LCL124, a Cationic Analog of Ceramide, Selectively Induces Pancreatic Cancer Cell Death by Accumulating in Mitochondria^S

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ABSTRACT

Treatment of pancreatic cancer that cannot be surgically resected currently relies on minimally beneficial cytotoxic chemotherapy with gemcitabine. As the fourth leading cause of cancer-related death in the United States with dismal survival statistics, pancreatic cancer demands new and more effective treatment approaches. Resistance to gemcitabine is nearly universal and appears to involve defects in the intrinsic/mitochondrial apoptotic pathway. The bioactive sphingolipid ceramide is a critical mediator of apoptosis initiated by a number of therapeutic modalities. It is noteworthy that insufficient ceramide accumulation has been linked to gemcitabine resistance in multiple cancer types, including pancreatic cancer. Taking advantage of the fact that cancer cells frequently have more negatively charged mitochondria, we investigated a means to circumvent resistance to gemcitabine by targeting delivery of a cationic ceramide (L-t-C6-CCPS [LCL124: ((2S,3S,4E)-2-N-

[6'-(1"-pyridinium)-hexanoyl-sphingosine bromide)]) to cancer cell mitochondria. LCL124 was effective in initiating apoptosis by causing mitochondrial depolarization in pancreatic cancer cells but demonstrated significantly less activity against non-malignant pancreatic ductal epithelial cells. Furthermore, we demonstrate that the mitochondrial membrane potentials of the cancer cells were more negative than nonmalignant cells and that dissipation of this potential abrogated cell killing by LCL124, establishing that the effectiveness of this compound is potential-dependent. LCL124 selectively accumulated in and inhibited the growth of xenografts in vivo, confirming the tumor selectivity and therapeutic potential of cationic ceramides in pancreatic cancer. It is noteworthy that gemcitabine-resistant pancreatic cancer cells became more sensitive to subsequent treatment with LCL124, suggesting that this compound may be a uniquely suited to overcome gemcitabine resistance in pancreatic cancer.

Introduction

Pancreatic tumors are notoriously treatment resistant (Jaffee et al., 2002), and pancreatic cancer is predicted to

affect 43,920 patients and cause 37,390 deaths in 2012 (www.cancer.gov), making it the fourth leading cause of cancer-related death in the United States. Gemcitabine (GMZ) has been the standard treatment of advanced pancreatic cancer for the past decade (Rao and Cunningham, 2002; Van Cutsem et al., 2004) based on marginal improvement in disease-related symptoms and minimal survival benefit over 5-fluorouracil (5-FU; 5.6 vs. 4.4 months); however, resistance develops rapidly in almost all patients (Burris et al., 1997). Recently, a regimen consisting of oxaliplatin, irinotecan, fluorouracil, and leucovorin (Folfinirinox) was compared with GMZ, resulting in an overall survival of 11.1 months compared with 6.8 months with GMZ. Unfortunately, this

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ABBREVIATIONS: C6-CCPS, L-t- ω -pyridinium C6-ceramide; 5-FU, 5-fluorouracil; ECAR, extracellular acidification rate; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazide; GMZ, gemcitabine; HNSCC, head and neck squamous cell carcinoma; JC-1, 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazole-carbocyanine iodide; LCL87, L-t-C16- ω -CCPS; LCL89, L-t-C12- ω -CCPS; LCL124, ((2S,3S,4E)-2-N-[6'-(1"-pyridinium)-hexanoyl-sphingosine bromide]; MALDI, matrix-assisted laser desorption ionization; MG132, *N*-benzoyloxycarbonyl (Z)-Leu-Leu-leucinal; MS, mass spectrometry; mt-DNA, mitochondrial DNA; OCR, oxygen consumption rate; PARP, poly(ADP-ribose) polymerase; S1P, sphingosine 1-phosphate; TMRM, tetramethylrhodamine methyl ester; $\Delta\psi_m$, mitochondrial membrane potential; ψ , electrical potential in millivolts.

regimen represents only a marginal improvement, because it improved survival but increased toxicity compared with GMZ in the phase III trial (Conroy et al., 2011).

Cancer cells have been shown to have a shift in the balance between proapoptotic ceramide and antiapoptotic sphingosine 1-phosphate (S1P), often favoring production of oncogenic S1P. This phenomenon is associated with cancer progression and poor therapeutic outcomes (Ogretmen and Hannun, 2004; Liu et al., 2009; Beckham et al., 2010). Similar to other cancers, dysregulation of sphingolipid metabolism has been observed in pancreatic cancer (Yu et al., 2003). Further studies suggest that ceramide generation and accumulation is a critical determinant of pancreatic cancer cell apoptosis in response to cytotoxic agents, including GMZ (Modrak et al., 2004, 2009). Likewise, enhanced expression of enzymes involved in the catabolism of ceramide (and, frequently, production of S1P) contributes to drug resistance in pancreatic cancer (Modrak et al., 2006). In another study, response to treatment of the ceramide to S1P ratio was correlated with the sensitivity and, conversely, the resistance of pancreatic cancer cells to GMZ (Guillemet-Guibert et al., 2009). Whereas cell lines with a low ceramide to S1P ratio required high concentrations of GMZ to induce apoptosis, cell lines with more favorable ceramide to S1P ratios were up to 10-fold more sensitive. Significantly, it was shown that Bcl-xl and inhibition of the mitochondrial apoptosis pathway played a primary role in resistance to GMZ-induced pancreatic cell apoptosis (Schniewind et al., 2004). These data suggest that mitochondrial apoptosis and a favorable sphingolipid response to treatment are necessary components of GMZ-induced cell death in pancreatic cancer. Furthermore, these data highlight the potential of manipulating these pathways to overcome the resistance of pancreatic cancer to current therapy.

The cationic ceramides (L- ω -pyridinium Cn-ceramide, generally termed Cn-CCPS) were designed to preferentially localize into negatively charged intracellular compartments because of the positive charge created by the pyridinium ring (Szulc et al., 2006). Many types of cancer cells have more negatively charged mitochondria (Chen, 1988; Modica-Napolitano and Aprile, 2001) compared with normal cells. It is reasonable to predict that cationic ceramides would preferentially accumulate in the mitochondria of cancer cells based on their increased negative charge. Indeed, the efficacy of cationic ceramides on tumor regression has been confirmed in multiple tumor models (Novgorodov et al., 2005; Senkal et al., 2006; Dahm et al., 2008). Specifically, LCL29 and 124 (D-e- and L-t-stereoisomers of C6-CCPS) (Senkal et al., 2006; Szulc et al., 2006) have been shown to have antiproliferative effects in MCF7 and head and neck squamous cell carcinoma (HNSCC) cell lines (Rossi et al., 2005). The synergistic effects of LCL124 in combination with GMZ on the inhibition of cell growth were also demonstrated in HNSCC cells in vitro (Rossi et al., 2005) and on HNSCC tumors in vivo (Senkal et al., 2006). Because resistance to GMZ in pancreatic cancer leads to poor management of the disease, developing new therapeutic agents that can bypass this resistance is desirable. In this study, we compared the growth inhibitory and cell death properties of three *N*-acyl-chain length *L*-*threo*-homologs on pancreatic cells: LCL124 (C6-CCPS), LCL89 (C12-CCPS), and LCL87 (C16-CCPS). We conclusively demonstrate that LCL124 is able to target mitochondria in a potential

dependent manner and selectively induce pancreatic cancer cell death at 4–28-fold lower concentration than in normal cells. It is noteworthy that, although most human pancreatic cancer lines are resistant to 5-FU or GMZ, they are uniformly sensitive to LCL124. The accumulation of LCL124 in mitochondria causes a decrease in mitochondrial membrane potential, leading to cytochrome *c* release and apoptosis. Unlike in HNSCC (Senkal et al., 2006), there was no synergistic effect observed with LCL124 combined with GMZ under in vitro conditions; however, GMZ-resistant cells became severalfold more sensitive to LCL124-induced cell killing, augmenting its potential as a candidate to circumvent GMZ resistance in pancreatic cancer.

Materials and Methods

Cell Lines, Culture, and Reagents. Aspc-1, MIA, Panc-01, and SK-MES pancreatic cancer cell lines (ATCC; Manassas, VA) and Panc-02 (a kind gift from Dr. Cole at the Medical University of SC) were regularly cultured at 37°C in 5% CO₂ in RPMI 1640 medium and DMEM (Thermo Scientific HyClone; Thermo Scientific, Logan, UT) containing 10% bovine growth serum (Thermo Scientific HyClone) and 1% penicillin/streptomycin solution (Mediatech; Manassas, VA). DT-PD59 cells were kindly provided by Dr. Ouellette at University of Nebraska Medical Center and have been described previously (Lee et al., 2005). Gemcitabine and 5-FU were obtained from Sigma-Aldrich (St Louis, MO). Cn-CCPS LCL124, LCL89, LCL87, and 17C-LCL124 were synthesized using the MUSC Lipidomics Shared Resources (Charleston, SC) as previously described (Szulc et al., 2006). LCL124 and 17C-LCL124 were prepared directly in sterilized phosphate-buffered saline (PBS) containing 137 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, and 2 mM KH₂PO₄, pH 7.4). LCL89 and LCL87 were dissolved in sterilized PBS (described as above) ethanol 4/1 (v/v) to final stock solution of 20 mM and stored at –20°C.

Carbonylcyanide *p*-trifluoromethoxyphenylhydrazone (FCCP) was obtained from Prof. Beeson at Medical University of South Carolina. Antibodies used include poly(ADP-ribose) polymerase (PARP) (Santa Cruz Biotechnology; Santa Cruz, CA), cytochrome *c* (Cell Signaling Technology, Beverly, MA), caspase 3 (PharMingen, San Diego, CA), anti-rabbit IgG-HRP (Santa Cruz Biotechnology), and anti-mouse IgG-HRP (Santa Cruz Biotechnology).

MTS Cytotoxicity Assays. Cell viability was assessed using the CellTiter 96 Aqueous One Solution Cell Proliferation Assay Kit (Promega; Madison, WI). Cells were plated at 5×10^3 cells per well in 96-well plates and incubated overnight. The following day, media were replaced with desired treatment, and after incubation, the assay was performed in accordance with the manufacturer's instructions. EC₅₀ was calculated using Prism 4 software (GraphPad Software, Inc., San Diego, CA). For experiments using inhibitors, cells were pretreated with inhibitors for 1 hour (FCCP for 15 minutes) at 37°C before adding media containing vehicle or LCL124. The remainder of the assay was performed as described above.

Mitochondrial Fractionation. A total of 5×10^6 cells were seeded per 150-mm dish. Treatment was initiated when cells reached 90% confluence, and cells were collected at the indicated times. Cell fractionation was performed using the Mitochondrial Fractionation Kit (Active Motif North America, Carlsbad, CA) according to the manufacturer's instructions, and the purity of the fractions was examined by Western blot with use of glyceraldehyde-3-phosphate dehydrogenase and cytochrome *c*.

Western Blotting. Cells were seeded in 60-mm plates as described above and treated as indicated. Cells were lifted by gently scraping the plates, washed once with ice-cold PBS and then lysed by incubation on ice for 30 minutes in radioimmunoprecipitation assay buffer with Complete Mini Protease Inhibitor Cocktail Tablet (Roche; Indianapolis, IN). Insoluble material was removed by centrifugation

at 14,000 rpm for 20 minutes at 4°C. The supernatants were supplemented with sodium dodecyl sulfate at a final concentration of 2% and stored at -80°C. Protein concentration was determined using the BCA Protein Assay kit (Pierce; Rockford, IL) according to the manufacturer's instructions. Protein lysates (50 µg per sample unless otherwise indicated) were resolved on NuPAGE 4–12% Bis-Tris gels (Life Technologies, Carlsbad, CA) and transferred to nitrocellulose membranes. Target proteins were detected using the indicated antibodies and Millipore Chemiluminescent HRP substrate (Millipore Corporation, Billerica, MA).

Cytochrome c Release. Aspc-1, MIA, and DT-PD59 cells were treated with LCL124 at indicated concentrations and time points before cell fractionation. Cytosolic fractions were obtained and analyzed for cytochrome c by immunoblotting as described above.

Live-Cell Mitochondrial $\Delta\Psi$ m Microscopy. Cells grown in 35-mm glass-bottom dishes (MatTek Corporation, Ashland, MA) were incubated in regular growth medium with 250 nM tetramethylrhodamine methyl ester (TMRM, kindly obtained from Dr. Beeson at the Medical University of South Carolina) for 30 minutes. The cells were then rinsed in prewarmed PBS, and fresh medium containing 50 nM TMRM was replaced. Ten minutes later, live cell images were acquired on an Olympus FV10i LIV laser scanning confocal microscope (Olympus, Tokyo, Japan) at 37°C. TMRM was excited at 543 nm, and the resulting fluorescence was collected with an emission barrier filter of 590 ± 25 nm. An image of background of each field of view was acquired by focusing on the coverslip. Acquired images were analyzed using Adobe Photoshop, and relative electrical potential of the mitochondria was calculated using the following formula: $\Psi = -59 \times \log(F_{in}/F_{out})$, where Ψ is electrical potential in millivolts, F_{out} is the average fluorophore concentration in the extracellular space (electrical ground), and F_{in} is the average fluorophore concentration at any point within the cell. To display the distribution of the Ψ , colors were assigned to specific millivolt ranges of the Ψ , and a pseudocolored image was created (Lemasters and Ramsdesh, 2007).

Measurement of Mitochondrial Membrane Depolarization by JC-1 Staining. Mitochondrial membrane potential ($\Delta\Psi$ m) was measured qualitatively using the lipophilic fluorescent probe 5, 5', 6, 6'-tetrachloro-1,1', 3,3'-tetraethylbenzimidazol-carbocyanine iodide (JC-1; Cayman Chemicals, MI). A total of 8×10^5 Aspc-1 and MIA cells were plated on 60-mm dishes. After overnight incubation, cells were treated with the indicated compounds in fresh medium containing 5% bovine growth serum. Two hours after treatment, cells were washed with PBS, and $\Delta\Psi$ m was examined using a JC-1 Assay Kit according to manufacturer's instructions. JC-1 fluorescence was measured using a Becton Dickinson FACScalibur analytical flow cytometer (BD Biosciences, San Jose, CA) in the MUSC Hollings Cancer Center Flow Cytometry & Cell Sorting Core Facility. The ratio of red (530 nm) to green (590 nm) fluorescence of JC-1 was calculated.

Measurements of Oxygen Consumption. Oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) were measured in real time with use of a Seahorse Bioscience XF24 extracellular flux analyzer (Seahorse Bioscience, North Billerica, MA), as previously described (Beeson et al., 2010). On the day before the experiment, the sensor cartridge was placed in the calibration buffer supplied by Seahorse Bioscience to hydrate overnight. After optimization of cell number, cells were seeded in XF 24-well microplates (35,000/well for Aspc-1, 40,000/well for MIA, and 22,000/well for DT-PD59). After an overnight incubation, assays were initiated by replacing the growth medium with 800 µl of assay medium (50:50 mixture of Dulbecco's modified Eagle's essential medium and Ham's F12 nutrient mix without phenol red supplemented with 15 mM NaHCO₃, 0.2 mM glycine, and 6 mM sodium lactate). After establishing baseline OCRs, LCL124 was introduced at injection A, and measurements continued for 90 minutes. Additional measurements were performed after injection of rotenone (final concentration 1 µM) at injection B. For any one treatment, the rates from three or four wells were used.

High-Performance Liquid Chromatography-MS/MS Analysis. Analyses of sphingolipid species and test compound were performed using the MUSC Lipidomics Shared Resources on a Thermo Finnigan TSQ 7000 (Thermo Fisher Scientific, Waltham, MA), triple-stage quadrupole mass spectrometer operating in a Multiple Reaction Monitoring positive ionization mode as described elsewhere (Bielawski et al., 2006). In brief, samples were fortified with internal standards and extracted into a one-phase solvent system with ethyl acetate/isopropanol/water (60%:30%:10%, v/v). Four milliliters was separated, followed by evaporation under nitrogen. After reconstitution in 100 µl of acidified (0.2% formic acid) methanol, samples were injected on the HP1100/TSQ 7000 LC/MS system and gradient-eluted from the BDS Hypersil C8, 150×3.2 mm, 3 µm particle size column, with 1.0 mM methanolic ammonium formate/2 mM aqueous ammonium formate mobile phase system. Peaks corresponding to the target analytes and IS were collected and processed using the Xcalibur software (Xcalibur, Inc., Arlington, VA).

Tumor Xenografts. Pathogen-free 4-week-old female Athymic NCr-nu/nu mice were purchased from the National Cancer Institute Frederick Cancer Research Center (Frederick, MD). The mice were maintained under standard conditions according to the institutional guidelines for animal care. All animal experiments were approved by the Committee for the Care and Use of Laboratory Animals of Medical University of South Carolina; 5×10^6 Aspc-1 cells in 100 µl PBS were injected subcutaneously in right flanks. Tumor formation was monitored twice per week by measuring the width, depth, and length of the mass, and tumor volume was calculated by the equation v (mm³) = $\pi/6 \times (a \times b \times c)$, or $v = \pi/6 \times a \times b^2$, with a as the smallest diameter and b as the largest, if depth is not measurable. After tumors reached a mean volume of at least 100 mm³, animals were treated every other day for 2 weeks with PBS and LCL124 (40 mg/kg) via intraperitoneal injection. Animals were weighed two times per week, and tumor size was evaluated by digital caliper measurements.

MALDI-MS Tissue Imaging. Tissue sections from LCL124-treated and untreated xenograft tumors (10 µm thick) were cut on a cryostat (Thermo Microm HM550), thaw mounted on Bruker ITO conductive slides, washed with 100 mM ammonium acetate, and placed in a vacuum dessicator for 5 minutes before matrix application. Tissue slices were coated with 2, 5-dihydroxybenzoic acid (DHB) matrix at 30 mg/ml in 50% methanol and 1% TFA with use of an Image Prep (Bruker Daltronics, Billerica, MA). ImagePrep matrix thickness was closely monitored and tailored to our matrix of choice. After matrix application and before MS analysis, the slides were placed in the dessicator for 15 minutes. Mass spectra were acquired using a Bruker Solarix 7T Dual Source MALDI/electrospray ionization Fourier transform ion cyclotron resonance with the laser focused to a diameter of 250 µm. Data were acquired and analyzed using Flex Imaging 3.0 software (Bruker Daltonics, Billerica, MA). Mass spectra were accumulated from 200 laser shots acquired from each spot on the tissue within the m/z 200–1500 range. Distribution of LCL124 within the tumor tissue was done using the CASI function (continuous accumulation of selected ions), which allows specific isolation of the 475.4 ion within an m/z 2 range. The spectra were normalized within the Flex Imaging software with use of root mean squares. Collision-induced fragmentation of the m/z 475.4 drug metabolite ion was done in the external quadrupole of the FT-ICR instrument. The fragmentation pattern for purified LCL124 was obtained by mixing the sample 1:1 in 50% acetonitrile and direct injection via electrospray into the FT.

Statistical Analysis. Statistical analyses were performed by one-way analysis of variance and unpaired one-tailed t test, using Prism (version 4.0) from GraphPad Software, Inc. The level of significance was set at $P < 0.01$ and $P < 0.05$ in the figures. $P < 0.05$ was considered to be statistically significant.

Results

Induction of apoptosis is generally divided into the extrinsic death receptor pathway and the intrinsic mitochondrial

pathway, with the latter accounting for most chemotherapy-induced apoptosis. One mechanism of GMZ-induced apoptosis requires activation of the intrinsic pathway with depolarization of the inner mitochondrial transmembrane potential ($\Delta\Psi_m$) with concomitant release of cytochrome *c* and activation of downstream executioner caspases. Development of an impaired intrinsic apoptotic pathway in tumors results in resistance to GMZ and, ultimately, unsuccessful management of pancreatic cancer (Friess et al., 1998; Xu et al., 2002; Schniewind et al., 2004). To improve therapeutic efficacy to pancreatic cancer, a group of drugs was designed to bypass upstream GMZ resistance by directly targeting mitochondria. Three candidate compounds that differ by the length of their *N*-acyl-chain (LCL124: C6-CCPS, LCL89: C12-CCPS, and LCL87: C16-CCPS, previously described [Szulc et al., 2006]) were tested in this study.

LCL124 Preferentially Kills Tumor Cells. To test the efficacy of cationic ceramides in pancreatic cancer, the EC_{50} (drug concentration effecting 50% cell death) of the three candidate compounds was determined based on the MTS assay. As depicted in Fig. 1, all 3 compounds had an inhibitory effect on Aspc-1 and MIA cells. MIA were most sensitive to LCL124 (EC_{50} , 2.295 μ M) (Fig. 1A), and Aspc-1 cells were the most sensitive to LCL89 (EC_{50} , 10.80 μ M) (Fig. 1B). Toxicity from chemotherapy is frequently attributable to its lack of tumor specificity, making tumor-targeted delivery of anti-cancer drugs one of the most important steps for the development of chemotherapeutic agents. Because cationic ceramides are designed to target more negatively charged mitochondria in tumor cells, we aimed to test their tumor

selectivity by using the immortalized normal pancreatic cell line DT-PD59. As shown in Fig. 1C, although DT-PD59 cells exhibited greater EC_{50} to LCL124 (EC_{50} , 62.75 μ M), which indicated a wider selectivity window, particularly against cancer cell lines, compared with normal cells, the EC_{50} of DT-PD59 to LCL89 and LCL87 (14.76 and 9.511 μ M, respectively) was nearly equivalent to the EC_{50} in tumor cells (MIA: 9.993 and 10.69 μ M, respectively; Aspc-1: 10.80 and 23.68 μ M, respectively). Of note, there was no apparent dose response when cells were treated using LCL89 and LCL87, compared with LCL124, potentially because of their poor solubility. In fact, LCL124 is readily soluble in water, whereas the other two compounds required an organic cosolvent. These data led us to choose LCL124 as our lead compound for further study.

LCL124 Is a Potent Agent for Killing Pancreatic Cells. Individual cancer cell lines have different disruptions in cell death and survival pathways that result in nonuniform response to therapeutic regimens. To test the efficacy of LCL124 for treatment of pancreatic cancer, the sensitivity of a panel of pancreatic cancer cell lines MIA, Aspc-1, Panc-01, and SK-MES1 to LCL124 and nonmodified C6-ceramide was evaluated. As shown in Fig. 1D, there is a wide variation of response to C6-ceramide in these cells, likely because of differences in expression, regulation, and functionality of apoptosis effectors. It is noteworthy that there was a much narrower range of sensitivity to LCL124, with MIA being the most sensitive and Aspc-1 being the least. The sensitivity to LCL124 is substantially higher than that to the nonmodified C6-ceramide, suggesting increased potency and uniform

Fig. 1. Cationic ceramides candidates induce cell death in pancreatic cancer cells. Growth inhibitory effects of cationic ceramides in MIA (A), Aspc-1 (B), and immortalized DT-PD59 normal cells (C) were assessed by MTS assay after 48 hours of treatment of cells with increasing concentrations of the indicated compounds. (D) EC_{50} values for pancreatic cell lines to C6-ceramide and LCL124. EC_{50} values were obtained from 10 concentrations based on individual drug, with four replicates for each cell line. EC_{50} was calculated and graphed using Prism 4.

responsiveness to LCL124 regardless of existing defects in death pathways.

LCL124 Accumulates in Mitochondria and Disrupts Cellular Respiration. To examine the intracellular distribution of LCL124, Aspc-1 and Panc02 pancreatic cells were treated with 20 μ M LCL124 for 24 hours. Cells were then fractionated into mitochondrial, cytosolic, and nuclear compartments with purity assessed by presence and absence of cytochrome *c*, glyceraldehyde-3-phosphate dehydrogenase, and lamin B (Fig. 2B). Fractions were then analyzed for LCL124. As shown in Fig. 2A, the mitochondrial compartment accumulated substantially more LCL124 than did the nucleus

or cytoplasm. At 24 hours, the nuclear compartment, which has a slight negative charge (Aronov et al., 2004), was also accumulating drug but only at half the concentration of that in the mitochondria. A similar result was observed in Panc02 cells (Supplemental Fig. 1A), in which we also observed accumulation of endogenous total ceramide in the cell mitochondria, whereas total cellular ceramide did not change appreciably (Supplemental Fig. 1B). To determine whether LCL124 is metabolized by endogenous enzymes, we treated cells with 17-Carbon LCL124 (instead of endogenous sphingosine, which has 18 carbons) and measured C17-sphingolipid species from 15 minutes to 24 hours after treatment. It is

Fig. 2. LCL124 accumulates in mitochondria and reduces mitochondrial respiration. Aspc1 cells were treated with 20 μ M LCL124. Cells were washed and collected at indicated time points. Nuclear, cytosolic, and mitochondrial fractions were isolated. (A) fractionated lysates (normalized by protein concentration) were analyzed for the level of LCL124 by mass spectrometry. (B) purity of cell fractions was examined by Western blot. OCR in Aspc-1 (C) and MIA (D) was determined by Seahorse XF-24 Metabolic Flux analyzer. Vertical lines indicate time of addition of (a) LCL124 or (b) rotenone (1 μ M) (* P < 0.05, ** P < 0.01 versus the vehicle control group by Student's *t* test). Data are represented as mean $\pm$ S.D.

noteworthy that LCL124 progressively accumulated inside the cells as expected, but no C17 metabolites were detected in cells or in the medium (Supplemental Fig. 1C). Thus, we conclude that LCL124 is not metabolized by cancer cell enzymes.

Although cancer cells frequently use glycolysis for ATP production (Dang, 2010), functional mitochondria appear to be crucial for cancer cell survival, and disruption of mitochondrial function has been suggested as a potential avenue for cancer therapy (Pilkington et al., 2008). Here, we proceeded to determine the effect of LCL124 treatment on mitochondrial bioenergetics (OCR and ECAR) with use of MIA, Aspc-1, and DT-PD59 cells. We first observed that basal OCR was approximately two-fold higher in MIA and Aspc-1 cancer cell lines, compared with DT-PD59 normal cells (data not shown), which indicates that the mitochondria in cancer cells are more metabolically active than are the mitochondria in normal cells. Furthermore, the decrease in OCR in the presence of LCL124 is consistent with LCL124 targeting mitochondria and, thereby, disrupting aerobic respiration. As shown in Fig. 2, C and D, OCR decreased in a dose-dependent manner within 30 minutes after administration of LCL124 in both Aspc-1 and MIA cells.

LCL124 Causes Mitochondrial Depolarization and Apoptosis in Pancreatic Cancer Cells. On the basis of the believed mechanism of action of this class of drugs (Szulc et al., 2006), we were interested to see whether a decrease in $\Delta\Psi_m$ could be detected in cells treated with cationic ceramides. JC-1 exhibits potential-dependent accumulation in mitochondria of living cells, leading to the formation of red

fluorescent JC-1 aggregates. If mitochondria are depolarized, there is a reduction in red staining after monomer binding that results in green fluorescence. In our study, LCL124-treated cells exhibited a dramatic dose-dependent decrease in their mitochondrial potential in both Aspc-1 (Fig. 3A) and MIA (Fig. 3B) cells, indicating depolarization. In contrast, GMZ-treated cells had a minimal decrease in potential (Fig. 3; Supplemental Fig. 2). These results suggest that these cells have defects in intrinsic apoptosis in response to GMZ that are overcome by treatment with LCL124. MIA and Aspc-1 cancer cells also demonstrate dose-dependent cytosolic accumulation of cytochrome *c*, consistent with depolarization of mitochondria. Of note, the normal cells DT-PD59 do not demonstrate LCL124-induced release of cytochrome *c*, consistent with the hypothesis that this drug is selective for the more negatively charged mitochondria of cancer cells (Fig. 3C).

Having determined that LCL124 induces mitochondrial depolarization, we sought to more fully analyze the mechanism of LCL124-induced cell death. Pretreatment of MIA cells with either an inhibitor of ceramide synthase, fumonisin B1 (FB1), or an inhibitor of serine palmitoyltransferase, myriocin, suppressed LCL124-mediated cell death (Supplemental Fig. 3A), suggesting that LCL124 exerts its effects on cell death in part through elevation of cellular ceramide. A caspase 3/7 activity assay showed a 20-fold increase in caspase 3/7 activity after LCL124 treatment, compared with untreated cells, suggesting critical involvement of caspases in LCL124-induced cell death (Supplemental Fig. 3B). Treatment of MIA and Aspc-1 pancreatic cell lines with LCL124 in

Fig. 3. Loss of mitochondrial membrane potential in pancreatic cell lines after LCL124 treatment: Aspc-1 (A) and MIA (B) cells were incubated in the presence of indicated concentration of LCL124 or GMZ for 2 hours, and mitochondrial depolarization was determined by JC-1 flow cytometry. Bar graph represents percentage of green fluorescent cells and red fluorescent cells. (C) MIA, Aspc-1, and DT-PD59 cells were treated with increased doses of LCL124. Twenty-four hours after treatment, cells were collected and the cytosolic fraction was analyzed by Western blot. Data are representative of three independent experiments. Cyt-C, cytochrome *c*; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

combination with the proteasome inhibitor *N*-benzoyloxycarbonyl (*Z*)-Leu-Leu-leucinal (MG132), a cathepsin B inhibitor CA074me or a pan-caspase inhibitor ZVAD revealed that ZVAD had the most pronounced inhibitory effect (Supplemental Fig. 3C), further implicating the involvement of the caspase cascade in the LCL124-induced death pathway. In comparison, the caspase 8 inhibitor had less of an effect on cell death, and the caspase 3 inhibitor blocked it in an intermediate fashion, which is consistent with the mitochondrial pathway being the chief mediator of LCL124-induced apoptosis (Supplemental Fig. 3D).

LCL124-Induced Cell Killing Is Dependent on Mitochondrial Membrane Potential. Tumor cells have been reported to have more negatively charged mitochondria (Chen, 1988), resulting in our hypothesis that they should be more sensitive to cationic LCL124. To examine whether the sensitivity to LCL124 is associated with $\Delta\Psi_m$, we analyzed the $\Delta\Psi_m$ of tumor cells and of immortalized noncancer cells by using a monovalent cationic fluorescent dye TMRM. Because $\Delta\Psi_m$ is correlated to TMRM uptake, using the Nernst equation (Lemasters and Ramshesh, 2007), we calculated the relative value of the electrical potential in those cells. The pseudocolor images depicted in Fig. 4A indicate the distribution of the electrical potential in pancreatic cancer cells (Aspc-1 and MIA) and in DT-PD59. Aspc-1 and MIA tumor cells have mean mitochondrial electrical potentials ranging from -110 mV to -130 mV, compared with normal cells in which the Ψ_m is about -50 mV, indicating that the mitochondrial membrane bears a more negative charge in cancer cells, compared with normal pancreatic ductal cells. To determine whether cells with higher $\Delta\Psi_m$ result in more LCL124 accumulation in mitochondria, MIA and DT-PD59 were treated with LCL124 and the mitochondrial compartment was fractionated and subjected to mass spectrometry for compound measurement. Remarkably, in Fig. 4B, we saw a significantly higher level of LCL124 in MIA cells, compared with DT-PD59 normal cells (4298 ± 641.9 pmol/500 μ g protein in MIA vs. 810 ± 165.6 pmol/500 μ g protein in DT-PD59). It is noteworthy that mitochondrial LCL124 in MIA cells is 1.5-fold higher (6643.7 ± 897 pmol/500 μ g protein) than that in the whole cells (4298 ± 641.9 pmol/500 μ g protein), suggesting that LCL124 has a preference to accumulate in the mitochondria of cancer cells.

Next, to evaluate whether increased drug uptake leads to LCL124 sensitivity in cancer cells, we examined apoptotic mediators in cells treated with LCL124. Treatment of MIA and Aspc-1 cells with LCL124 at doses selected based on the EC_{50} of each drug resulted in significant PARP cleavage (Fig. 4C) and cytochrome *c* release (Fig. 3C), in addition to the increased caspase 3/7 activity assay in MIA cells (Supplemental Fig. 3B), indicating an intact apoptotic response to LCL124. In contrast, LCL124 does not significantly activate mitochondrial apoptosis as detected by PARP cleavage or cytochrome *c* release in DT-PD59 cells. These observations are consistent with the EC_{50} data, in which LCL124 is 4- to -28 -fold more potent in the pancreatic cancer cells, compared with immortalized noncancer cells (EC_{50} Aspc-1: 14.06 μ M; MIA: 2.261 μ M; DT-PD59: 62.75 μ M) (Fig. 4D). The application of FCCP, a mitochondrial uncoupling agent that dissipates $\Delta\Psi_m$, appears to delay LCL124-induced cell killing in both Aspc-1 and MIA in a dose responsive manner (Fig. 4, E and F), confirming that LCL124-induced cell death is

mediated by $\Delta\Psi_m$. These results indicate that LCL124 induces killing in cells with higher $\Delta\Psi_m$, suggesting potential tumor selectivity.

Induction of GMZ Resistance in Pancreatic Cells Sensitizes Them to LCL124. Because it was previously reported that LCL124 acted in synergy with GMZ to kill squamous carcinoma of the head and neck (Senkal et al., 2006), a similar experiment was performed in Panc-02 cells. No synergy was observed when GMZ and LCL124 were combined or when GMZ was given 2 hours before LCL124 treatment (Supplemental Fig. 4, A and B). To our surprise, cells selected to become GMZ resistant (Supplemental Fig. 4C), as described previously (Shi et al., 2002), became 2.5-fold more sensitive to LCL124 (Fig. 5A). It is noteworthy that these same GMZ-resistant cells were not more sensitive to 5-FU, cisplatin, etoposide, or doxorubicin (Fig. 5B), suggesting that GMZ-induced sensitivity to LCL124 was specific. To explore whether GMZ resistance resulted in increased drug uptake, levels of LCL124 were determined in both wild-type and GMZ-resistant cells. Compared with parent cells, GMZ-resistant cells had an $\sim 30\%$ increase in accumulation of LCL124 (15390.8 vs. 12037.3 pmol/500 μ g protein). This observation is consistent with a previous study that showed that treatment with GMZ enhanced the accumulation of LCL124 in the HNSCC tumor model by an unknown mechanism (Senkal et al., 2006).

Because cationic drug uptake into mitochondria is potential-dependent, we sought to investigate whether GMZ alters the $\Delta\Psi_m$ of mitochondria when cells became GMZ resistant. In Fig. 5C, GMZ-resistant cells displayed stronger mitochondrial TMRM staining, compared with wild-type control cells, as shown in the pseudocolored image indicating more negatively charged mitochondria. Calculating $\Delta\Psi_m$ based on the method of Lemasters and Ramshesh (2007) also revealed that GMZ-resistant cells have a mean $\Delta\Psi_m$ of -110 mV, and $\Delta\Psi_m$ on wild-type cells is about -80 mV. This increase in negative $\Delta\Psi_m$ offers an explanation as to why GMZ-resistant cells accumulate more LCL124 and exhibit increased sensitivity to LCL124.

LCL124 Inhibits Pancreatic Cancer Xenograft Growth and Enhances Animal Survival In Vivo. In preparation for evaluating the in vivo anti-tumor activity of the compound on tumor cell xenograft growth in nude mice, LCL124 was injected at 20, 40, or 80 mg/kg doses, and blood chemistry was analyzed 7 days after injection to determine toxicity (Supplemental Fig. 5). On the basis of these data, it was determined that 40 mg/kg did not significantly alter blood chemistry, and this dose was chosen for administration to mice bearing Aspc-1 xenografts. As can be seen in Fig. 6, LCL124 effectively inhibited tumor growth. At 40 mg/kg once every other day administration for 15 days, LCL124 inhibited tumor growth by 50% in Aspc-1 xenografts compared with the vehicle-treated group (Fig. 6A). It is noteworthy that the survival curve demonstrates that there is an advantage to the animal based on treatment with LCL124. LCL124 treatment significantly increases survival, compared with untreated animals (Fig. 6B). There was no observed weight loss in LCL124-treated animals over this period (Supplemental Fig. 6B).

To examine whether LCL124 preferentially accumulates in tumor tissues, the distribution of LCL124 after multiple treatment was analyzed by mass spectrometry in mice

Fig. 4. LCL124 selectively kills pancreatic cancer cells. (A) distribution of electrical potential in pancreatic cancer cells and normal cells. Cells were seeded on glass bottom 35-mm dishes. After overnight incubation, cells were loaded with 200 nM TMRM for 30 minutes at 37°C and then washed and imaged in medium containing 50 nM TMRM. The distribution of electrical potential was determined by laser scanning confocal microscopy using 543-nm excitation from a He-Ne laser and a 590 $\pm$ 25-nm emission barrier filter. (B) LCL124 levels in whole cells (W.C.) and in mitochondria were determined by mass spectrometry. Purity of mitochondria (Mito.) was determined by Western blotting. (C) Aspc-1, MIA, and PD-DT59 cells were treated with LCL124 at the indicated doses, which were chosen based on the EC₅₀ for each cell line as determined in Figure 1. Cells were collected after 24 hours, and protein lysates were prepared to examine apoptotic mediators by Western blot. (D) EC₅₀ of LCL124 in Aspc-1, MIA, and DT-PD59 cells. Pancreatic cancer cells were pretreated with FCCP for 15 minutes, followed by administration of LCL124. Cell viability was examined by a MTT assay for Aspc-1 (E) and MIA cells (F). * P < 0.05, ** P < 0.01, compared with no FCCP pretreatment by Student's t test. Data are represented as mean $\pm$ S.D. Cyto-C, cytochrome c .

bearing tumor xenografts. Consistent with what we have seen in our clearance study, LCL124 levels were high in the kidney, suggesting that kidneys may be the main organ for clearance. Remarkably, the level of LCL124 in tumor tissues was approximately 500 pmol/mg protein, which was much higher than liver, spleen, brain, intestine, and lung (all <110 pmol/mg), revealing a significantly increased uptake of LCL124 in tumor tissue. Although the heart is known to be enriched in mitochondria, accumulation in this organ was 10-fold less than in the tumor (Fig. 6C). Tumor tissues were directly analyzed by MALDI mass spectrometry imaging (Cornett et al., 2008; Nilsson et al., 2010) for the presence and distribution of LCL124. The spatial distribution of the m/z 475.4 LCL124 in tumor tissue is shown in Fig. 6D and was

readily detectable. There was no ion detected at this mass in untreated tumor tissues (Fig. 6E). The drug could also be detected in kidney tissues of LCL124-treated animals (data not shown). Confirmation that this m/z 475.4 ion in tissues is LCL124 was done by isolation and collision-induced fragmentation (Supplemental Fig. 6C–E). Analysis of the effect of LCL124 on endogenous level of sphingolipids in kidney and tumor, the two organs that demonstrated accumulation of LCL124, showed that both organs had significant increased total ceramide levels (Fig. 6F); however, different ceramide species were altered, with long chain ceramides (C-14, C-16, and C-18) elevated in tumors and very long chain ceramide C-26 elevated in the kidney (Supplemental Fig. 6A). Tumor tissues also demonstrated a greater elevation in sphingosine

Fig. 5. GMZ-resistant pancreatic cells become more susceptible to LCL124. (A) GMZ-resistant Panc-02 cells were treated with different doses of LCL124, and EC₅₀ was obtained. (B) GMZ-resistant Panc-02 cells were treated with cisplatin (1 μ g/ml), 5-FU (15 μ g/ml), etoposide (15 μ M), and doxorubicin (0.6 μ g/ml). Forty-eight hours after treatment, cell viability was assessed using an MTS cell viability assay. (C) 1×10^5 wild-type MIA cells and GMZ-resistant MIA cells were seeded on 35-mm dishes. After overnight incubation, cells were loaded with 200 nM TMRM for 30 minutes in culture medium at 37°C and then switched to 50 nM TMRM for imaging. The distribution of $\Delta\Psi_m$ was determined by laser scanning confocal microscopy using 543-nm excitation from a He-Ne laser and a 590 ± 25 -nm emission barrier filter on an Olympus FV10i. The relative value of $\Delta\Psi_m$ was calculated.

levels in response to LCL124; however, there was no significant change detected in S1P (Fig. 6F).

Discussion

The drug-resistant and rapidly progressing nature of pancreatic cancer benchmarks the need for new therapies. Clinical trials have combined the standard therapy for pancreatic cancer, GMZ, with numerous agents, with uniformly disappointing results (Rao and Cunningham, 2002; Van Cutsem et al., 2004; Oberstein and Saif, 2011). A recent trial of folfinorox versus GMZ exhibited an improvement in progression-free survival (Conroy et al., 2011). Unfortunately, folfinorox, a combination of several toxic agents, reduced overall quality of life, a critically important parameter for patients undergoing palliative treatment of advanced cancer.

Recent studies have demonstrated that resistance to GMZ is mainly attributed to an altered intrinsic apoptotic threshold, suggesting an essential role for the mitochondrial compartment in pancreatic cancer cell death (Schniewind et al., 2004). Although abnormalities in ceramide-mediated cell death have been studied in multiple models, including pancreatic cancer (Modrak et al., 2004, 2005; Ogretmen and

Hannun, 2004; Guillermet-Guibert et al., 2009), sphingolipid metabolism has been inadequately explored as an avenue for pancreatic cancer therapy. Exogenous ceramides induce apoptosis primarily through the mitochondrial pathway, with activation of the caspase 9-3/7 executioner mechanism (Lin et al., 2005, 2007; Yu et al., 2010). Ceramide accumulation is a hallmark of multiple modalities of apoptosis-inducing cancer therapies (Huwiler and Zangemeister-Wittke, 2007). Likewise, resistance to therapy has been linked to lack of ceramide accumulation, by reduced generation (Chmura et al., 1997; Holland et al., 2007) and by accelerated metabolism (Liu et al., 2008, 2009). Despite the favorable signaling responses elicited in vitro, treating patients with exogenous ceramides presents major challenges: 1) poor water solubility and cellular uptake, 2) intracellular metabolism, and 3) lack of tumor-targeted delivery. Because mitochondria in cancer cells are typically more negatively charged than in normal cells, novel ceramide analogs have been developed with greater water solubility, cell-membrane permeability, and cellular-uptake profiles, in comparison with native ceramides (Szulc et al., 2006). LCL124 belongs to a group of ceramide analogs with a fixed positive charge that targets them to negatively charged organelles, predominantly

Fig. 6. LCL124 inhibits pancreatic xenograft growth in vivo. A total of 5×10^6 cells/100 μ l were subcutaneously injected into right flanks of nude mice. LCL124 was administered intraperitoneally at 40 mg/kg in PBS every other day for 2 weeks, and tumor volume was measured with calipers. Compound distribution and sphingolipids levels (Sph) were analyzed by mass spectrometry. (A) in vivo therapeutic effect of LCL124 on pancreatic tumor growth ($n = 5$ for each group). (B) survival rate of the animal in response to LCL124, compared with nontreated group ($n = 5$ for each group). (C) compound distribution in animals treated with LCL124 ($n = 3$). (D and E) frozen xenograft tissue (10- μ m slice) treated with LCL124 (D) or sham treated (E) analyzed by MALDI-MS imaging. Shown in the spectra (m/z 300–700 range) is the signal of the primary LCL124 ion at m/z 475.4. The bottom left image panel shows the spatial distribution of the m/z 475.4 ion in the tissue, using a color bar to link peak intensity with pixel color. An hematoxylin and eosin stain of the tissue is shown in the bottom right panel. (F) relative sphingolipid alterations in kidney and tumor tissues as determined by mass spectrometry ($n = 3$). Values are expressed as a percentage change in LCL124 treated, compared with PBS treated. * $P < 0.05$. Results are expressed as mean $\pm$ S.D.

NT: No Treatment.

mitochondria (Senkal et al., 2006). We examined LCL124, LCL89, and LCL87 in pancreatic cancer and in immortalized pancreatic cells. These studies show that LCL124 is the most efficacious compound for killing cancer cells, regardless of their pre-existing defects in death-inducing pathway and is relatively nontoxic to normal cells. This is consistent with a previous study in which keratinocytes demonstrated less sensitivity to LCL124, compared with HNSCC cells, making this drug appear to be preferentially capable of inducing cell death in tumors (Senkal et al., 2006). In addition, compared with LCL89 and LCL87, LCL124 demonstrated more solubility in aqueous solutions, which will aid in formulation and delivery to patients.

LCL124 selectively targets mitochondria because of the affinity of the cationic pyridinium moiety for the negative $\Delta\Psi_m$. Tumor cells are known to have both increased

numbers and increased negative charge in their mitochondria (Chen, 1988; Modica-Napolitano and Aprille, 2001). Thus, we hypothesized that LCL124 would accumulate more readily in cancer cells than in normal cells. To answer this question, a cationic fluorescent dye TMRM was used to evaluate $\Delta\Psi_m$ in normal cells compared with tumor cells. As hypothesized, pancreatic cancer cell lines MIA and Aspc-1 demonstrated a more negative $\Delta\Psi_m$ (Fig. 4A). We also observed markedly amplified but poorly organized mitochondria in these two cancer cell lines, which is also frequently seen in transformed cells (Han et al., 2002; Lee and Wei, 2005; Kim and Dang, 2006). The more negative $\Delta\Psi_m$ results in the preferential accumulation of LCL124 in cancer cells and in mitochondria, compared with normal cells (Fig. 4B). This is consistent with our observation that tumor cell lines were much more susceptible to LCL124 treatment, compared with the

immortalized normal cell line DT-PD59, as determined by measuring the EC₅₀, cytochrome *c* release, and PARP cleavage (Fig. 3C; Fig. 4, C and D). Consistent with LCL124 preferentially accumulating in tumor cells, we detected significant accumulation of LCL124 in Aspc-1 xenografts (Fig. 6C). LCL124 also accumulated in the kidney, suggesting renal clearance. It is noteworthy that, in tumors, we observed increases in long-chain ceramides (C-14, C-16, and C-18), which have been identified as proapoptotic (Bielawska et al., 2008), whereas kidney had a substantial increase in a very long-chain ceramide (C-26), which has been shown to have antiapoptotic roles (Bielawska et al., 2008) (Supplemental Fig. 6A).

Because mitochondria account for the majority of cellular oxygen consumption, OCR serves as a direct indicator of mitochondrial function (Hussain et al., 2008). In this study, we were able to determine that LCL124 affected oxygen consumption in pancreatic cells. As expected, we also demonstrated that untreated cancer cells had significantly higher OCR and ECAR relative to normal cells, reflecting increased metabolism and mitochondrial function. OCR was significantly reduced by LCL124 treatment in a dose-dependent manner (Fig. 2, C and D). In particular, a robust OCR reduction in cancer cells, compared with normal cells, further suggests the selective mitochondrial targeting of this compound (data not shown). It is difficult to interpret O₂ consumption in cells with viabilities less than ~95%, because cells experiencing active death can produce confounding respiration rates. Thus, we have only measured respiration after short drug exposure times when viabilities are still high, and under these conditions, significant reductions in respiration were only detected at higher concentrations. In addition, we observed that the response of tumor cells to LCL124 could be delayed by adding FCCP, a proton ionophore that uncouples oxidation from phosphorylation by dissipating $\Delta\Psi_m$ (Mitchell and Moyle, 1967; Van Blerkom et al., 2003) (Fig. 4, E and F). This strongly suggests that the $\Delta\Psi_m$ is required for LCL124-mediated cell killing.

Mitochondrial depolarization is a hallmark of apoptosis, reflecting increased membrane permeability resulting in cytochrome *c* release and activation of the downstream caspase cascade. We used JC-1, a cationic dye that forms fluorescent aggregates in proportion to the integrity of the mitochondrial membrane, to analyze the impact of LCL124 on mitochondrial membrane depolarization. Similar to ceramide-treated cells (Arora et al., 1997; Novgorodov et al., 2005; Stoica et al., 2005) treatment with LCL124 promoted an increase in the ratio of fluorescence at 530:590 nm, indicating that LCL124 caused mitochondrial depolarization, a hallmark of intrinsic apoptosis. Cells treated with GMZ did not exhibit alterations in JC-1 staining, indicating that GMZ did not promote mitochondrial depolarization in these models (Fig. 3, A and B). As such, LCL124 may be able to cause tumor cell death in drug-resistant cells, because it bypasses the upstream mechanisms of resistance that impair induction of intrinsic apoptosis (Bold et al., 1999; Bai et al., 2005) and functions directly at the mitochondria to induce mitochondrial membrane depolarization, leading to reactivation of intrinsic apoptosis. It is noteworthy that our data demonstrated that LCL124 is not metabolized by cancer cell enzymes (Supplemental Fig. 1C) but appears to elevate endogenous ceramide, specifically in the mitochondria (Supplemental Fig. 1, A and B), as part of its cell killing mechanism.

It is noteworthy that we observed increased sensitivity to LCL124 in GMZ-resistant pancreatic cancer cells, cells that did not show increased sensitivity to other cytotoxic chemotherapy drugs (Fig. 5, A and B), suggesting that a specific mechanism of sensitivity to LCL124 might be induced by GMZ. GMZ can be phosphorylated and incorporated into mitochondrial DNA (mt-DNA), interfering with mt-DNA replication (Zhu et al., 2000; Mini et al., 2006; Fowler et al., 2008). Thus, long-term GMZ exposure may gradually cause mt-DNA depletion and eventually affect mitochondrial function. mt-DNA-depleted cells, so called ρ^0 cells, have been produced by prolonged treatment of cells with agents that prohibit replication of mt-DNA (King and Attardi, 1989). Of interest, some lines of ρ^0 cells have a high negative $\Delta\Psi_m$ (Garcia et al., 2000), prompting us to hypothesize that GMZ-induced resistance might create cells with hyperpolarized mitochondria, which promote uptake of LCL124. Our current findings demonstrate a significant difference in mitochondrial TMRM dye uptake in wild-type MIA pancreatic cancer cells and in the GMZ-resistant MIA model (Fig. 5C). We observed an increase in the total amount of mitochondria in resistant cells and increased $\Delta\Psi_m$. Although the precise mechanism of mitochondrial hyperpolarization induced by GMZ remains to be elucidated, these observations present a rational explanation for why GMZ-resistant cells became more susceptible to subsequent LCL124 treatment, because hyperpolarized mitochondria of GMZ resistant cells accumulate more LCL124. Increased sensitivity to LCL124 after development of GMZ resistance is very encouraging, because development of GMZ resistance is almost inevitable during pancreatic cancer therapy, and agents that perform on resistant tumors would be a significant clinical advancement.

Here, we demonstrated that LCL124 1) preferentially kills pancreatic cancer cells through initiation of mitochondrial depolarization and apoptosis, 2) accumulates in cancer cell mitochondria bypassing GMZ resistance to induce potential dependent cell killing, 3) GMZ-resistant cells accumulate more LCL124 and are more susceptible to LCL124, and 4) LCL124 accumulates in xenografts and suppresses tumor growth. Taken together, our studies provide strong evidence that LCL124 may be a potential agent to overcome GMZ resistance and induce cell death in pancreatic cancer.

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Authorship contributions

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Conducted experiments: Lu, Jones, Marrison, Lewis, G. Beeson, Liu.

Contributed new reagents or analytical tools: Jones, Ramshesh, Drake, A. Bielawska, J. Bielawski, Szulc, Ogretmen.

Performed data analysis: Beckham, Jones, Ramshesh, Liu.

Wrote or contributed to the writing of the manuscript: Beckham, Drake, Norris, Liu.

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