LCL124, a cationic analog of ceramide, selectively induces pancreatic cancer cell death by accumulating in mitochondria*


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Nonstandard abbreviations used: GMZ: Gemcitabine, S1P: (sphingosine 1-phosphate), 5-FU: 5-fluorouracil, LCL 124: (2S, 3S,4E) C6-CCPS; L-t-ω-pyridinium C6-ceramide, HNSCC: Head and neck squamous cell carcinoma, TMRM: tetramethylrhodamine methyl ester, ΔΨm: mitochondrial membrane potential, Ψ: electrical potential in millivolts, JC-1: 5,5’,6,6’-tetrachloro-1,1’,3,3’-tetraethylbenzimidazole-carbocyanine iodide, OCR: oxygen consumption rate, ECAR: extracellular acidification rate, mt-DNA: mitochondrial DNA

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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 4th leading cause of cancer 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. Interestingly, insufficient ceramide accumulation has been linked to gemcitabine resistance in multiple cancer types, including pancreatic. 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) to cancer cell mitochondria. LCL124 was effective in initiating apoptosis by causing mitochondrial depolarization in pancreatic cancer cells but demonstrated significantly less activity against nonmalignant 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. Interestingly, 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 (cancer.gov) making it the fourth leading cause of cancer death in the United States. Gemcitabine (GMZ) has been the standard treatment for 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-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 (FOLFIRINOX) was compared with GMZ, resulting in an overall survival of 11.1 months vs. 6.8 with GMZ. Unfortunately, this regimen represents only a marginal improvement as it improved survival but increased toxicity versus GMZ in the phase III trial (Conroy et al., 2011).

Cancer cells have been shown to have a shift in the balance between pro-apoptotic ceramide and anti-apoptotic 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; Modrak et al., 2009). Similarly, 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/S1P ratio was correlated with the sensitivity and, conversely, the resistance of
pancreatic cancer cells to GMZ (Guillermet-Guibert et al., 2009). Whereas cell lines with a low ceramide/S1P ratio required high concentrations of GMZ to induce apoptosis, cell lines with more favorable ceramide/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-\(\omega\)-pyridinium Cn-ceramide, generally termed Cn-CCPS) were designed to preferentially localize into negatively charged intracellular compartments due to 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 Aprille, 2001) compared to 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 anti-proliferative effects in MCF7 and 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 \textit{in vitro} (Rossi et al., 2005), and on HNSCC tumors \textit{in vivo} (Senkal et al., 2006). As 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: LCL124 (C6-CCPS), LCL 89 (C12-CCPS) and LCL 87 (C16-CCPS)
on pancreatic cells. We conclusively demonstrate that LCL124 is able to target mitochondria in
a potential dependent manner and selectively induce pancreatic cancer cell death at 4 to 28-fold
lower concentration than in normal cells. Interestingly while 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 become several fold more sensitive to LCL124 induced cell killing,
augmenting its potential as a candidate to circumvent GMZ resistance in pancreatic cancer.

Methods:

Cell lines, culture and reagents
Aspc-1, MIA, Panc-01, SK-MES pancreatic cancer cell lines (ATCC; Manassas, VA, USA) 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 and DMEM (Thermo Scientific HyClone; Logan, UT, USA)
containing 10% bovine growth serum (BGS) (Thermo Scientific HyClone) and 1%
penicillin/streptomycin solution (Mediatech; Manassas, VA, USA). 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 (St Louis, MO,
USA). Cn-CCPS: LCL124, 89, 87 and 17C-LCL124 were synthesized by the MUSC Lipidomics
Shared Resources as previously described (Szulc et al., 2006) (Charleston, SC, USA). LCL124
and 17C-LCL124 were prepared directly in sterilized PBS containing 137 mmol/L NaCl, 2.7 mmol/L KCl, 10 mmol/L Na2HPO4 and 2 mmmol/L KH2PO4 (pH 7.4). LCL89 and LCL87 were dissolved in sterilized PBS (described as above) / ethanol 4/1 (v/v) to final stock solution 20 mM and stored at −20°C.

FCCP was obtained from Prof. Beeson at Medical University of South Carolina. Antibodies used include PARP (Santa Cruz Biotechnology; Santa Cruz, CA, USA), cytochrome c (Cell Signaling Technology, Beverly, MA, USA), Caspase 3 (PharMingen, San Diego, CA, USA), 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, USA). Cells were plated at 5 x10^3 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 carried out according to the manufacturer’s instructions. EC50 was calculated using Prism 4 software. For experiments using inhibitors, cells were pretreated with inhibitors for 1 h (FCCP for 15 minutes) at 37°C before adding media containing vehicle or LCL124. The remainder of the assay was carried out as described above.

**Mitochondrial fractionation**

5x10^6 cells were seeded per 150mm 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, USA) according to manufacturer’s instructions and the purity of the fractions was examined by western blot using GAPDH and cytochrome c.

**Western blotting**
Cells were seeded in 60mm plates as described above and treated as indicated. Cells were lifted by gently scraping the plates, washed once with ice-cold phosphate-buffered saline (PBS) and then lysed by incubation on ice for 30 minutes in RIPA buffer with Complete Mini Protease Inhibitor Cocktail Tablet (Roche; Indianapolis, IN, USA). 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, USA) 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.

**Cytochrome c release**

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

**Live-Cell mitochondrial ΔΨm microscopy**

Cells grown in 35mm glass-bottom dishes (MatTek Corporation, Ashland, MA, USA) were incubated in regular growth medium with 250 nM tetramethylrhodamine methyl ester (TMRM, kindly obtained from Dr. Beeson at the Medical University of SC) for 30 min. The cells were then rinsed in pre-warmed 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) 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 formula: $\Psi = -59 \times \log (\text{Fin}/\text{Fout})$ where $\Psi$ is electrical potential in millivolts, Fout is the average fluorophore concentration in the extracellular space (electrical ground), and Fin is the average fluorophore concentration at any point within the cell. To display the distribution of the $\Psi$, colors were assigned to specific millivolt ranges of the $\Psi$ and a pseudocolored image was created (Lemasters and Ramshesh, 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, Michigan, USA). 8x10^5 Aspc-1 and MIA cells were plated on 60mm dishes. Following overnight incubation, cells were treated with the indicated compounds in fresh medium containing 5% BGS. Two hours after treatment cells were washed with PBS, and $\Delta \Psi_m$ was examined using JC-1 Assay Kit according to manufacturer instructions. JC-1 fluorescence was measured using a Becton Dickinson FACScalibur analytical flow cytometer (BD Biosciences, San Jose, CA, USA) 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 using a Seahorse Bioscience XF24 extracellular flux analyzer (Seahorse Bioscience, North Billerica, MA, USA) as previous described (Beeson et al., 2010). On the day before the
experiment, the sensor cartridge was placed into 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). Following 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 min. 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.

**HPLC–MS/MS analysis**

Analyses of sphingolipid species and test compound were performed by the MUSC Lipidomics Shared Resources on a Thermo Finnigan TSQ 7000, triple-stage quadrupole mass spectrometer operating in a Multiple Reaction Monitoring positive ionization mode as described (Bielawski et al., 2006). Briefly, samples were fortified with internal standards and extracted into a one-phase solvent system with ethyl acetate/isopropanol/water (60:30:10%, v/v). 4 ml 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.

**Tumor xenografts**
Pathogen-free four-week-old female Athymic NCr-nu/nu mice were purchased from the NCI Frederick Cancer Research Center (Frederick, MD, USA). 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 SC. \(5 \times 10^6\) Aspc-1 cells in 100 ul PBS were injected subcutaneously in right flanks. Tumor formation was monitored twice a week by measuring the width, depth and length of the mass, and tumor volume was calculated by the formula \(v (\text{mm}^3) = \pi / 6 \times (a \times b \times c)\), 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 an average volume of at least 100 mm\(^3\), 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 \(\mu\)m 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 five minutes prior to matrix application. Tissue slices were coated with 2, 5- dihydroxybenzoic acid (DHB) matrix at 30mg/ml in 50% methanol and 1% TFA using an Image Prep (Bruker Daltronics). ImagePrep matrix thickness was closely monitored and tailored to our matrix of choice. Post matrix application and prior to MS analysis the the slides were placed in the dessicator for 15 minutes. Mass spectra were acquired using a Bruker Solarix 7T Dual Source MALDI/ESI FT-ICR MS with the laser focused to a diameter of 250 \(\mu\)m. Data were acquired and analyzed using Flex Imaging 3.0 software. Mass spectra were accumulated from 200 laser shots acquired from each spot on the tissue within the 200-1500 m/z 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 a 2 m/z range. The spectra were normalized within the Flex Imaging software using root mean squares. Collision-induced fragmentation of the 475.4 m/z 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 ANOVA and unpaired one-tailed t test, using Prism (version 4.0) from GraphPad. The level of significance was set at **, < 0.01 and *, < 0.05 in figures. \( p < 0.05 \) was considered significant.

Results:

Induction of apoptosis is generally divided into the extrinsic death receptor pathway and the intrinsic mitochondrial pathway with the later 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). In order 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 EC50s (drug concentration effecting 50% cell death) of the three candidate compounds were determined based on the MTS assay. As depicted in Figure 1, all 3 compounds had an inhibitory effect on Aspc-1 and MIA cells. MIA were most sensitive to LCL124 (EC50 2.295μM, Figure 1A) and Aspc-1 cells were the most sensitive to LCL89 (EC50 10.80μM, Figure 1B). Toxicity from chemotherapy is frequently attributable to its lack of tumor specificity, making tumor-targeted delivery of anticancer drugs one of the most important steps for the development of chemotherapeutic agents. As 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 Figure 1C, while DT-PD59 cells exhibited greater EC50 to LCL124 (EC50 62.75μM), which indicated a wider selectivity window particularly against cancer cell lines compared with normal cells, the EC50s of DT-PD59 to LCL89 and LCL87 (14.76 and 9.511 μM, respectively) were nearly equivalent to their EC50s in tumor cells (MIA: 9.993 and 10.69 μM, respectively. Aspc-1: 10.80 and 23.68 μM, respectively). It is of note that there was no apparent dose response when cells were treated using LCL89 and LCL87 in comparison to LCL124, potentially due to their poor solubility. In fact, LCL124 is readily soluble in water, whereas the other two compounds required an organic co-solvent. 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 non-uniform 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 non-modified C6-ceramide was evaluated. As shown in Figure 1D, there is a wide
variation of response to C6-ceramide in these cells likely due to differences in expression, regulation, and functionality of apoptosis effectors. Interestingly, 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 to the non-modified C6-ceramide suggesting increased potency and uniform 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 μM LCL124 for 24 hours. Cells were then fractionated into mitochondrial, cytosolic and nuclear compartments with purity assessed by presence and absence of cytochrome c, GAPDH, and lamin B (Figure 2B). Fractions were then analyzed for LCL124. As evidenced in Figure 2A, the mitochondrial compartment accumulated substantially more LCL124 than 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 (Suppl. 1A), in which we also observed accumulation of endogenous total ceramide in the cell mitochondria, whereas total cellular ceramide did not change appreciably (Suppl. 1B). In order to determine whether LCL124 is metabolized by endogenous enzymes, we treated cells with 17-Carbon LCL124 (vs. endogenous sphingosine which has 18 Carbons) and measured C17-sphingolipid species from 15 minutes to 24 hours after treatment. Interestingly, LCL124 progressively accumulated inside the cells as expected, but no C17 metabolites were detected in cells or in the medium (Suppl. 1 C). Thus, we conclude that LCL124 is not metabolized by cancer cell enzymes.
Although cancer cells frequently utilize 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 (oxygen consumption (OCR) and extracellular acidification (ECAR)) using 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 when compared to DT-PD59 normal cells (data not shown), which indicates that the mitochondria in cancer cells are more metabolically active than the mitochondria in normal cells. Furthermore, the drop of OCR in the presence of LCL124 is consistent with LCL124 targeting mitochondria and thereby disrupting aerobic respiration. As shown in Figure 2C and 2D, OCR decreased in a dose-dependent manner within 30 minutes following administration of LCL124 in both Aspc-1 and MIA cells.

**LCL124 causes mitochondrial depolarization and apoptosis in pancreatic cancer cells.**

Based on the believed mechanism of action of this class of drugs (Szulc et al., 2006) we were interested to see whether a drop in ΔΨm 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 following by monomer binding that result in green fluorescence. In our study, LCL124 treated cells exhibited a dramatic dose-dependent decrease in their mitochondrial potential in both Aspc-1 (Figure 3A) and MIA (Figure 3B) cells, indicating depolarization. In contrast, GMZ-treated cells had a minimal drop in potential (Figure 3 and Suppl. 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 (Figure 3C).

Having determined that LCL124 induces mitochondrial depolarization, we sought to more fully analyze the mechanism of LCL124-induced cell death. Pre-treatment of MIA cells with either an inhibitor of ceramide synthase, fumonisin B1 (FB1), or an inhibitor of serine palmitoyltransferase, myriocin, both suppressed LCL124 mediated cell death (Suppl. 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 following LCL124 treatment compared to untreated cells, suggesting critical involvement of caspases in LCL124-induced cell death (Suppl. 3B). Treatment of MIA and Aspc-1 pancreatic cell lines with LCL124 in combination with the proteosome inhibitor MG132, a cathepsin B inhibitor CA074me, or a pan-caspase inhibitor ZVAD revealed that ZVAD had the most pronounced inhibitory effect (Suppl. 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 while 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 (Suppl. 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. In order to examine whether the sensitivity to LCL124 is associated with ΔΨ\text{m}, we analyzed the ΔΨ\text{m} of tumor cells
and of immortalized non-cancer cells by using a monovalent cationic fluorescent dye TMRM. Since ΔΨm 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 Figure 4A indicates 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 average mitochondrial electrical potentials ranging from -110 mV to -130 mV compared to normal cells in which the Ψm 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 ΔΨm results 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 Figure 4B, we saw significantly higher level of LCL124 in MIA cells compared to DT-PD59 normal cells (4298 ± 641.9 pmole/500ug protein in MIA vs 810 ± 165.6 pmole/500ug protein in DT-PD59). Importantly, mitochondrial LCL124 in MIA cells is 1.5 fold higher (6643.7± 897pmole/500ug protein) than that in the whole cells (4298 ± 641.9 pmole/500ug protein), suggesting LCL124 has a preference to accumulate in the mitochondria of cancer cells.

Next, to evaluate whether increase 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 EC50 of each drug resulted in significant PARP cleavage (Figure 4C), and cytochrome c release (Figure 3C) in addition to the increased Caspase 3/7 activity assay in MIA cells (Suppl 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
EC50 data where LCL124 is 4 to 28 fold more potent in the pancreatic cancer cells compared with immortalized non-cancer cells (EC50 Aspc-1: 14.06µM, MIA: 2.261µM, DT-PD59: 62.75µM), Figure 4D). The application of FCCP, a mitochondrial uncoupling agent that dissipates ΔΨm, appears to delay LCL124-induced cell killing in both Aspc-1 and MIA in a dose responsive manner (Figure 4E, 4F) confirming that LCL124-induced cell death is mediated by ΔΨm. These results indicate that LCL124 induces killing in cells with higher ΔΨm, suggesting potential tumor selectivity.

**Induction of GMZ resistance in pancreatic cells sensitizes them to LCL124.** Since 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 carried out in Panc-02 cells. No synergy was observed when GMZ and LCL124 were combined, or when GMZ was given 2 hours prior to LCL124 treatment (Supp 4A and 4B). To our surprise, cells selected to become GMZ resistant (Supp 4C), as described previously (Shi et al., 2002), became 2.5-fold more sensitive to LCL124 (Figure 5A). Interestingly these same GMZ resistant cells were not more sensitive to 5-FU, cisplatin, etoposide or doxorubicin (Figure 5B) suggesting that GMZ-induced sensitivity to LCL124 was specific. To explore if GMZ resistance resulted in increased drug uptake, levels of LCL124 were determined in both wild type cells and in GMZ resistance cell. Compared with parent cells, GMZ-resistant cells had approximately 30% increase in accumulation of LCL124 (15390.8 vs 12037.3 pmole/500ug protein). This observation is consistent with a previous study which showed that treatment with GMZ enhanced the accumulation of LCL124 in the HNSCC tumor model by an unknown mechanism (Senkal et al., 2006).
Since 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 Figure 5C, GMZ resistant cells displayed stronger mitochondrial TMRM staining compared with wild type control cells as illustrated in the pseudocolored image indicating more negatively charged mitochondria. Calculating $\Delta \Psi_m$ based on the method of Lemasters, et al (Lemasters and Ramshesh, 2007) also revealed that GMZ resistant cells have an average $\Delta \Psi_m$ of -110 mV while $\Delta \Psi_m$ 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 seven days post injection to determine toxicity (Suppl. 5). Based on 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 Figure 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 (Figure 6A). Importantly, the survival curve demonstrates that there is an advantage to the animal based on treatment with LCL124. LCL124 treatment significantly increases survival compared to untreated animals (Figure 6B). There was no observed weight loss in LCL124 treated animals over this time period (Suppl. 6B).

To examine whether LCL124 preferentially accumulates in tumor tissues, the distribution of LCL124 after multiple treatment was analyzed by mass spectrometry in mice 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 pmole/mg protein which was much higher than liver, spleen, brain, intestine and lung (all <110 pmole/mg) revealing a significant increase 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 (Figure 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 475.4 m/z LCL124 in tumor tissue is shown in Figure 6D and was readily detectable. There was no ion detected at this mass in untreated tumor tissues (Figure 6E). The drug could also be detected in kidney tissues of LCL124 treated animals (data not shown). Confirmation that this 475.4 m/z ion in tissues is LCL124 was done by isolation and collision-induced fragmentation (Suppl, 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 (Figure 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 (Suppl. 6A). Tumor tissues also demonstrated a greater elevation in sphingosine levels in response to LCL124, however, there was no significant change detected in S1P (Figure 6F).

Discussion:

The drug-resistant and rapidly progressing nature of pancreatic cancer benchmark 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 FOLFIRINOX verses GMZ did exhibit an improvement in progression-free survival (Conroy et al., 2011). Unfortunately, FOLFIRINOX, itself a combination of several toxic agents, reduced overall quality of life, a critically important parameter for patients undergoing palliative treatment for 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; Ogretmen and Hannun, 2004; Modrak et al., 2006; 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; Lin et al., 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; Liu et al., 2009). Despite the favorable signaling responses elicited in vitro, treating patients with exogenous ceramides presents major challenges: (a) poor water solubility and cellular uptake, (b) intracellular metabolism, and (c) lack of tumor-targeted delivery. Taking advantage of the fact that 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 to 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 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 where keratinocytes demonstrated less sensitivity to LCL124 compared to HNSCC cells, making this drug appear preferentially capable of inducing cell death in tumors (Senkal et al., 2006). In addition, compared to LCL89 and LCL87, LCL124 demonstrated more solubility in aqueous solutions, which will aid in formulation and delivery to patients.

LCL124 selectively targets to mitochondria due to 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 vs. tumor cells. As hypothesized, pancreatic cancer cell lines MIA and Aspc-1 demonstrated a more negative $\Delta\Psi_m$ (Figure 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 to normal cells (Figure 4B). This is consistent with our observation that tumor cell lines were much more susceptible to LCL124 treatment compared to the immortalized normal cell line DT-PD59 as determined by measuring the EC50, cytochrome c release, and
PARP cleavage (Figure 3C, 4C and 4D). Consistent with LCL124 preferentially accumulating in tumor cells, we detected significant accumulation of LCL124 in Aspc-1 xenografts (Figure 6C). LCL124 also accumulated in the kidney, suggesting renal clearance. Interestingly, in tumors we observed increases in long chain ceramides (C-14, C-16, and C-18) which have been identified as pro-apoptotic (Bielawska et al., 2008), while kidney had a substantial increase in a very long chain ceramide (C-26), which has been shown to have anti-apoptotic roles (Bielawska et al., 2008) (Suppl. 6A).

Given that 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 (Figure 2C, D). Particularly, a robust OCR reduction in cancer cells in comparison to 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 going through 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. Additionally, 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 ΔΨm (Mitchell and Moyle, 1967; Blerkom et al., 2003) (Figure 4E and 4F). This strongly suggests that the ΔΨm 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 utilized JC-1, a cationic dye which 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 530/590nm 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 (Figure 3A and B). As such, LCL124 may be able to cause tumor cell death in drug resistant cells since 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. Importantly, our data demonstrated that LCL124 is not metabolized by cancer cell enzymes (Suppl. 1C), but appears to elevate endogenous ceramide, specifically in the mitochondria (Suppl. 1A and B), as part of its cell killing mechanism.

Interestingly, we observed increased sensitivity to LCL124 in GMZ resistant pancreatic cancer cells, cells that did not show increased sensitivity to other cytotoxic chemotherapy drugs (Figure 5A and B) suggesting 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 $^{\rho}_0$ cells, have been produced by prolonged treatment
of cells with agents that prohibit replication of mt-DNA (King and Attardi, 1989). Interestingly, some lines of ρ0 cells have a high negative ΔΨm (Garcia et al., 2000) prompting us to hypothesize that GMZ induced resistance might create cells with hyperpolarized mitochondria that promotes 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 (Figure 5C). We observed an increase in the total amount of mitochondria in resistant cells as well as increased ΔΨm. 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, as hyperpolarized mitochondria of GMZ resistant cells accumulate more LCL124. Increased sensitivity to LCL124 following development of GMZ resistance is very encouraging, as 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 LCL124 a) preferentially kills pancreatic cancer cells through initiation of mitochondrial depolarization and apoptosis. b) accumulates in cancer cell mitochondria bypassing GMZ resistance to induce potential dependent cell killing. c) GMZ-resistant cells accumulate more LCL124 and are more susceptible to LCL124. d) 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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Core, and the Cell and Molecular Imaging facility. The small animal PD/PK study was supported in part by the Drug Metabolism and Clinical Pharmacology Shared Resource, Hollings Cancer Center, Medical University of South Carolina. MALDI tissue imaging was performed in the MUSC Proteomics Center. We are grateful to Professor Beeson and Gyda Beeson at Medical University for their kind assistance with the Seahorse Biosciences Academic Core Facility. We thank Dr. Ouellette at the University of Nebraska Medical Center for kindly proving us DT-PD59 cells.

**Authorship contributions:**

Participated in research design: Beckham, Marrison, Lewis, Liu, C. Beeson

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

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

Performed data analysis: Beckham, Ramshesh, Jones, Liu

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


Lee KM, Yasuda H, Hollingsworth MA and Ouellette MM (2005) Notch 2-positive progenitors with the intrinsic ability to give rise to pancreatic ductal cells. *Lab Invest* **85**:1003-1012.


Footnotes:

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3 College of Pharmacy / Pharmaceutical & Biomedical Sciences

4 College of Pharmacy Medical University of South Carolina
Legends for Figures:

**Figure 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. EC50 values for pancreatic cell lines to C6-ceramide and LCL124. EC50 values were obtained from 10 concentrations based on individual drug, with four replicates for each cell line. EC50 was calculated and graphed using Prism 4.

**Figure 2:** LCL124 accumulates in mitochondria and reduces mitochondrial respiration. Aspc1 cells were treated with 20 µM LCL124. Cells were washed and collected at indicated time points. Nuclear, cytosolic, and mitochondrial fractions were isolated. Fractionated lysates (normalized by protein concentration) were analyzed for the level of LCL124 (A) 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 μM) (*, p < 0.05, **, p < 0.01 versus the vehicle control group by student t-test). Data are represented as mean ± SD.

**Figure 3:** Loss of mitochondrial membrane potential in pancreatic cell lines following LCL124 treatment: Aspc-1 (A) and MIA (B) cells were incubated in the presence of indicated concentration of LCL124 or GMZ for 2 h, 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.
Figure 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 min 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±25 nm emission barrier filter. B. LCL124 levels in whole cells and in mitochondria were determined by mass spectrometry. Purity of mitochondria 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 using a MTS assay for Aspc-1 (E) and MIA cells (F). (* p, < 0.05 ** p, < 0.01, compared with no FCCP pretreatment by student t-test. Data are represented as mean ± SD.)

Figure 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 (1µg/ml), 5-FU (15µg/ml), etoposide (15µM) and doxorubicin (0.6µg/ml). 48 hours post-treatment, cell viability was assessed using an MTS cell viability assay. C. 1x10⁵ wild type MIA cells and GMZ resistance MIA cells were seeded on 35 mm dishes. Following overnight incubation, cells were loaded with 200 nM TMRM for 30 min in culture medium at 37 °C and then switched to 50 nM TMRM for imaging. The distribution of ΔΨm was determined by laser scanning confocal microscopy using 543-nm
excitation from a He-Ne laser and a 590±25nm emission barrier filter on an Olympus FV10i. The relative value of ΔΨm was calculated.

Figure 6: LCL124 inhibits pancreatic xenograft growth in vivo. 5X10^6 cells/100µl 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 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 to non-treated group (n=5 for each group). C. Compound distribution in animals treated with LCL124 (n=3). D-E. Frozen xenograft tissue (10 µm slice) treated with LCL124 (D) or sham treated (E) analyzed by MALDI MS imaging. Shown in the spectra (300-700 m/z range) is the signal of the primary LCL124 ion at 475.4 m/z. The bottom left image panel shows the spatial distribution of the 475.4 m/z ion in the tissue, using a color bar to link peak intensity with pixel color. An H&E 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 percent change in LCL124 treated versus PBS treated. (*, p < 0.05, **, p<0.01). Results are expressed as mean± SD.
Figure 1

A  EC50 in Aspc-1 Cells

B  EC50 in MIA Cells

C  EC50 in DT-PD59 Cells

D

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Figure 6

A. Tumor Growth Curve (IP injection every other day X7 n=5, p<0.001)

B. Survival Curve

C. 124 pmole/1 mg protein

D. Mass spectrum: 475.4 m/z LCL 124 t

E. Mass spectrum: 475.4 m/z untreated

F. % NT

Sph S1P Total Ceramide
**Supplementary 1**

Panc02 cells were treated with 20 μM LCL124 and collected at the indicated times. Cell fractionation was carried out and levels of LCL124 (A) and ceramide (B) were measured by Mass Spectrometry. C. MIA cells were treated with 17C-LCL124 at 1 μM concentration in serum free medium. Cells and medium were collected at indicated times and 17C-sphingolipids were measured by Mass Spectrometry.
Supplementary 1

LCL124, a cationic analog of ceramide selectively induces pancreatic cancer cell death by accumulating in mitochondria


### Concentration [pmole/total sample]

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### Concentration [pmole/total sample]

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*p > 0.05

*p > 0.05

*p > 0.05

*p > 0.05

*p > 0.05
Supplementary 2: Aspc-1 (A) and MIA (B) cells were plated and cultured overnight. Cells were then incubated in the presence of indicated concentration of LCL124 or GMZ for 2 h and mitochondrial depolarization was determined by JC-1 flow cytometry. Bar graph represents percentage of green fluorescent cells and red fluorescent cells. Data are representative of two independent experiments. Bar graph represents fold change of the fluorescence intensity 530 nm/590 nm.
LCL124, a cationic analog of ceramide selectively induces pancreatic cancer cell death by accumulating in mitochondria*

Thomas H. Beckham¹, Ping Lu¹, Elizabeth E. Jones², Tucker Morrison¹, Clayton S. Lewis³, Joseph C. Cheng¹, Venkat K. Ramshesh⁴, Gyda Beeson³, Craig C. Beeson³, Richard R. Drake³, Alicja Bielawska², Jacek Bielawski², Zdzislaw M. Szulc², Besim Ogretmen², James S Norris¹ and Xiang Liu¹
Supplementary 3: A. MIA cells were pre-treated for two hours with indicated inhibitors with indicated doses then treated with 2.5 µM of LCL124. Cell viability was assessed using an MTS assay 24 hours of the treatment. B. MIA cells were treated in the absence or presence compound and Caspase 3/7 activity was examined 12 hours after treatment. C. MIA cells were incubated overnight and pre-treated for 1 hr with the proteasome inhibitor MG132 (100nM), the cathepsin B inhibitor Came074 (10µM), 100µM pane Caspase inhibitor ZVAD prior to the treatment of LCL124. Cell viability was assessed using an MTS assay. D. MIA cells were pre-treated for one hour with indicated inhibitors then treated with increasing doses of LCL124.
Supplementary 4: Panc-02 cells were plated in 96-well plates at a density of 5000 cells per well. EC50 was determined according to the Material and Methods. A. Cells received simultaneously treatment with both GMZ and LCL124. B. Cells treated with GMZ 2 hours prior to LCL124. C. EC50 of GMZ in GMZ resistant Panc-02 cells. Results are expressed as mean±SD.
LCL124, a cationic analog of ceramide selectively induces pancreatic cancer cell death by accumulating in mitochondria.


### Supplementary 5

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<th>WBC (10^9/L) (SD)</th>
<th>LYM (10^9/L) (SD)</th>
<th>MON (10^9/L) (SD)</th>
<th>GRA (10^9/L) (SD)</th>
<th>LY%</th>
<th>MO%</th>
<th>GR%</th>
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<th>HCT (%) (SD)</th>
<th>MCV (fl) (SD)</th>
<th>MCH (pg) (SD)</th>
<th>MCHC (g/dL) (SD)</th>
<th>RDWc (%) (SD)</th>
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**Supplementary 5:** Determination of injection dose of LCL124 in vivo. BA1B/c mice were treated with 20 mg/kg, 40 mg/kg and 80 mg/kg of LCL124 intraperitoneally. Seven days after injection, mice were sacrificed and blood was collected (n=4).
LCL124, a cationic analog of ceramide selectively induces pancreatic cancer cell death by accumulating in mitochondria.


**Supplementary 6:** A. Aspc-1 tumor-bearing mice were injected with 40 mg/kg of LCL124 every other day intraperitoneally for two weeks. 24 hours after the last injection, tumors and organs were removed and sphingolipids levels were (n=3). B. Animal weight was monitored twice a week and percentage change of weight compared to the initial day was demonstrated (n=5). (*, p<0.05; ***, p<0.01 versus to PBS treatment). Results are expressed as mean± SD. C. MALDI MS spectra of pure LCL124. D. MALDI MS spectra of collision-induced fragmentation of pure LCL124. E. MALDI MS spectra of LCL124 in tumor xenograft tissue, enriched by CASI followed by fragmentation.
LCL124, a cationic analog of ceramide selectively induces pancreatic cancer cell death by accumulating in mitochondria.

LCL124, a cationic analog of ceramide selectively induces pancreatic cancer cell death by accumulating in mitochondria

Thomas H. Beckham¹, Ping Lu¹, Elizabeth E. Jones⁵, Tucker Morrison¹, Clayton S. Lewis⁴, Joseph C. Cheng⁴, Venkat K. Ramshesh⁴, Gyda Beeson³, Craig C. Beeson³, Richard R. Drake⁵, Alicja Bielawska², Jacek Bielawski², Zdzislaw M. Szulc², Besim Ogretmen², James S Norris¹ and Xiang Liu¹