A Novel Salicylanilide Derivative Induces Autophagy Cell Death in Castration-Resistant Prostate Cancer via ER Stress-Activated PERK Signaling Pathway

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ABSTRACT

Metastatic castration-resistant prostate cancer (CRPC) is currently incurable. Cancer growth and progression is intimately affected by its interaction with host microenvironment. Cotargeting of the stroma and prostate cancer is therefore an emerging therapeutic strategy for metastatic CRPC. Cancer-induced osteoclastogenesis is known to contribute to CRPC bone metastasis. This study is to extend pharmacologic value of our synthesized LCC03, a derivative of 5-((2′,4′-difuoro phenyl)-salicylanilide that has previously testified for its osteoclastogenesis activity, by exploring its additional cytotoxic properties and underlying mechanism in CRPC cells. LCC03 was chemically synthesized and examined for cell growth inhibition in a serial of CRPC cell lines. We demonstrated that LCC03 dose-dependently suppressed proliferation and retarded cell-cycle progression in CRPC cells. The classical autophagy features, including autophagosome formation and LC3-II conversion, were dramatically shown in LCC03-treated CRPC cells, and it was associated with the suppressed AKT/mTOR signaling pathways, a major negative regulator of autophagy. Moreover, an expanded morphology of the endoplasmic reticulum (ER), increased expression of the ER stress markers GRP78 and PERK, and eIF2α phosphorylation were observed. Blockage of autophagy and PERK pathways using small molecule inhibitors or shRNA knockdown reversed LCC03-induced autophagy and cell death, thus indicating that the PERK-eIF2α pathway contributed to the LCC03-induced autophagy. Furthermore, treatment of tumor-bearing mice with intraperitoneal administered LCC03 suppressed the growth of CRPC xenografts in mouse bone without systemic toxicity. The dual action of 5-((2′,4′-difuoro phenyl)-salicylanilide on targeting both the osteoclasts and the tumor cells strongly indicates that LCC03 is a promising anticancer candidate for preventing and treating metastatic CRPC.

Introduction

Despite high response rates to androgen deprivation therapy in men with advanced prostate cancer, nearly all types of prostate cancer are eventually progression to the androgen-independent stage, which is termed castration-resistant prostate cancer (CRPC). CRPC is an incurable stage of prostate cancer, in which approximately 90% of patients develop metastases, mainly in the skeletal environment, such as bone, lung, and brain (1). Bone metastasis and skeletal complications are the major contributing factors to morbidity and mortality in patients with prostate cancer. Over time, the conventional treatment is ineffective, and the patients die of CRPC; the survival time is less than 19 months. Although new therapies (and drugs), including tubulin targeting chemotherapy (cabazitaxel), immunotherapy (sipuleucel-T), the steroidogenesis inhibitor (abiraterone), AR antagonist (enzalutamide), and ω-emitting radiotherapy (radium-223), have shown promising results in impairing the tumor growth and extending survival, a considerable proportion of patients with CRPC become unresponsive or become resistant to these treatment after a short period (2). The development of novel therapeutics that target distinct mechanisms of action is necessary to overcome resistance in patients with CRPC.

The endoplasmic reticulum (ER) plays pivotal roles in cell homeostasis and survival, which involved in biosynthesis of lipids, regulation of intracellular calcium concentration and metabolism of carbohydrates, and synthesis and folding of proteins. The accumulation of misfolded proteins within the ER lumen induces ER stress that triggers the unfolded protein response (UPR), an evolutionarily adaptive mechanism for restoring ER homeostasis; thus, UPR protects cells against the toxic accumulation of misfolded proteins (3). Under environmental stress, such as nutrient deprivation or hypoxia, malignant cells are particularly prone to protein misfolding and UPR activation, which lead to tumor progression and survival (4). However, in contrast to cytoprotection, recent studies have highlighted the role of severe or unresolved ER stress in cell death (5); unsolved ER stress has been previously associated with various human diseases, including atherosclerosis (6), neurodegenerative disorders (7), and type 2 diabetes (8). Therefore, the pharmacologic modulation of cellular responses toward ER stress-induced cell death pathway may prevent...
tumor development and growth, thus representing an attractive therapy for different cancers (9), including prostate cancer.

Cancer growth and progression is intimately affected by its interaction with the adjacent host microenvironment, that comprised of various stromal cell types, growth factors, and extracellular matrices, and thus presents an attractive target for therapeutic intervention (10). Salicylanilides are a key class of aromatic pharmacophore, comprising amides of salicylic acid and aniline. Salicylanilides derivatives have been reported to possess myriad pharmacological activities, such as antimicrobial [against bacterial (11), mycobacteria (12), viruses (13), and fungi (14)], anti-inflammatory (15), and antitumor properties (16). Recently, a novel function of 5-(2′,4′-difluorophenyl)-salicylanilide derivatives as potent inhibitors of osteoclastogenesis has also been discovered (17–19). Because pathologic osteoclast activation is associated with an increased risk of tumor progression (20), subsequent skeletal complications and death due to metastatic CRPC, in this study, the additional pharmacological value of 5-(2′,4′-difluorophenyl)-salicylanilide was examined by determining its effect and elucidating mechanism of action underlying its direct cytotoxicity to CRPC cells.

Materials and Methods

Cell lines and cell culture

Human prostate cancer cell lines: PC3, DU145, C4-2, and CWR22Rv1 were originally obtained from ATCC. Human benign prostatic hyperplasia (BPH) epithelial cell line BPH-1 was a kind gift of Dr. Su-Hwa Lin (The University of Texas MD Anderson Cancer Center). CWR22Rv1 were originally obtained from ATCC. Human benign prostatic hyperplasia (BPH) epithelial cell line BPH-1 was a kind gift of Dr. Su-Hwa Lin (The University of Texas MD Anderson Cancer Center). Human prostate cancer cell lines: PC3, DU145, C4-2, and CWR22Rv1 were originally obtained from ATCC. Human benign prostatic hyperplasia (BPH) epithelial cell line BPH-1 was a kind gift of Dr. Su-Hwa Lin (The University of Texas MD Anderson Cancer Center). No further authentication was performed by authors. The cells were maintained in RPMI1640 medium (Thermo Fisher Scientific) containing 10% FBS (Gibco), 100 U/mL of penicillin, and 100 μg/mL of streptomycin (Thermo Fisher Scientific) at 37°C under 5% CO₂ for less than 25 passages after thawing and routinely testing negative for Mycoplasma contamination using a PCR Mycoplasma Detection Kit (Abcam) to conduct described experiments.

Chemicals

N-(3,4-difluorophenyl)-2′,4′-difluoro-4-hydroxy[1,1′-biphenyl]-3-carboxamide (C₂₀H₁₁F₄NO₂, LCC03) was chemically synthesized using previously published procedures (21). The chemical structure of LCC03 is shown in Fig. 1A. 3-Methyladenine (3-MA, PubChem CID:1235398661), an autophagy inhibitor was purchased from Sigma-Aldrich. Selective PERK inhibitor GSK2606414 (PubChem CID:53469448) was purchased from EMD Millipore.

Cell proliferation and viability assay

The cytotoxic effects of LCC03 was assayed using a WST-1 Cell Proliferation Assay Kit (Roche Applied Science) following the manufacturer’s protocol. The IC₅₀ was calculated as the concentration of sample needed to reduce 50% of the absorbance compared with the dimethyl sulfoxide (DMSO)-treated control. The viability of the PC3 and C4-2 cells treated with LCC03 in the absence or presence of 3-MA (2 μmol/L) for 72 hours was assessed through trypsin-blue exclusion (Thermo Fisher Scientific). All assays were done in 6 replicates on at least 4 independent experiments.

Western blot analysis

Protein expression was analyzed as performed previously (22). The primary antibodies were listed in Supplementary Table S1. After incubation with an HRP-conjugated secondary antibody (1:5,000; GE Healthcare Life Sciences), the corresponding bands were detected using an ECL Kit (Advanta) and an AI 600 chemiluminescent imaging and analysis system (GE Healthcare Life Sciences). Each blot was performed at least twice.

Gene knockdown

Small hairpin RNA (shRNA) expression plasmids, including pLKO.1-shPERK (TRCN0000062373, target sequence TGCATCT-GGCTTGTTAATTAA), pLKO.1-shAtg7 (TRCN0000007584, target sequence GCCTGCTAGGGAGCTCTCAT; TRCN0000007587, target sequence CCCAGCTATTGGAACACTGTA), and a mammalian non-targeting shRNA control pLKO.1-shGFP (sh-ctr, TRCN0000072178, target sequence, CAACGCGCAACGTCTATA-TAT) were obtained from the National RNAi Core Facility (Institute of Molecular Biology). The recombinant lentivirus was generated by transfecting shRNA plasmids along with the packaging plasmids pCMV-ΔR8.91(Gag/Pol/Rev) and pMD.G (VSV-G envelope) in 293FT cells and used for infecting the PC3 and C4-2 cells according to a previously described protocol (23). The extent of gene knockdown was determined through an immunoblotting assay.

Transmission electron microscopy

Sample preparation for TEM were prepared with aid from the Imaging Core at Taipei Medical University. Briefly, the cells grown on plastic chamber slides were fixed using 2% paraformaldehyde (PFA) and 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.4) for 30 minutes at room temperature and then subjected to postfixation with 2% osmium tetroxide. After dehydration in ascending grades of ethanol and propylene oxide, the samples were embedded in Epon. Epon polymerization was conducted at 62°C for 48 hours. Ultrathin sections (approximately 70 nm in thickness) were obtained using an ultramicrotome (Leica Ultracut UCT; Leica Microsystems GmbH) and were collected on 100 mesh copper grids. After double staining with uranyl acetate and lead citrate, the sections were examined under a Hitachi H-600 Transmission Electron Microscope operating at 100 kV.

Immunofluorescence staining

The PC3 and C4-2 cells transfected with the EGFP-LC3 (24) plasmid (a gift from Karla Kirkegaard; Addgene plasmid #11546) were grown on coverslips for overnight and treated with DMSO or LCC03 combined with GSK2606414 for 72 hours. After removing the culture media, the cells were fixed using 4% PFA and washed thrice with PBS. Subsequently, the cells were stained with DAPI and then mounted in ProLong Gold Antifade reagent (Thermo Fisher Scientific). Images of the cells were acquired using a Carl Zeiss LSM 510 META confocal microscope (Carl Zeiss) equipped with a Plan Apochromat 63× oil/1.4 NA DIC objective.

Animal studies

Animal experiments were approved by the Institutional Animal Care and Use Committee of Taipei Medical University (LAC-2016-0345) and complied with their regulations. Six-week-old male nude mice (BALB/CAnN.Cg-Foxn1nu/CrlNarl) were purchased from the National Laboratory Animal Center (Taipei, Taiwan) and maintained under the specific pathogen-free conditions and cared for according to the criteria outlined in the National Academy of Sciences Guide for the Care and Use of Laboratory Animals. To initiate experimental prostate bone metastasis in the xenograft models, the mice were anesthetized by Zoletil and Rompun mixture, and subjected to an intraosseous injection of 2 × 10⁶ PC3-Luc cells into the mouse tibia following our previously established protocol (23). One week after cell injection, the tumor-bearing mice were randomized and given the following intraperitoneal...
treatments twice per week for 4 weeks \((n = 10 \text{ for each group})\): (i) untreated, (ii) DMSO, (iii) LCC03 (20 mg/kg), and (iv) LCC03 (40 mg/kg). The tumor growing in the bone were monitored through bioluminescent imaging (BLI) weekly by using the IVIS 2000 system and the Living Image software (Caliper Life Sciences) for quantification of luminescence intensity. The body weights of all the mice were measured thrice per week for 4 weeks. The mice were sacrificed at 5 weeks after the initial treatment and the heart, lung, kidney, liver, spleen, and tibia were excised for histopathologic analyses. Before the animals were sacrificed, blood samples were collected to analyze biochemical parameters with aid from the TMU Animal Care Facility.

**IHC staining**

Bone specimens were fixed in 10% neutral buffered formalin for 24 hours. They were then decalcified using 14% EDTA (pH 7.2) for 14 days at room temperature and embedded in paraffin. IHC analyses were performed using the NovoLink Polymer Detection System (Leica Microsystems) with the following antibodies: mouse monoclonal anti-Ki-67 antibody (1:100; Clone GM010; Genemed Biotechnologies) and rabbit polyclonal anti-human LC3B (1:50; LS-B9807; LifeSpan BioSciences). The extent of apoptosis and osteoclastogenesis was evaluated using an Apo-BrdU-IHC In Situ DNA Fragmentation Assay Kit (BioVision, Inc.) and a TRACP &

**Figure 1.**

Effect of LCC03 on cell growth and cell-cycle progression in prostate cancer cell lines. A, Rational design synthesis and chemical structures of small molecule LCC03. B, Proliferation of benign (BPH) and malignant prostate cancer cell lines treated with a serial of doses of LCC03 for 48 hours was determined using the WST-1 assay. Relative cell proliferation is presented as the mean ± SD percentage of absorbance (at 450 nm) compared with the DMSO-vesicle-treated control (0 μmol/L LCC03) for at least 4 independent experiments \((n ≥ 4)\). C, Bar graphs of the percentages of G1, S, and G2/M populations in the PC3 and C4-2 cells treated with the DMSO vesicle control and the indicated concentrations of LCC03 for 48 hours. The results of 1 of 3 independent experiments are shown. D, Western blotting analysis of cell-cycle checkpoint markers. β-Actin is shown as the protein loading control. The expected molecular weight of each protein is indicated on the left side of the figure.
ALP Double-Stain Kit (TaKaRa), respectively following the manufacturer’s instructions.

Statistical analysis
Statistical analysis was performed using GraphPad Prism 6 (GraphPad Software). Data are presented as mean ± SD and % change versus controls. Difference between 2 groups was measured by Student t test. A probability value of P < 0.05 was considered statistically significant.

Results

**LCC03 induces growth retardation and cell-cycle arrest in prostate cancer cells**

Treating patients with CRPC remains a substantial clinical challenge. Thus, we assessed whether LCC03, a novel derivative of 5-(2',4'-difluorophenyl)salicylanilide (Fig. 1A), is potentially toxic to a series of CRPC-like cell lines, including androgen receptor (AR)-positive cell lines C4-2 and CWR22r v1 and the AR-negative cell lines PC3 and DU145. A cell proliferation assay revealed that the incubation of prostate cancer cells with LCC03 for 48 hours caused a marked and dose-dependent growth inhibition (Fig. 1B). The IC50 in different cell lines was 0.69 to 4.8 μmol/L (Table 1). By contrast, the cell proliferation of benign hyperplastic prostatic epithelial cells (BPH-1) was unaffected by LCC03 at a concentration of <5 μmol/L. The IC50 value of LCC03 for BPH-1 (5.7 μmol/L) cells was 3 to 20 times higher than that for the cancerous cells, thus indicating that LCC03 is selectively more toxic to cancerous than to noncancerous cells. The highest IC50 value of LCC03 was observed in the PC3 cell line, which was originally derived from different bone metastases. The lowest IC50 value of LCC03 was observed in the C4-2 cells; thus, the C4-2 cells were most sensitive to LCC03. These 2 cell lines were selected for subsequent investigation of the anticancer properties of LCC03.

We analyzed the cell-cycle distribution in the PC3 and C4-2 cells after 48 hours of incubation with LCC03 to characterize the anti-proliferative properties of LCC03 in detail. We observed dose-dependent accumulation of the PC3 cells in the G1- M phase on LCC03 exposure (Supplementary Fig. S1A and S1C, PC3). The increase in the (G1– M ) phase was coupled with a reduction in percentage of cells in the G0–G1 phase. In the C4-2 cells, LCC03 treatment increased the proportion of cells in the G1 phase and reduced the proportion of cells in the G2 phase (Supplementary Fig. S1A and S1C, C4-2). The effect of LCC03 on cell cycle progression was also supported by the result of mRNA array-based gene profiling analysis, which showed a marked change in the cell-cycle pathway in the PC3 cells after treatment with LCC03 (Supplementary Fig. S1B). Western blotting analysis further confirmed the time- and dose-dependent repression of cyclin D1 as well as the cdc2/cyclin B complex activity by LCC03 in both cell lines (Fig. 1D), thus indicating a strong effect of LCC03 on the cell-cycle checkpoints.

**LCC03 causes cytotoxicity activity through autophagy but not via an apoptosis-dependent pathway**

The cytotoxic effect of LCC03 on prostate cancer cell survival was assessed through the trypan blue assay after incubating the cells for 3 days with LCC03. LCC03 treatment reduced cell viability in a dose-dependent manner (Fig. 2A), which was similar to the effect of LCC03 on cell proliferation. We then assessed whether the cytotoxic effect of LCC03 on prostate cancer cells was correlated with increased apoptosis. Notably, the percentage of annexin V-positive cells increased only slightly among the PC3 cells and decreased among the C4-2 cells, when the cells were treated with LCC03 (up to their respective IC50 concentrations) for 72 hours (Supplementary Fig. S2A). Moreover, Western blotting analysis revealed that only minimal cleavage of PARP and caspase 3 was detectable in LCC03-treated cells (Supplementary Fig. S2B). Collectively, these data indicate that LCC03-induced cytotoxicity was not closely associated with the apoptotic cell death.

Recent studies have identified autophagy as the major mechanism of cell death in addition to apoptosis in response to cellular stress. Hence, we examined whether autophagy is induced by LCC03 and whether it has a critical role in mediating cell death. Cyto-ID green fluorescent dye was used to stain autophagic vacuoles in the cells and the fluorescence intensity was quantified through flow cytometry. Similar to the treatment with the autophagy inducers tunicamycin and rapamycin, an increase in fluorescent intensity was observed in the PC3 and C4-2 cells after treatment of LCC03 for 72 hours, thus suggesting the induction of autophagy (Fig. 2B). To confirm this result, TEM was performed for ultrastructural analysis. Numerous autophagic vacuoles and empty vacuoles were observed in the LCC03-treated PC3 cells, and most of the autophagic vacuoles contained intact lamellar structure and/or residual digested materials (Fig. 2C). The DMSO-vesicle treated control exhibited only a few autophagic features. The Western blot analysis results (Fig. 2D) revealed a time- and dose-dependent increase in the conversion of LC3B-I to LC3B-II, which is a hallmark of macroautophagy. LCC03 also induced a higher amount of the autophagy factor Beclin1-Atg5 conjugate between 48 and 72 hours after treatment, and its induction was associated with increased LC3B conversion in both cell lines. Notably, Beclin-1 protein, a key regulator complexed with class III PI3K for autophagosome formation through dissociation from the anti-apoptotic protein Bcl2, was upregulated concomitantly with a decreased in Bcl2 in the C4-2 cells after treatment with LCC03. However, no significant changes were observed in the PC3 cells, thus suggesting that LCC03 elicited a canonical or noncanonical autophagic pathway in a cell-type–dependent manner. Because Bcl-2 is a prosurvival protein, the increased expression of Bcl-2 seen in PC3 cells after the longer exposure (72 hours) of LCC03 might contribute to helping cell surviving under treatment, by which PC3 cells acquire more resistance than C4-2 cells to the cytotoxic effects of LCC03. In addition, blockade of autophagosome formation by using 3-MA, a PI3K inhibitor, or genetically knocking down autophagy-related 7 (ATG7) expression significantly attenuated the induction of autophagosome marker expression and restored the viability of cells treated with LCC03 (Fig. 2E and F), thus confirming that LCC03 induced cytotoxicity in prostate cancer cells through activation of autophagy.

### Table 1. Summary of IC50 values for LCC03 in 1 human benign prostate hyperplasia (BPH) and 4 prostate cancer cell lines after 48 hours' treatment.

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Mean IC50 ± SD (μmol/L)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>BPH-1</td>
<td>15.7322 ± 8.944</td>
</tr>
<tr>
<td>C4-2</td>
<td>0.688 ± 0.061</td>
</tr>
<tr>
<td>CWR22r v1</td>
<td>2.57 ± 0.35</td>
</tr>
<tr>
<td>PC3</td>
<td>4.48 ± 2.58</td>
</tr>
<tr>
<td>DU145</td>
<td>2.64 ± 0.85</td>
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*All values were presented as mean ± SD of 6 replicates in 4 independent experiments.
LCC03 activates ER-stress-induced PERK-eIF2α-ATF4 signaling pathway

To gain a better insight into the LCC03-induced autophagic pathways, we identified autophagy-related signaling molecules. The kinase mTOR is a critical regulator of autophagy induction. The phosphorylation of mTOR suppresses autophagy, whereas the dephosphorylation of mTOR promotes autophagy. Our results identified significant downregulation of p-mTOR level in C4-2 and PC3 cells treated with LCC03 (Fig. 3A). This downregulation of p-mTOR was strongly associated with Akt inactivation by dephosphorylation alone in the PC3 cells or together with reduced expression of total Akt protein in the C4-2 cells. The expression of AMPK, a negative regulator of mTOR, although did not differ significantly between the LCC03- and DMSO-treated cells, the phosphorylation of AMPK-α was massively induced upon LCC03 treatment. Collectively, these findings indicate that LCC03 triggered autophagy activation in the prostate cancer cells via an AMPK- and Akt-dependent mTOR pathway.

Several reports have reported that ER-stress-activated UPR can trigger several signaling pathways that cause autophagy (25, 26). In addition to the autophagosome-like vesicles, TEM revealed that the LCC03-treated cells exhibited abnormal ER expansion (Fig. 3B). Therefore, we determined whether LCC03 could induce an ER stress response and subsequently regulate autophagy. The Western blot analysis results revealed upregulation of GRP78 expression, which indicated the activation of ER stress (Fig. 3C). Moreover, LCC03 treatment considerably increased the expression of the ER stress sensor PERK, the levels of phosphorylated eIF2α and ATF4, and the ATF4 downstream target genes CHOP (a transcription factor known to induce autophagy) and TRIB3 (a negative regulator of Akt activation) in a time- and dose-dependent manner. These results demonstrated that the PERK-eIF2α-signaling pathway was activated during LCC03-induced ER stress.

Blockage of the PERK activation attenuates the LCC03-induced autophagic cell death

We investigated whether LCC03-induced autophagic cell death in the tested cells was mechanistically related to ER stress signaling pathways by using pharmacological and genetic approaches to inhibit...
ER stress sensor PERK activity. Stable transfection of EIF2AK3-targeting shRNA (sh-PERK) significantly suppressed PERK expression, blocked LCC03-induced upregulation of LC3B conversion, and inhibited eIF2α phosphorylation compared with cells transfected with negative control shRNA (Fig. 4A). Similarly, inhibition of PERK kinase activity by using the compound GSK2606414 slightly increased the basal levels of PERK; however, it prevented the phosphorylation of eIF2α and the conversion of LC3B-I to LC3B-II when the cells were exposed to LCC03 (Fig. 4B). We further analyzed autophagosome formation in the PC3 and C4-2 cells that were transfected with a plasmid expressing autophagosome-associated LC3 protein fused to green fluorescent protein (GFP-LC3). In the GFP-LC3 transfected cells, treatment with LCC03 resulted in an increase in the redistribution of green fluorescence from diffused to a punctum pattern in the perinuclear region. Treatment with GSK2606414 also suppressed the LCC03-induced autophagosome formation, which was indicated by

Figure 3.

Time and dose effect of LCC03 on ER stress activation in CRPC cells. A and C, Western blotting analysis of signaling molecules involved in (A) autophagy and (C) ER stress activation in the PC3 and C4-2 cells. β-Actin is shown as the protein loading control. The expected molecular weight of each protein is indicated on the left side of the figure. B, Ultrastructural analysis of ER stress activation in the PC3 cells treated with DMSO or 10 μmol/L LCC03 for 48 hours through TEM. Asterisks indicate the enlarged ER. Images are representative of 3 independent treatments.

ER stress sensor PERK activity. Stable transfection of IF2AK3-targeting shRNA (sh-PERK) significantly suppressed PERK expression, blocked LCC03-induced upregulation of LC3B conversion, and inhibited eIF2α phosphorylation compared with cells transfected with negative control shRNA (Fig. 4A). Similarly, inhibition of PERK kinase activity by using the compound GSK2606414 slightly increased the basal levels of PERK; however, it prevented the phosphorylation of eIF2α and the conversion of LC3B-I to LC3B-II when the cells were exposed to LCC03 (Fig. 4B). We further analyzed autophagosome formation in the PC3 and C4-2 cells that were transfected with a plasmid expressing autophagosome-associated LC3 protein fused to green fluorescent protein (GFP-LC3). In the GFP-LC3 transfected cells, treatment with LCC03 resulted in an increase in the redistribution of green fluorescence from diffused to a punctum pattern in the perinuclear region. Treatment with GSK2606414 also suppressed the LCC03-induced autophagosome formation, which was indicated by
the reduced appearance of GFP-LC3 puncta (Fig. 4C). The PERK signaling pathway attenuated by either shRNA or small molecule inhibitors restored cell survival in response to LCC03 treatment, thus confirming the critical role of PERK-mediated ER-stress-signaling pathway in LCC03-induced cell death (Fig. 4D). Interestingly, knocked down of EIF2AK3 did not reverse the LCC03-mediated mTOR dephosphorylation (Fig. 4A). Conversely, activation of mTOR by MHY1485 decreased PERK expression in LCC03-treated PC3 cells (Supplementary Fig. S3), implying that PERK-mediated autophagy by LCC03, at least in part, is regulated via mTOR pathway.

LCC03 treatment suppresses the growth of osteolytic prostate cancer metastasis in mouse bone

To investigate the therapeutic potential of LCC03 for prostate cancer, particularly targeting CRPC bone metastases and the associated osteoclast activation, PC3 cells that are relatively highly resistant to LCC03 and produce a pure osteolytic reaction in the bone were used in an experimental animal model. Luciferase expressing-PC3 cells (PC3-Luc) were injected directly into the tibia of the nude mice and allowed to form tumors for 10 days, as demonstrated through BLI. The tumor-bearing mice received intraperitoneal administration of LCC03 at a low (20 mg/kg) and high (40 mg/kg) dose or DMSO vesicle every other day (QOD) for 4 weeks. A control group received no treatment. Simultaneously, the body weights of all the mice were also recorded. The responsiveness of the PC3-Luc tumors to this therapy was monitored through weekly BLI. During the 5-week monitoring period, the bioluminescence signal gradually increased with time in the untreated control group, which indicated that the tumors progressed aggressively (Fig. 5A, imaging). A similar imaging pattern was observed in the mice treated with DMSO vesicles and the low-dose LCC03, which indicated that 20 mg/kg QOD of LCC03 was not effective at suppressing tumor growth. By contrast, the mice treated with high-dose (40 mg/kg) LCC03 exhibited a relatively constant or slowly changing profile of bioluminescence signals over time. A quantitative analysis of the BLI data revealed that the average signal intensity in the mice treated with high-dose LCC03 was approximately 75% less than that in the untreated and DMSO-treated control mice (Fig. 5A); thus confirmed a significant anticancer effect of LCC03.
effect on tumor growth. These results confirmed dose-dependent inhibition of tumor growth by LCC03 and demonstrated that 40 mg/kg QOD for 4 weeks are the efficacious dose to induce regression.

In addition, the high-dose treatment was not associated with any observed adverse effect in the body weight and cellular structure of nontarget organ tissues (Fig. 5B). The biochemical parameters, including BUN (blood urea nitrogen), CREA (creatinine), ALT (alanine aminotransferase), ALP (alkaline phosphatase), and TBIL (total bilirubin) were all within the reference range and no significant changes ($P > 0.1$) in mice before and after treatment with LCC03 (40 mg/kg) for 5 weeks (Supplementary Table S2), which indicated that the administered dose of LCC03 was safe for therapeutic use.

Histologic analysis (Fig. 5C, H&E) of the affected legs from the sacrificed mice after 5 weeks of treatment revealed healthy and packed tumor cells growing in the marrow cavity of the control groups (no treatment or DMSO-vesicle treatment). The highly proliferative nature of the tumor cells in these control groups was confirmed through ki-67 expression (Fig. 5C, ki-67). By contrast, extensive necrotic regions were found in the tumors excised from the mice treated with high-dose LCC03, and only a few ki-67-stained cancer cells were detected in these tumors. IHC staining of LC3B indicated a strong increase in LC3B expression in the tumors from the mice treated with high-dose LCC03. TUNEL staining results of the tumors indicated that apoptotic cells were absent (Fig. 5C, LC3B and TUNEL). Collectively, these results confirmed that the considerable tumor regression caused by systemic LCC03 treatment was associated with autophagy but not apoptosis. Moreover, the tartrateresistant acid phosphatase (TRAP)-positive osteoclasts were distributed mainly in osteolytic bone lesions of the control groups but barely detected in the LCC03-treated tumor sections (Fig. 5C, TRAP), further confirming the dual targeting of prostate cancer cells and osteoclastogenesis by LCC03.

**Discussion**

The NF-kB ligand (RANKL) pathways participate in the activation and survival of osteoclasts; therefore, they represent a therapeutic target for osteoclast-induced bone destruction in treatment- and metastatic cancer-induced osteolysis. The RANKL inhibitor denosumab is recently approved by the U.S. FDA for the prevention of skeletal...
related events in men with metastatic CRPC, but studies on these compounds have not demonstrated a survival benefit (27). Moreover, recent drug discovery efforts have identified small molecules that impair the ER function for therapeutic intervention. For example, bortezomib, the first FDA-approved proteasome inhibitor that induces severe ER stress and causes apoptosis, was used to treat patients with multiple myeloma (28). However, the ineffectiveness of the aforementioned small molecules against several solid tumors including prostate cancer emphasized the need for the development effective drugs. Autophagy can be stimulated in response to multiple forms of cellular stress, such as intracellular pathogens, nutrient and growth factor deprivation, hypoxia, damaged organelles, and ER stress (29). In the present study, we provided the first evidence that LCC03, a derivative of 5-(2′,4′-difluorophenyl)-salicylanilides that was originated reported as a small molecular inhibitor of RANKL-induced osteoclastogenesis, exhibits strong activity against prostate cancer cell growth through ER-stress-mediated autophagy via the PERK/eIF2α signaling pathway. Other salicylanilide derivatives, such as Niclosamide (30) and nitro-substituted hydroxybenzamides (31–33) that share structure similarities with gefitinib and erlotinib, a class of small molecule inhibitors of the epidermal growth factor receptor (EGFR) exhibited antiproliferative and/or proapoptotic activities against a spectrum of human cancer cell lines. However, therapies targeting the EGFR by using gefitinib, and erlotinib showed nonsignificant clinical benefit in patients with CRPC (34). The dual action of 5-(2′,4′-difluorophenyl)-salicylanilides on both the osteoclasts and various CRPC cell lines (both AR-positive and AR-negative) may make it a superior anticancer candidate to EGFR tyrosine kinase inhibitors and AR-targeting agents to prevent and treat metastatic CRPC.

We demonstrated that LCC03 exerts a robust antiproliferative effect, induces cell-cycle arrest and reduces the viability in a dose-dependent manner in various prostate cancer cell lines. The tumor suppressor protein p53 is a master regulator with pleiotropic effects on metabolism, anti-oxidant defense, genomic stability, proliferation, senescence, and cell death. The activation of p53 was shown to inhibit mTOR activity and transactivate autophagy-induced genes (35). Four core autophagy genes: ATG4B, ATG4D, ULK1, and ULK2 were also recently defined as AR-targeting genes (36). The higher susceptibility of C4-2 to LCC03 compared with the cell lines DU145 (AR-null and p53-mutant cells), CWR22r-v1 (AR-positive and p53-mutant cells), and PC3 (AR-null and p53-null cells) may be attributable to the expression of both functional p53 and AR to induce autophagy. Thomas and colleagues (37) have demonstrated a 2-signal model for the regulation of cell proliferation during ER stress. The Thomas and colleagues (37) has demonstrated a 2-signal model for the regulation of cell proliferation during ER stress. The regulation of cell proliferation during ER stress, requires functional p53 and AR, and erlotinib showed nonsignificant and/or proapoptotic activities against a spectrum of human cancer cell lines. However, therapies targeting the EGFR by using gefitinib, and erlotinib showed nonsignificant clinical benefit in patients with CRPC (34). The dual action of 5-(2′,4′-difluorophenyl)-salicylanilides on both the osteoclasts and various CRPC cell lines (both AR-positive and AR-negative) may make it a superior anticancer candidate to EGFR tyrosine kinase inhibitors and AR-targeting agents to prevent and treat metastatic CRPC.

Aluminum hydroxide, as a small molecular inhibitor of RANKL-induced osteoclastogenesis, extends strong activity against prostate cancer cell growth through ER-stress-mediated autophagy via the PERK/eIF2α signaling pathway. Other salicylanilide derivatives, such as Niclosamide (30) and nitro-substituted hydroxybenzamides (31–33) that share structure similarities with gefitinib and erlotinib, a class of small molecule inhibitors of the epidermal growth factor receptor (EGFR) exhibited antiproliferative and/or proapoptotic activities against a spectrum of human cancer cell lines. However, therapies targeting the EGFR by using gefitinib, and erlotinib showed nonsignificant clinical benefit in patients with CRPC (34). The dual action of 5-(2′,4′-difluorophenyl)-salicylanilides on both the osteoclasts and various CRPC cell lines (both AR-positive and AR-negative) may make it a superior anticancer candidate to EGFR tyrosine kinase inhibitors and AR-targeting agents to prevent and treat metastatic CRPC.

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Numerous studies have suggested that autophagy induction is a cell survival mechanism of chemoresistance (40, 41); therefore, blocking autophagy during cancer treatment can improve clinical outcomes. However, other studies have indicated that autophagy induction sensitizes cancer cells to chemotherapy and radiation (42, 43), suggesting autophagy stimulation for tumor-targeted therapy. Because autophagy is negatively regulated by mTOR, the pharmacological anticancer agents, including rapamycin, everolimus, and temsirolimus, which operate at the level of the mTOR pathway level are currently under investigation for clinical induction of autophagy. In the present study, we defined LCC03 as a novel ER stressor that initiates cell death in prostate cancer cells through autophagy induction. Although class III PI3K activation directly affects autophagy, our results revealed that in addition to AMPK pathway, LCC03 considerably suppressed class I PI3K/Akt activation that regulates autophagy indirectly via the mTOR pathway in both AR-positive and AR-negative prostate cancer cell populations. Thus, it provides the mechanistic rationale for the efficacy of LCC03 in autophagy induction against prostate cancer and may extend to other tumor types.

Three signaling proteins, IRE1α, ATF6, and PERK, are involved in the initiation of ER-stress response (5). PERK was associated with ER stress-induced autophagy. Once activated, this kinase directly phosphorylates eIF2α to block translation initiation, leading to the upregulation of autophagy-related genes and inhibition of autophagy suppressors. Although the involvement of the IRE1α and ATF6 pathways in the anticancer property of LCC03 was not addressed in this study, we determined that blockage of PERK expression by sequence-specific siRNA or selective small molecule inhibitors rendered prostate cancer cells resistant to LCC03 to a similar degree as the untreated group, thus implicating PERK as a major effector of LCC03-induced cell death. Notably, a basal level of LC3-B conversion in response to LCC03 treatment was still retained even when the cell viability was completely restored by PERK antagonist, suggesting that the other ER-stress signaling pathways, such as the IRE1α and ATF6 pathways, may contribute to cytotoxic rather than cytotoxic autophagy induction. Our current knowledge and future work in the area of autophagy regulation should facilitate the development of improved therapeutic approaches for cancer.

CRPC is well recognized to be resistant to conventional therapies, mainly due to its typical feature of apoptosis resistance (44). Our in vivo proof of principle study showed the clinical benefit and safety of LCC03 treatment in CRPC bone metastases in the clinically relevant animal models. Thus, strongly support the translational application of LCC03 [5′-(2′,4′-difluorophenyl)-salicylanilide derivative] for potential monotherapy and combination therapy against metastatic CRPC. Additional optimization studies for therapeutic index and biologics formulation are warranted to improve its potency and pharmacological properties.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors’ Contributions

Conception and design: C.-L. Hsieh, H.-S. Huang, K.-C. Chen, L.W.K. Chung, S.-Y. Sung


Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): K.-C. Chen, T. Saka, C.-Y. Chiang

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): H.-S. Huang, T. Saka, C.-Y. Chiang, S.-Y. Sung

Writing, review, and/or revision of the manuscript: H.-S. Huang, K.-C. Chen, S.-Y. Sung

Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): H.-S. Huang

Study supervision: C.-L. Hsieh, H.-S. Huang.
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