an increase in ceramide levels in DU145 cells, which is consistent with a loss of AC activity, as there is an accumulation of enzyme substrate (ceramide) and a decrease in product (sphingosine). Similar results were found in leukemia cell lines, although sphingosine levels were not measured in those studies [8, 12]. Analysis of AC protein levels in response to LCL204 treatment revealed that the loss of AC activity was due to loss of the protein itself in a dose- and time-dependent manner, with no effect on mRNA level (Fig. 3). These results indicated a post-transcriptional event, such as proteolytic degradation. We did not detect significant changes in ceramide levels in PPC-1 cells, however the sphingosine levels declined similarly to those in DU145 cells. Ceramide accumulation in this cell line may be kept low by metabolism via different biochemical pathways, such as glucosylceramide synthase. Cytotoxic effects of LCL204 were observed in PPC-1 cells regardless of the increase in measurable ceramide, suggesting the involvement of alternate pathways resulting in cell death in these cells. Additional studies will need to be pursued in order to resolve these disparate results.

In order to determine if cytotoxicity of PCa cells in response to LCL204 treatment involved caspases we pretreated cells with the broad-spectrum caspase inhibitor zVAD-fmk. Although we detected caspase activity and a loss of cytotoxicity in response to LCL204 when cells were pretreated with the caspase inhibitor, these results were gradually reduced with increasing concentrations of LCL204, and were abrogated at 20 μM LCL204 (Fig. 4a, b). In addition, AC degradation in response to LCL204 was at least partially blocked when cells were pretreated with the caspase inhibitor, the protease inhibitor MG132, or the cathepsin B inhibitor CA074Me (Fig. 4c). These results suggest the involvement of caspase-independent apoptotic pathways as well. This is consistent with what has been reported previously for leukemic cell lines [8, 12]. ASMase activity was also reduced in PCa cells in response to LCL204, and was partially restored following pretreatment with CA047Me (Fig. 4d, e). As AC and ASMase are known to reside in the lysosome, we hypothesized the observed effects in response to LCL204 included lysosomal degradation.

These results led us to investigate the role of cathepsin B in PCa cells in response to LCL204. We observed increased cathepsin B activity and its translocation to the cytosol in response to LCL204 treatment. Analysis of lysosomal pH in response to LCL204 showed a dose- and time-dependent increase in lysosomal pH, which was not observed with B13 or a short-chain ceramide mimic (Fig. 5). Thus, LCL204 not only rapidly elevates lysosomal pH but also affects the membrane integrity of the lysosomes as indicated by translocation of cathepsin B to the cytosol. One major structural difference between B13 and LCL204 is that B13 does not have an amino group. The apoptotic consequences of LCL204-induced lysosomal rupture could not be reproduced with other agents that strictly alter lysosomal pH such as bafilomycin A_1 or NH_4Cl (not shown), suggesting that the hydrophobic lipid structure of LCL204 may be necessary for exerting these effects. Similar observations were made for other aromatic N-alkyl-amino—analogs of LCL204 (Z. M. Szulc et al., Submitted for publication). Collectively, these experiments strongly suggest that the amino group carried by LCL204 confers lysosomotropic properties to the molecule, placing it under the umbrella of amphiphilic drugs. As apoptosis signaling following LMP often follows the intrinsic apoptotic pathway, we analyzed parameters of this pathway as well as involvement of the

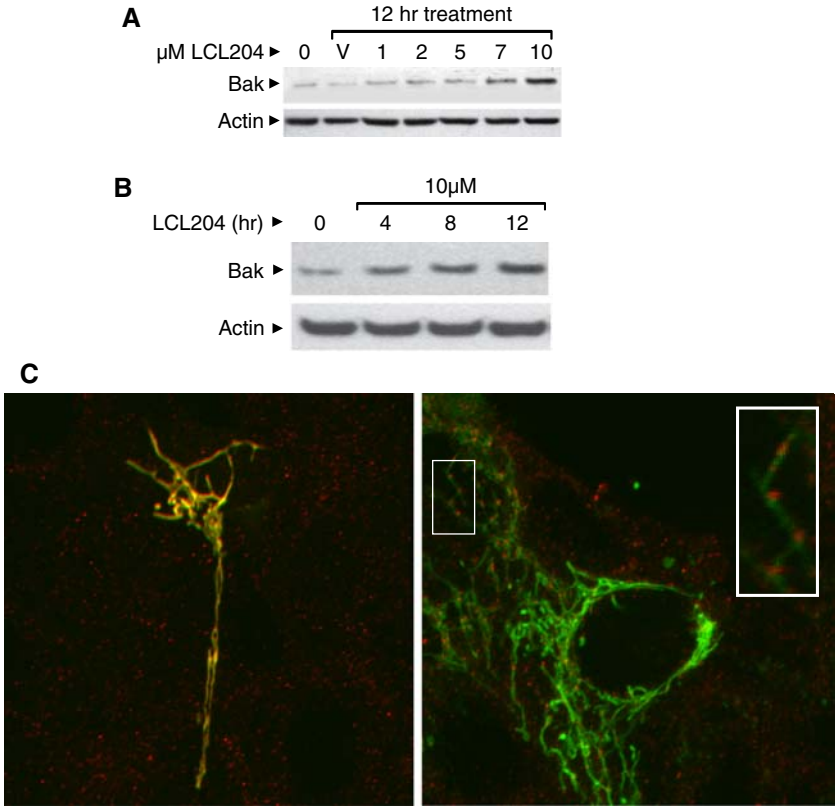

Fig. 7 LCL204 induces pro-apoptotic Bcl-2 family members and formation of Bak foci. DU145 cells were treated for 12 h with indicated concentrations of LCL204 (**a**) or with 10 μM LCL204 for indicated time (**b**) and cell lysates were analyzed by Western blot for Bak expression. **c** Visualization of LCL204-induced Bak foci using confocal microscopy. DU145 cells transfected with YFP-mito (*left panel*) were treated with 15 μM LCL205 (*right panel*). Bak was immunostained *red*, while mitochondria showed *green* fluorescence. Overlay of the two is *yellow*. Large inset: zoom of Bak foci in smaller inset. *V* vehicle only. Results are representative of three independent experiments

pro-apoptotic Bcl-2 family member Bak. We observed a loss of mitochondrial membrane potential and release of cytochrome c into the cytosol in response to LCL204, confirming involvement of the intrinsic apoptotic pathway (Fig. 6). In addition, we observed a dose- and time-dependent increase in Bak concentration following LCL204 treatment and the formation of Bak foci, an observation consistent with cells undergoing apoptosis [24] (Fig. 7). Bak can be activated by a number of different mechanisms ranging from elevated ER Ca^{2+} levels [26, 33] to kinase fragments [22] to Bid cleavage [7, 41]. It has been reported previously that Bak is sequestered by Mcl-1 and Bcl-xL, and that following activation of the BH3-only pro-apoptotic family members, Bak is displaced from Bcl-xL or Mcl-1 and self-associates, which leads to mitochondrial membrane permeabilization [43]. Thus, treatment with LCL204 releases Bak from its associated BH3-only family member(s), resulting in its activation and subsequent mitochondrial membrane permeabilization.

Targeting altered sphingolipid metabolism pathways in order to reset intrinsic apoptotic mechanisms represents a unique therapeutic strategy for treatment of PCa. Collectively, the results presented here show that the AC inhibitor LCL204 induces apoptosis in PCa cells via multiple pathways, and with more dramatic results than the parent compound B13. The fact that LCL204 activates multiple pathways makes it a potent cytotoxic agent for destroying cancer cells. This is apparent in the PCa cell line PPC-1 where, although ceramide levels were not measurably increased, LCL204 still had a toxic effect. The overall toxicity levels of LCL204 against a normal prostate epithelial cell line were lower (LD_{50} 16–18 μM) than the averages of all PCa cell lines tested (LD_{50} 7–12 μM) (data not shown). Additionally, preliminary in vivo studies found LCL204 to have no ill side effects in mice by intraperitoneal injections at concentrations up to 75 mg/kg body weight [20]. This may indicate LCL204 has a higher toxicity against malignant cells than normal cells. Although the inhibition of AC by LCL204 was anticipated, the complete degradation of AC was surprising. The tricyclic antidepressant desipramine is known to induce degradation of ASMase, which can be blocked using the protease inhibitor leupeptin [10, 14]. It was proposed that desipramine potentially induces a conformational change in ASMase, which is anchored within the lysosomal membrane. This was thought to result in exposure of proteolytic cleavage site(s) to the lysosomal lumen, allowing for its degradation by lysosomal proteases. A similar scenario could support LCL204-induced AC/ASMase degradation in PCa cells, as both LCL204 and desipramine are lipophilic aromatic N-alkylamines.

Based on the results reported here, we conclude that treatment of PCa cells with LCL204 leads to increased ceramide levels and activation of apoptotic pathways. Loss of lysosomal membrane integrity leads to release of cathepsin B

into the cytosol and consequently mitochondrial membrane permeabilization and cytochrome c release. These events culminate in the enzymatic activation of caspase 9 and 3. In addition, amplification loops may be involved as well. Further studies to elucidate the pathways and mediators activated in response to LCL204 in PCa cells and the potential of LCL204 to treat PCa in vivo are ongoing.

Acknowledgements We thank Rick Peppler of the MUSC Flow Cytometry Facility for acquisition of flow cytometry data. We would also like to thank the MUSC Lipidomics Core for the synthesis of sphingolipid reagents and sphingolipid analysis. This work was supported by NIH/NCI PO1 CA97132 and HCC/DOD N6311601MD10004.

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