RESEARCH PAPER

Identification of an old antibiotic clofoctol as a novel activator of unfolded protein response pathways and an inhibitor of prostate cancer

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BACKGROUND AND PURPOSE

Finding new indications for existing drugs, also known as drug repositioning or repurposing, is a powerful approach to accelerate drug discovery and development. The unfolded protein response pathways have been proposed to be a viable target for developing new anticancer drugs.

EXPERIMENTAL APPROACH

We screened the Johns Hopkins Drug Library for inhibitors of prostate cancer cell proliferation to identify new antiprostate cancer treatments among known drugs. We systematically investigated the mechanism underlying the anticancer activity of a hit and assessed its efficacy in blocking prostate tumour growth in a mouse model.

KEY RESULTS

The antibacterial drug clofoctol was identified as a novel inhibitor of prostate cancer cell proliferation. Morphologically, cells treated with clofoctol were found to undergo massive vacuolization, reminiscent of endoplasmic reticulum stress. Indeed, all three unfolded protein response pathways including inositol requiring enzyme 1, double-stranded RNA-activated PK-like ER kinase and activating transcription factor 6 were found to be activated by clofoctol. Activation of unfolded protein response pathways by clofoctol led to the inhibition of protein translation in cells and the induction of G_1 cell cycle arrest in prostate cancer cells. Clofoctol also inhibited prostate cancer xenograft growth *in vivo* without apparent toxicity.

CONCLUSION AND IMPLICATIONS

Our findings revealed clofoctol as a novel activator of the unfolded protein response pathways and a promising inhibitor of prostate cancer. As clofoctol has been used in the clinic for years, it is ready for clinical evaluation as a novel antiprostate cancer drug candidate.

Abbreviations

ATF6, activating transcription factor 6; CHOP, transcription factor C/EBP homologous protein; eIF2α, eukaryotic translation initiation factor 2α; IRE1, inositol requiring enzyme 1; JHDL, Johns Hopkins Drug Library; LC3B, microtubule-associated protein light chain 3B; PERK, double-stranded RNA-activated PK-like ER kinase; UPR, unfolded protein response; XBP-1, X-box binding protein-1

Table of Links

TARGETS	LIGANDS
eIF2α	Thapsigargin
IRE1	SP600125
PERK	Cycloheximide

This Table lists key protein targets and ligands in this document, which are hyperlinked to corresponding entries in http://www.guidetopharmacology.org, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY (Pawson *et al.*, 2014) and are permanently archived in the Concise Guide to PHARMACOLOGY 2013/14 (Alexander *et al.*, 2013).

Introduction

Drug discovery and development is costly and a time-consuming process. Discovering new pharmacological activity among known drugs, or drug repositioning, allows for dramatic acceleration of the discovery and development of new drugs (Ashburn and Thor, 2004). Existing drugs have favourable pharmacokinetic and pharmacodynamic properties with tolerable side effects. Thus, old drugs can quickly enter human clinical testing for newly identified indications using the existing drug dosage regimen. Over 10 years ago, we began to assemble a library of clinical drugs, dubbed the Johns Hopkins Drug Library (JHDL), which contains approximately 3000 drugs that have been approved by either the US Food and Drug Administration or its foreign equivalents, or are under the late phases of clinical trials (Chong *et al.*, 2006b). We have screened the JHDL in a variety of biological assays and disease models, including cancers, diabetes and infectious diseases, and identified a number of promising hits (Chong *et al.*, 2006a,b; 2007; McMahon *et al.*, 2007; Ren *et al.*, 2008; Zhang *et al.*, 2008; 2009; Lee *et al.*, 2009; Kim *et al.*, 2010; Shim *et al.*, 2010; 2012; Lin *et al.*, 2011; Platz *et al.*, 2011; Rovira *et al.*, 2011; Liu-Chittenden *et al.*, 2012; Choi *et al.*, 2013). Among the hits is the antifungal drug itraconazole that was identified as a potent inhibitor of angiogenesis and the hedgehog signalling pathway respectively (Chong *et al.*, 2007; Kim *et al.*, 2010). To date, itraconazole has entered multiple phase 2 clinical studies and shown promise in treating both non-small cell lung cancer and prostate cancer (Antonarakis *et al.*, 2011; Rudin *et al.*, 2013). One of the most potent hits from our screen for inhibitors of prostate cancer is digoxin, a cardiac glycoside, which was also identified as a potent inhibitor of hypoxia-inducible factor-1 alpha (HIF-1α) (Zhang *et al.*, 2008; Platz *et al.*, 2011). These findings prompted us to conduct a large-scale cohort study with male patients having heart diseases who have taken digoxin for 5–10 years. Digoxin was found to significantly reduce prostate cancer incidence among patients who have

used it compared with the control group. These findings suggest that digoxin is a promising drug candidate for the prevention and treatment of prostate cancer. Emerging from the same screen for inhibitors of prostate cancer is the antibacterial drug clofoctol that is likely to work through the activation of the unfolded protein response (UPR) pathway in the endoplasmic reticulum (ER).

The ER is a major cellular organelle in which folding and maturation of secretory proteins take place (Rutkowski and Kaufman, 2004; Ron and Walter, 2007). It plays an important role in the quality control of proteins, determining whether proteins are properly folded, which allows proteins to reach their final destination. When proteins are misfolded, they are retained in the ER and delivered into cytosol for proteosomal degradation in a process known as ER-associated degradation (Bernasconi and Molinari, 2011). If misfolded proteins accumulate over the limit of the capacity of ER-associated degradation, a process called UPR is activated (Sidrauski *et al.*, 1998). UPR is a cellular protective mechanism to cope with an excess of misfolded proteins in the ER. UPR is activated by one of three sensing proteins including inositol requiring enzyme 1 (IRE1), double-stranded RNA-activated PK-like ER kinase (PERK) and activating transcription factor 6 (ATF6) (Walter and Ron, 2011). These three UPR pathways activate the transcription of UPR target genes which increase the protein folding capacity in the ER. In addition to transcriptional activation, they also activate signalling pathways which down-regulate protein translation and enhance the degradation of ER-bound mRNAs to eventually reduce the ER folding load (Harding *et al.*, 2000).

The inhibition of protein translation under UPR conditions is governed by PERK that phosphorylates eukaryotic translation initiation factor 2α (eIF2α) (Harding *et al.*, 2000). The phosphorylation of eIF2α inhibits the formation of the 43S translation initiation complex and, thereby blocking global translation. Inhibition of protein translation largely affects short-lived proteins such as cyclin D1, a rate-limiting regulator of cell cycle progression during the G_1 phase. Activation of UPR decreases cyclin D1 translation, thereby

inhibiting cyclin D-dependent kinase activity. This also leads to a concomitant inhibition of cyclin E- and A-dependent kinases, which culminate in G_1 phase cell cycle arrest (Brewer et al., 1999). Upon prolonged activation of UPR, cells cannot alleviate ER stress and undergo cell death. If cells fail to establish homeostasis under ER stress, UPR activates apoptotic and/or autophagic cell death to remove these cells from the body to protect the entire organism (Woehlbier and Hetz, 2011). Highly proliferative cancer cells have a higher demand for ER to synthesize more proteins to keep up with the increased metabolic demand compared with normal cells (Suh et al., 2012). The more proteins the ER processes, the larger the amount of misfolded proteins that accumulate in the ER. Several lines of evidence suggest that many types of cancer cells have higher levels of ER stress and as a consequence, the UPR pathways are already active to maintain cellular homeostasis (Luo and Lee, 2013). Therefore, further increasing the ER stress, to overload the machinery of stress responses in cancer cells that can in turn induce cell cycle arrest or cell death, is recognized as an alternative approach to selectively inhibit cancer cells (Leleu et al., 2009; Suh et al., 2012). Several anticancer agents under development, including heat shock protein 90 (HSP90) inhibitors and proteasome inhibitors, have been shown to induce ER stress in cancer cells and have entered phase I/II clinical trials for cancer therapy (Obeng et al., 2006; Davenport et al., 2007).

Clofoctol is an antibiotic that has been used for the treatment of upper respiratory tract infections in France and Italy among other countries for decades (Danesi and Del Tacca, 1985). The proposed mechanism for its antibacterial activity is the alteration of bacterial membrane permeability owing to its hydrophobic nature (Yablonsky, 1983). Recently, it was reported that clofoctol inhibits protein translation in mammalian cells and as such, could serve as a potential anticancer drug (Pelletier et al., 2007). However, the mechanism by which clofoctol inhibits translation and whether this inhibition of translation is responsible for its anticancer activity is not known. In the present study, we demonstrated that clofoctol inhibits the growth of prostate cancer cells at clinically achievable concentrations. It was also found to induce ER stress and activate all three UPR pathways in prostate cancer cells, which led to indirect inhibition of protein translation. Inhibition of protein translation by clofoctol caused a decrease in the levels of cyclins that are critical for G_1/S transition, leading to G_1 cell cycle arrest. We further demonstrated that clofoctol was effective in an animal xenograft model of prostate cancer. Taken together, these results suggest that clofoctol is a novel small molecule activator of the UPR pathway that has potential as a new therapeutic agent for cancer.

Methods

Cell culture

LNCaP, C42B, PC-3 and CWR22Rv1 were grown in RPMI 1640 media plus 10% FBS (Life Technologies, Grand Island, NY, USA). DU145 were grown in DMEM with 10% FBS (Life Technologies). LAPC4 cells were grown in Iscove's media with 10% FBS (Life Technologies) and 1 nM R1881. All cell lines were maintained at 37°C in a humidified incubator adjusted with 5% CO_2. Authentication of these cell lines was carried out by the Powerplex 2.1 STR genotyping assay (Promega, Madison, WI, USA) as described previously (Platz et al., 2011).

Cell viability assay

Approximately 2×10^3 cells per well were seeded in 96-well plates with a total media volume of 100 μL per well and allowed to adhere overnight. The cells were treated with various concentrations of clofoctol for 72 h before incubation with 10 μL per well of alamarBlue dye (Life Technologies) for 2 h. The alamarBlue fluorescence from cells was measured at 560EX nm/590EM nm using a BMG FLUOStar OPTIMA plate reader (BMG Labtech, Cary, NC, USA). The IC_{50} values were calculated using the GraphPad Prism 4.0 software (GraphPad Software, San Diego, CA, USA).

Cell cycle analysis

PC3 cells were plated in a 6-well plate or in 100-mm dishes and allowed to adhere overnight. After treatment with drugs for 24 h, the cells were harvested by trypsinization. The cell were then fixed with ice-cold 70% ethanol with gentle vortexing and stained with 20 μg·mL^{-1} propidium iodide in the presence of 0.2 μg·mL^{-1} RNase A for 30 min. The cells were passed through a mesh filter and subjected to cell cycle analysis using BD FACScan System (BD Biosciences, San Jose, CA, USA).

In vitro *translation assay*

In vitro translation of luciferase mRNA was conducted using Flexi® Rabbit Reticulocyte Lysate System (Promega) according to the manufacturer's instructions. Briefly, rabbit reticulocyte lysate was incubated with 20 μM amino acid mixture, 70 mM KCl, 2 mM DTT, 0.4 U RNase inhibitor and 20 μg·mL^{-1} luciferase mRNA. Drugs (clofoctol or cycloheximide) were added to the reaction mixture and the reaction was continued at 30°C for 30 min before lysis buffer was added to stop the reaction. An aliquot of the reaction mixture was mixed with the luciferase substrate solution and the luciferase activity was measured using the MicroBeta luminescence plate reader (PerkinElmer, Waltham, MA, USA).

In vivo *translational assay*

PC3 cells (6×10^4 cells per well) were plated in 12-well plates and allowed to adhere overnight. The cells were then treated with clofoctol at the indicated concentrations. The cells were metabolically labelled with [^{35}S]-methionine by adding the mixture of 1 μCi [^{35}S]-methionine/Cys (PerkinElmer), per well, for 15 min. The cells were lysed with RIPA buffer containing 50 mM Tris-HCl, pH 7.4, 1% NP-40, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM EDTA, 1 mM PMSF, 1× protease inhibitor cocktail, 1 mM Na_3VO_4 and 1 mM NaF. Equal amounts of total proteins were resolved by SDS-PAGE and were stained with Coomassie brilliant blue. The gels were destained and dried and subjected to the autoradiography.

X-box binding protein-1 (XBP-1) *splicing assay*

PC3 cells (1.5×10^5 cells per well) were plated in 6-well plates and allowed to adhere overnight. The cells were treated with various concentrations of clofoctol or 1 μM thapsigargin

for 24 h. Total RNA were harvested using RNeasy mini kit (Qiagen, Valencia, CA, USA) and subjected to a reverse transcriptase-PCR analysis using specific primer pairs for XBP-1 (forward: 5′-AAACAGAGTAGCAGCTCAGACTGC-3′ and reverse: 5′-TCCTTCTGGGTAGACCTCTGGGAG-3′). The PCR products were resolved on 1.5% agarose gel and observed under the Kodak Image Station 440CF (Kodak, Rochester, NY, USA). To further distinguish the unspliced XBP-1 mRNA from the spliced form, the PCR products of XBP-1 mRNA were digested with PstI before gel electrophoresis.

Western blot analysis

Cells were lysed by adding 1 volume of 2× Laemmli buffer followed by boiling in water for 10 min. The samples were separated by SDS-PAGE and transferred onto nitrocellulose membrane (Bio-Rad, Hercules, CA, USA). Proteins were detected using primary antibodies for eIF2α, transcription factor C/EBP homologous protein (CHOP), BiP, phospho-c-Jun, microtubule-associated protein light chain 3B (LC3B), PARP (Cell Signaling Technology, Danvers, MA, USA), phospho-eIF2α (Enzo Life Sciences, Farmingdale, NY, USA) and α-tubulin (Santa Cruz Biotechnology, Santa Cruz, CA, USA); followed by incubation with HRP–conjugated anti-mouse or antirabbit antibodies (GE Healthcare, Pittsburg, PA, USA) and enhanced chemiluminescence (GE Healthcare).

Confocal microscopy

Human pEGFP-LC3 (Addgene plasmid 24920) was obtained from Addgene (Cambridge, MA, USA) (Lee *et al.*, 2008). PC3 cells (1×10^5) were seeded on a MatTek glass bottom culture dish (Fisher Scientific, Pittsburgh, PA, USA) and allowed to adhere for 24 h. The cells were transiently transfected with pEGFP-LC3 using the SuperFect transfection reagent (Qiagen) for 24 h. The cells were then treated with either DMSO or drugs for 6 h and the cellular EGFP fluorescence was observed under the Zeiss 510 Meta multiphoton confocal microscope (Carl Zeiss, Thornwood, NY, USA).

In vivo *prostate cancer xenograft assay*

Male athymic nude mice (BALB/c, nu/nu-NCr) aged 4–6 weeks were purchased from the National Cancer Institute (Frederick, MD, USA) and treated in accordance with Johns Hopkins Animal Care and Use Committee procedures. The study protocol was approved by the Johns Hopkins University Animal Care and Use Committee (MO10M221). All studies involving animals were conducted and reported in accordance with the ARRIVE guidelines for reporting experiments involving animals (Kilkenny *et al.*, 2010; McGrath *et al.*, 2010). Animals were housed in temperature- and humidity-controlled rooms on a 12 h light-dark cycle and had free access to food and water. A total of 13 animals were used in the experiments described herein. PC3 cells (1 million cells per injection) were implanted s.c. into the mice. After the tumours had become palpable, mice were randomly assigned to two groups. One group of mice ($n = 6$) were treated with vehicle (peanut oil with 5% DMSO) and the other group ($n = 7$) were treated with clofoctol (175 mg·kg^{-1}, injected i.p.) every day. The tumour volume was measured periodically using a vernier caliper and calculated according to the modified ellipsoid formula: tumour

volume (mm^3) = (short axis)^2 × (long axis) × π/6. After 37 days of treatment, mice were killed by an i.p. injection of 0.5 mL of 250 mg·kg^{-1} tribromoethanol (Sigma-Aldrich, St. Louis, MO, USA), followed by treatment with 100% CO_2 when fully anaesthetized. Death was verified by an observed lack of breathing, below normal body temperature and absence of a palpable heartbeat. The tumours were harvested for wet weight measurement.

Statistical analysis

The statistical significance of differences between control and experimental groups was determined by Student's *t*-test. All statistical tests were two-sided and *P* values less than 0.05 were considered statistically significant.

Results

Clofoctol inhibited prostate cancer cell growth and cell cycle progression

We have previously screened JHDL using two prostate cancer cell lines, LNCaP and PC3, to identify potential antiprostate cancer drugs (Platz *et al.*, 2011). Clofoctol, an antibacterial drug, was among the hits that showed inhibitory activity against prostate cancer cell proliferation (Supporting Information Figure S1A). We thus tested clofoctol on six different prostate cancer cell lines including LNCaP, DU145, PC3, LAPC4, CWR22Rv1 and C42B. Clofoctol was active against all six prostate cancer cell lines with IC$_{50}$ values ranging from 10 to 15 μM (Figure 1A and B). We further determined the effect of clofoctol on cell cycle progression of the PC3 line and found that it induced a G$_1$ arrest (Figure 1C). Although the potency of clofoctol for inhibition of prostate cancer cell proliferation appeared relatively low in comparison with other hits (Platz *et al.*, 2011), the average plasma concentrations of clofoctol in humans are significantly higher than its IC$_{50}$ values for inhibition of prostate cancer cell growth (Del Tacca *et al.*, 1987; Danesi *et al.*, 1988), giving rise to a sufficiently wide therapeutic window and prompting us to characterize the antiproliferative activity on prostate cancer further.

Clofoctol induced ER stress and activated UPR pathways

To investigate the cellular and molecular mechanisms underlying the anticancer activity of clofoctol, we began by assessing the morphological changes of PC3 cells upon treatment with clofoctol. Clofoctol induced massive vacuolization in PC3 cells upon treatment for 24 h (Supporting Information Figure S1B). The induction of extensive cellular vacuolization and the G$_1$ cell cycle arrest (Figure 1C) caused by clofoctol are both hallmarks of ER stress. We thus determined the effect of clofoctol on the activity of UPR pathways using ER stress markers in PC3 cells. We first determined the effect of clofoctol on the processing of XBP-1, which is a transcription factor that regulates expression of genes important for ER stress response (Calfon *et al.*, 2002). When UPR is activated, IRE1 oligomerizes and activates its ribonuclease domain which catalyzes splicing of ubiquitously expressed form of XBP-1 mRNA (XBP-1u). This process will generate a spliced isoform of XBP-1 mRNA (XBP-1s), which serves as a marker
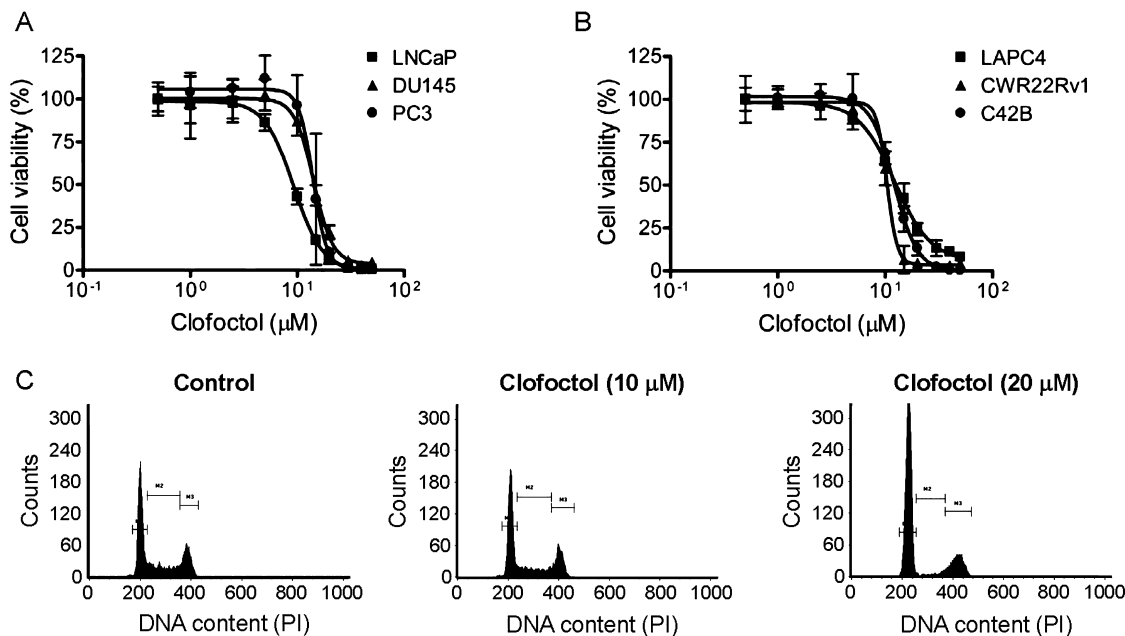
Figure 1

Clofoctol inhibited prostate cancer cell growth and cell cycle. (A and B) Six prostate cancer cell lines were grown in 96-well plates and treated with various concentrations of clofoctol for 72 h. An AlamarBlue assay was conducted to assess cell viability. Data represent mean + 95% confidence interval from three independent experiments performed in triplicate. (C) PC3 cells were grown in a 6-well plate and treated with clofoctol at indicated concentrations for 24 h. The cells were then harvested and subjected to cell cycle analysis. Representative cell cycle histograms from three independent experiments with similar results are shown.

of IRE1 activation. Using reverse transcriptase-PCR, we observed that clofoctol increased the splicing of XBP-1 mRNA in PC3 cells in a dose-dependent manner (Figure 2A). Unspliced form of XBP-1 mRNA contains a PstI digestion site that is removed upon splicing. We observed that the PstI digestion products from XBP-1 mRNA were dose-dependently decreased by clofoctol (Figure 2B). These results demonstrate that clofoctol activates IRE1 pathway in PC3 cells.

The second branch of UPR is mediated by PERK. PERK undergoes oligomerization and phosphorylates eIF2α, thereby inhibiting protein translation. In PC3 cells, clofoctol dose- and time-dependently increased the level of phosphorylated eIF2α (Figure 2C and D). It is known that when eIF2α is inhibited, certain mRNA, such as ATF4, that contains short open reading frames in their 5'-untranslated region are preferentially translated. ATF4 is a transcription factor that drives the expression of CHOP (Walter and Ron, 2011). In parallel with the induction of eIF2α phosphorylation, clofoctol up-regulated CHOP expression in a dose- and time-dependent manner (Figure 2C and D) consistent with the activation of the PERK-eIF2α-ATF4-CHOP signalling pathway.

The third branch of UPR involves the activation of ATF6 by two Golgi proteases, S1P and S2P that sequentially remove the luminal domain and the transmembrane anchor of ATF6 (Ye *et al.*, 2000). The activated N-terminal fragment of ATF6 translocates into the nucleus and activates its target genes such as BiP. As shown in Figure 2C and D, clofoctol increased the expression of BiP in dose- and time-dependent manner. Activation of ATF6 pathway by clofoctol was further demonstrated by the nuclear translocation of the ATF6 fragment in

PC3 cells (Supporting Information Figure S2). Together, these results indicate that clofoctol induces ER stress and activates all three UPR pathways in PC3 cells.

Clofoctol did not induce apoptosis or autophagy

Activation of IRE1 is known to activate JNK pathway, which eventually leads to an induction of apoptosis or autophagy during ER stress. We thus determined if clofoctol activated JNK pathway and autophagy in PC3 cells. Treatment of PC3 cells with clofoctol dose-dependently increased the level of phosphorylated c-Jun (Figure 3A). Conversion of LC3B-I to LC3B-II, a marker of autophagy induction, was also observed by increasing concentrations of clofoctol. In contrast, cleavage of PARP, a marker of caspase-dependent apoptosis, was not observed upon clofoctol treatment. To further determine whether clofoctol induced autophagy, we detected the autophagosome formation using EGFP-LC3-overexpressing PC3 cells. An increase in fluorescently labelled LC3 puncta together with formation of massive vacuoles was observed in the presence of clofoctol (Figure 3B). We next examined whether the induction of autophagy is mediated by JNK activation. PC3 cells were treated with a specific JNK inhibitor, SP600125 and the levels of phosphorylated c-Jun and LC3B were measured. The level of phosphorylated c-Jun was significantly increased by clofoctol and this increase was completely blocked by addition of SP600125 (Figure 3C). The conversion of LC3B by clofoctol, however, was not blocked by SP600125 (Figure 3C). These results suggested that JNK
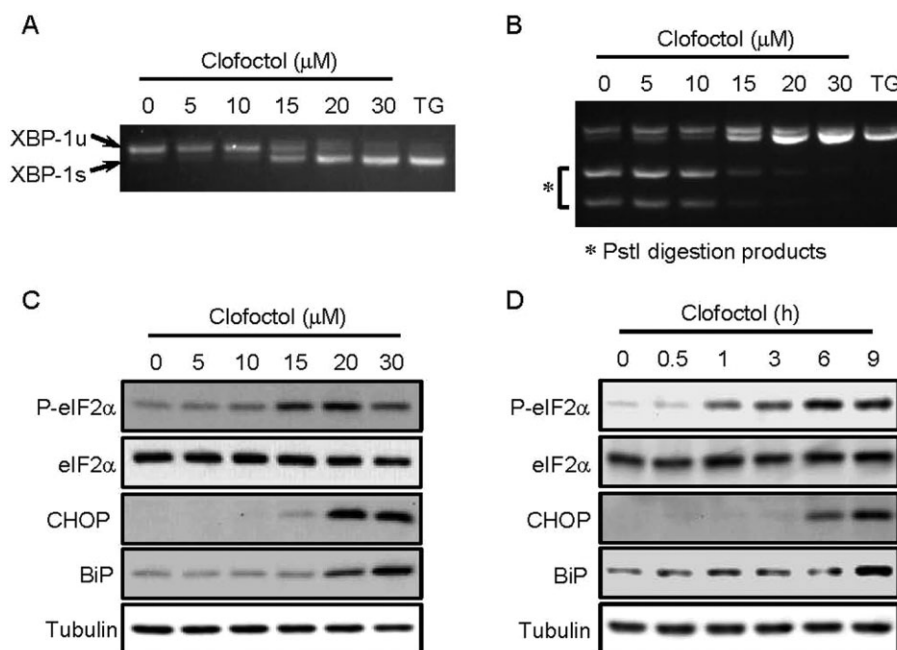
Figure 2

Clofoctol activates three UPR pathways in PC3 cells. (A and B) PC3 cells were treated with the indicated concentrations of clofoctol or thapsigargin (TG, 1 µM) for 24 h. RT-PCR analysis of XBP-1 mRNA was performed to observe the spliced form (XBP-1s) and unspliced form (XBP-1u) of XBP-1 in PC3 cells (A). Thapsigargin was used as a positive control compound for XBP-1 splicing. The RT-PCR products of XBP-1 mRNA were digested with PstI to further distinguish the XBP-1s from XBP-1u. XBP-1s does not contain a PstI digestion site, thus showing no digestion products (B). (C and D) PC3 cells were treated with various concentrations of clofoctol for 24 h (C). PC3 cells were treated with 20 µM clofoctol for indicated time points (D). The levels of phosphorylated and total proteins were assessed by Western blot analysis.

was not involved in the clofoctol-induced LC3B conversion. As inhibition of JNK did not affect growth inhibitory activity of clofoctol (Supporting Information Figure S3C), we wondered whether clofoctol-induced LC3B conversion was the indication of bona fide autophagy induction.

The autophagosome formation is a dynamic process as it is balanced by turnover through fusion with lysosomes and subsequent lysosome-mediated degradation. Inhibition of either fusion with lysosomes or lysosome-mediated degradation should prevent LC3 from degradation, which also causes increase in LC3 staining. Under these conditions, basal autophagic flux is inhibited but LC3 is accumulated, which often leads to misinterpretation of the autophagy-inducing activity of compounds. Thapsigargin, for example, had been known to induce autophagy as it is capable of increasing LC3 punta (Ogata *et al.*, 2006). Subsequently, it was found to inhibit fusion between autophagosomes and lysosomes, thereby blocking basal autophagy (Ganley *et al.*, 2011). An inhibitor of vacuolar type H⁺-ATPase (V-ATPase), bafilomycin A1, is known to raise lysosomal pH and inhibit lysosome-mediated degradation (Klionsky *et al.*, 2008). Bafilomycin A1 also blocks basal autophagy while significantly increasing LC3 punta. We thus investigated whether clofoctol is a genuine inducer of autophagy. PC3 cells treated with bafilomycin increased LC3B conversion, indicating blockade of LC3 degradation (Figure 3D). Tunicamycin, an inhibitor of N-linked glycosylation and a genuine autophagy inducer, slightly increased the conversion of LC3B. The LC3B conversion

induced by tunicamycin was further enhanced by bafilomycin, indicating that tunicamycin induced autophagic flux with active LC3B turnover. In contrast, thapsigargin and clofoctol strongly increased LC3B conversion with no further enhancement by bafilomycin. Similar results were observed from the LC3 staining. Cells treated with tunicamycin increased the number of LC3 puncta which were further enhanced by bafilomycin (Figure 3E). However, there was no significant enhancement in the number of LC3 puncta by bafilomycin in the cells treated with either thapsigargin or clofoctol. These results suggested that the increase in LC3B conversion by clofoctol was due to a blockade of basal autophagic flux rather than an induction of autophagy.

Clofoctol inhibited prostate cancer cell growth through induction of ER stress

To further investigate the underlying mechanism of anticancer activity of clofoctol, we applied several pathway inhibitors together with clofoctol and determined if they could rescue growth inhibitory activity of clofoctol. Thus, neither a specific autophagy inhibitor, 3-methyladenine nor apoptosis inhibitor, zVAD-fmk, affected the growth inhibition by clofoctol (Supporting Information Figure S3A and B), indicating that neither autophagy nor caspase-dependent apoptosis was involved in the anticancer activity of clofoctol. As mentioned previously, a JNK inhibitor SP600125 failed to rescue the growth inhibition by clofoctol (Supporting Information Figure S3C), suggesting that JNK pathway activation was not
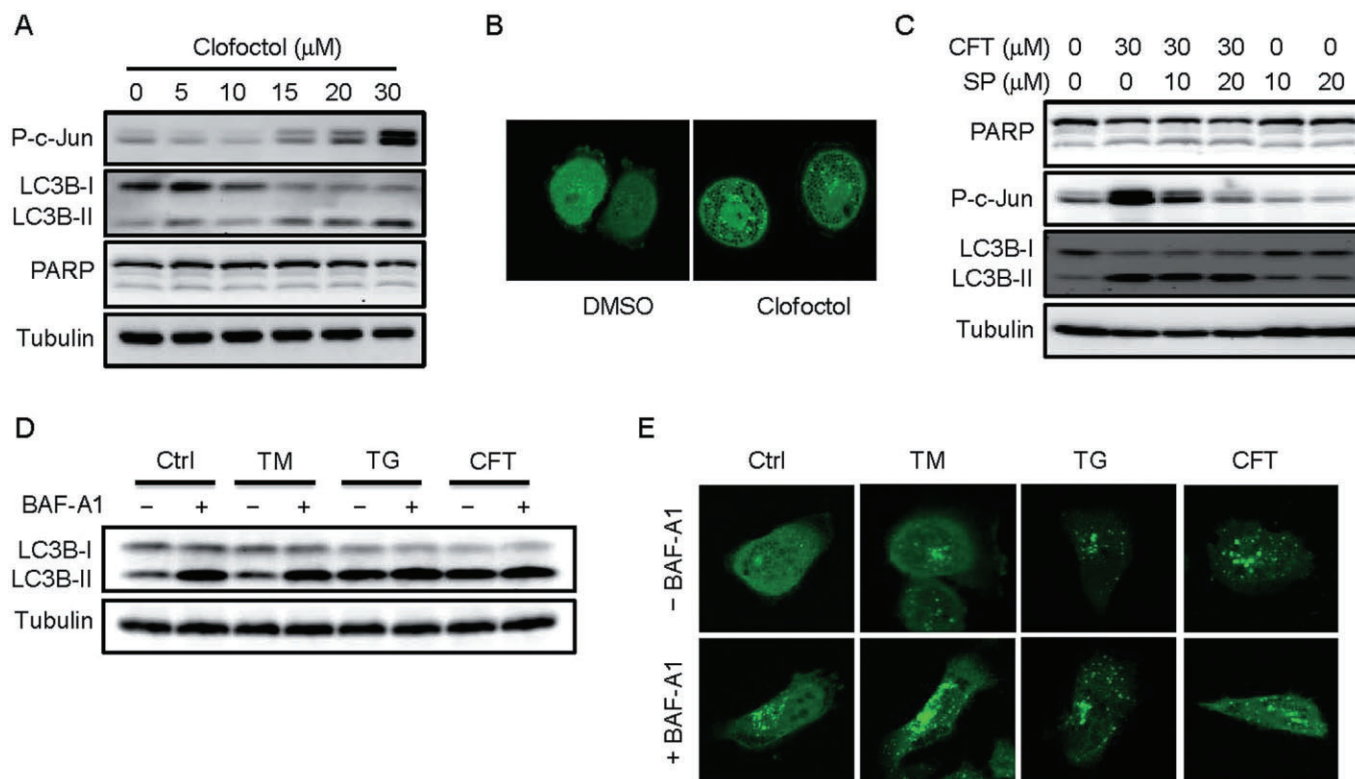
Figure 3

Clofoctol inhibits autophagic flux in PC3 cells. (A) PC3 cells were treated with the indicated concentrations of clofoctol for 24 h. The protein levels of phosphorylated c-Jun, LC3B-I/II, PARP and tubulin were assessed by western blot analysis. (B) PC3 cells were transiently transfected with pEGFP-LC3 for 24 h and were treated with DMSO or clofoctol (20 μM) for 6 h. The EGFP-LC3 fluorescence was observed under the confocal microscope. Intense green dots are indicative of LC3 puncta. (C) PC3 cells were treated with indicated concentrations of clofoctol (CFT) and a JNK inhibitor, SP600125 (SP) for 24 h. The protein levels of PARP, phosphorylated c-Jun, LC3B-I/II and tubulin were assessed by Western blot analysis. (D) PC3 cells were treated with either DMSO (Ctrl), 20 μg·mL^{-1} tunicamycin (TM), 3 μM thapsigargin (TG) or 25 μM clofoctol (CFT) for 6 h and then were harvested for Western blot analysis of LC3B-I/II. Bafilomycin A1 (BAF-A1, 100 nM) was added 2 h before harvesting the cells. (E) PC3 cells were transiently transfected with pEGFP-LC3 for 24 h and were treated with either DMSO (Ctrl), 20 μg·mL^{-1} tunicamycin (TM), 3 μM thapsigargin (TG) or 25 μM clofoctol (CFT) for 6 h. Either DMSO (–BAF-A1) or bafilomycin A1 (+BAF-A1) was added 2 h before processing for the confocal microscope analysis of EGFP-LC3.

critical for anticancer activity of clofoctol. Similarly, both necroptosis inhibitor (necrostatin-1) and ferroptosis inhibitor (ferrostatin-1) failed to rescue the growth inhibition by clofoctol (Supporting Information Figure S3D and E), indicating that none of the pertinent cell death mechanisms are involved in the clofoctol activity. Lastly, we determined the effect of an ER stress inhibitor, phenylbutyrate, on the activity of clofoctol. Phenylbutyrate is the most popular chemical chaperone that can reduce cellular ER stress by correcting protein misfolding (Yam *et al.*, 2007). We observed that 2 mM of phenylbutyrate partially, but significantly, reversed the growth inhibition of PC3 cells by clofoctol (Figure 4A). We further confirmed the reversal of growth inhibition by phenylbutyrate in the cells treated with a lower concentration of clofoctol (Supporting Information Figure S4). Reduction of ER stress by phenylbutyrate, that is decrease in the levels of phosphorylated eIF2α and CHOP, was observed in the cells treated with clofoctol (Figure 4B). These results suggested that anticancer activity of clofoctol is, at least in part, mediated through the induction of ER stress.

Clofoctol inhibited translation

Clofoctol has been reported to inhibit translation, which is consistent with the induction of phosphorylation of eIF2α we observed (Pelletier *et al.*, 2007). To determine the effect of clofoctol on translation in prostate cancer cells, total proteins in PC3 cells were metabolically labelled with [³⁵S]-methionine in the presence or absence of clofoctol. Clofoctol inhibited global protein synthesis in a dose- and time-dependent manner, as judged by the amounts of [³⁵S]-methionine incorporated into newly synthesized proteins (Figure 5A and B). In contrast, no obvious difference in total protein levels as visualized by Coomassie brilliant blue staining between control and clofoctol-treated cells was observed within the time frame of the experiments, suggesting that newly synthesized protein levels were selectively reduced by clofoctol (Supporting Information Figure S5). To determine if the inhibition of translation is a direct effect by clofoctol, *in vitro* translation assay was conducted using luciferase mRNA and rabbit reticulocyte lysates. In this assay system, clofoctol showed no
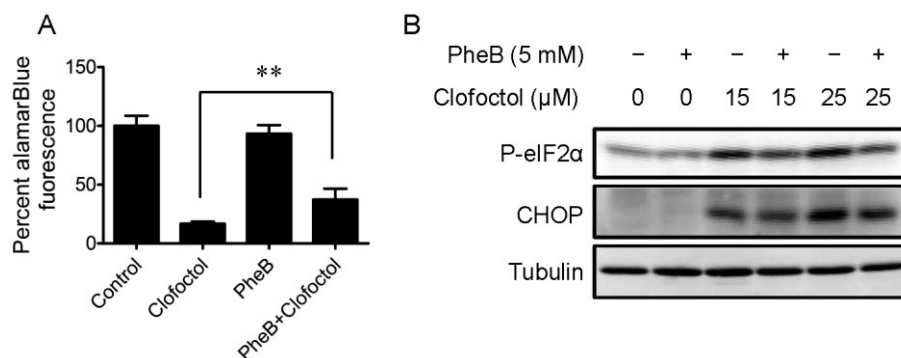
Figure 4

Clofoctol inhibits prostate cancer growth through induction of ER stress. (A) PC3 cells were grown in 96-well plates and treated with 20 μM clofoctol and 2 mM phenylbutyrate (PheB) for 72 h. An AlamarBlue assay was conducted to assess the cell viability. Data represent mean ± 95% confidence interval from three independent experiments (**$P < 0.001$). (B) PC3 cells were treated with the indicated concentrations of phenylbutyrate (PheB) and clofoctol for 24 h. Cells were then harvested for Western blot analysis of phosphorylated eIF2α and CHOP.
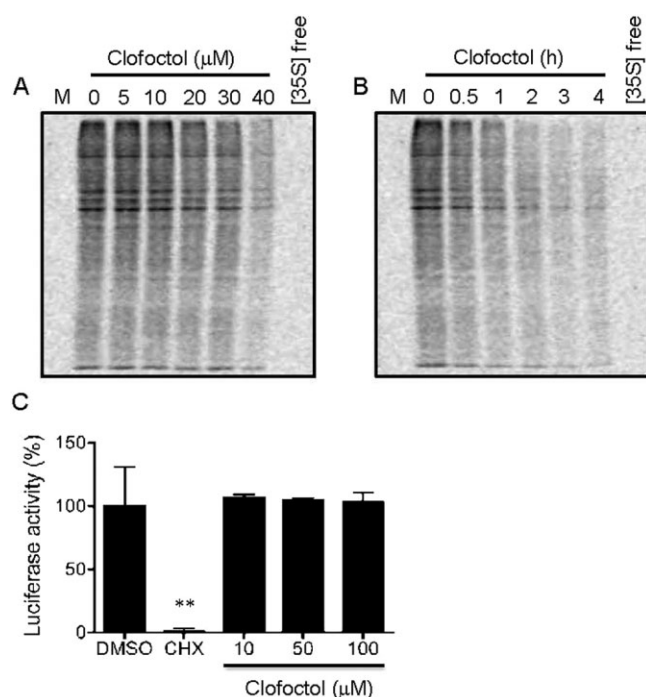

Figure 5

Clofoctol inhibits *in vivo* translation. (A and B) Total proteins in PC3 cells were metabolically labelled with [^{35}S]-Met. The cells were then treated with various concentrations of clofoctol for 24 h (A) or treated with 30 μM clofoctol for indicated time points (B). The total proteins were resolved by the SDS-PAGE. ^{35}S-labelled proteins were visualized by autoradiography. Cells without treatment with [^{35}S]-Met ([^{35}S] free) were used as a negative control. (C) The luciferase mRNA was translated *in vitro* using a rabbit reticulocyte lysate system. The reaction mixture was treated with either clofoctol or cycloheximide (CHX, 10 μM) and the luciferase activity assay was conducted to assess the *in vitro* translation of luciferase mRNA. Cycloheximide was used as a positive control compound to inhibit *in vitro* translation. Data represent mean + 95% confidence interval from three independent experiments performed in triplicate (**$P < 0.001$ vs. DMSO).

inhibition of the translation of luciferase mRNA at concentrations up to 100 μM. In contrast, cycloheximide, a known inhibitor of elongation, completely inhibited the translation of luciferase (Figure 5C). These results suggest that clofoctol inhibited *in vivo* translation indirectly through induction of the phosphorylation of eIF2α following ER stress induction in PC3 cells.

Clofoctol induced G$_1$ phase cell cycle arrest through activation of UPR

Pharmacological activation of UPR leads to a G$_1$ cell cycle arrest through inhibition of translation of cyclins that are responsible for G$_1$/S transition (Brewer *et al.*, 1999). Indeed, we observed that clofoctol induced G$_1$ cell cycle arrest in PC3 cells (Figure 1C). Next, we determined whether clofoctol affected the levels of G$_1$/S cyclins in PC3 cells. Treatment of PC3 cells with clofoctol for 24 h led to a dose-dependent decrease in the levels of cyclin A and cyclin D1 (Figure 6A). A subtle decrease in the level of cyclin E was also observed. Concurrently, the levels of phosphorylated eIF2α and CHOP were strongly increased. These results demonstrated that activation of UPR by clofoctol accompanied down-regulation of G$_1$/S cyclins. As shown previously, an ER stress inhibitor phenylbutyrate could partially reverse inhibition of PC3 cell growth by clofoctol (Figure 4A; Supporting Information Figure S4). We thus examined whether phenylbutyrate could also reverse the clofoctol-induced G$_1$ arrest in PC3 cells. As shown in Figure 6B, clofoctol increased the cell population in G$_1$ phase, while phenylbutyrate partially increased the G$_2$/M population. When applied together, phenylbutyrate reversed G$_1$ cell population to the control level compared with that in clofoctol-treated cells (Figure 6B). These results suggested that the G$_1$ cell cycle arrest caused by clofoctol is mediated through activation of UPR.

Clofoctol inhibited tumour growth in a mouse xenograft model

A mouse model of human prostate cancer xenograft was employed to determine the *in vivo* anticancer activity of clofoctol. PC3 cells were implanted s.c. into athymic nude mice and the mice were treated with either vehicle or clofoctol for
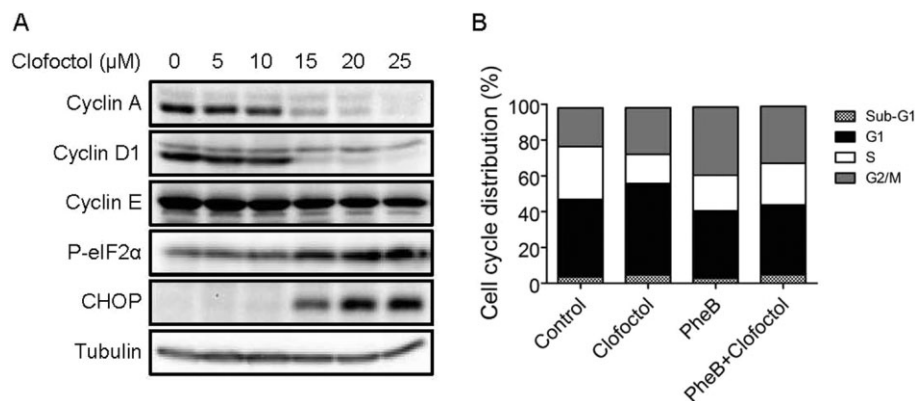
Figure 6

Clofoctol decreases G_1/S cyclin levels. (A) PC3 cells were treated with indicated concentrations of clofoctol for 24 h. Cells were then harvested for Western blot analysis of cyclin A, cyclin D1, cyclin E, phosphorylated eIF2α and CHOP. (B) PC3 cells were treated with clofoctol and/or phenylbutyrate (PheB) for 24 h. The cells were then harvested and subjected to a cell cycle analysis. The % of cell population in each cell cycle phase is shown as a bar graph.
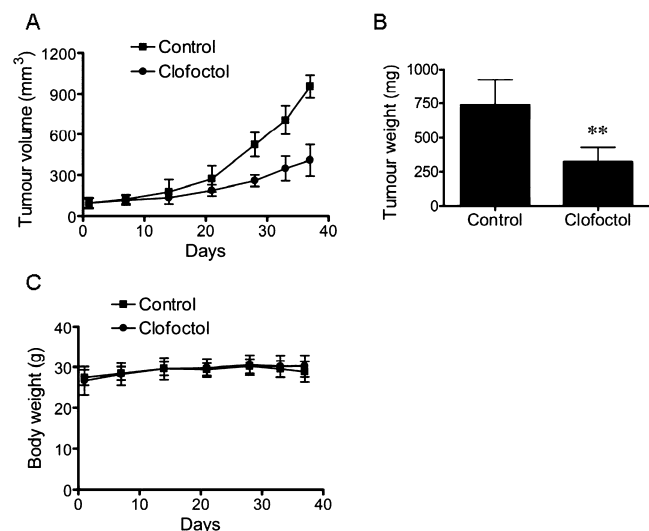

Figure 7

Clofoctol inhibits the growth of PC3 tumour xenografts in mice. (A–C) PC3 cells were implanted s.c. into nude mice. The mice were given vehicle ($n = 6$) or clofoctol (175 mg·kg^{-1}) ($n = 7$) once daily via i.p. injection for 37 days. The tumour size was measured periodically and the tumour volume was calculated using a modified ellipsoid formula (A). After mice were killed, tumours were isolated from mice and the tumour wet weight was measured (B). Mice body weight was measured periodically to assess drug toxicity (C). In A–C, data represent mean + 95% confidence interval (**$P < 0.001$ vs. control).

37 days. Tumour volume was measured during the course of the treatment. As shown in Figure 7A, PC3 tumour growth was significantly inhibited by clofoctol. Tumour weight was also reduced by 60% by clofoctol (Figure 7B). There was no significant change in mice body weight during the course of the drug treatment, suggesting that clofoctol did not have gross toxicity at the dose applied (Figure 7C). These results demonstrate that clofoctol is capable of blocking tumour growth *in vivo* without apparent toxicity.

Discussion

In this study, we identified clofoctol as an inhibitor of prostate cancer growth from JHDL with great potential as a translatable lead for developing antiprostate cancer drugs. Clofoctol is an antibacterial drug that has been used to treat upper respiratory tract infections in European countries for years. It is administered either orally or rectally. It showed relatively high peak plasma (Cmax: 38.1 µg·mL^{-1} or 104 µM) and lung (Cmax: 93.1 mg·kg^{-1}) levels (Del Tacca *et al.*, 1987; Danesi *et al.*, 1988). In the present work, we found that clofoctol inhibited prostate cancer cell growth with IC$_{50}$ values ranging from 10 to 15 µM, which are far below the clinically achievable plasma concentrations *in vivo*. It also inhibited the growth of a prostate cancer xenograft in mice with no apparent toxicity. These results suggest that clofoctol should have anticancer activity under its current antibacterial drug regimen.

Clofoctol is quite unique in its mechanism of action on cancer cells – it inhibits prostate cancer cell growth through activation of the ER stress and all three branches of the UPR pathway including IRE1, PERK and ATF6. As the three upstream UPR sensor protein receptors have distinct specificity, it is unlikely that clofoctol acts at the level of those UPR sensors. It is likely that clofoctol acts on a target that lies upstream of the UPR sensors which leads to the generation of unfolded proteins in the ER lumen. Prolonged induction of ER stress and activation of UPR result in cell growth arrest. It is well established that UPR uses PERK to coordinate inhibition of global protein translation by phosphorylating eIF2α (Harding *et al.*, 2000). Inhibition of global translation causes a rapid reduction of short-lived cell cycle regulatory proteins, such as cyclin D1, which eventually leads to inhibition of G_1/S transition (Brewer *et al.*, 1999).

Clofoctol indeed inhibited global protein translation and reduced the levels of cyclins A and D1 that are critical for G_1/S transition. Furthermore, clofoctol induction of G_1 arrest was partially reversed by an ER stress inhibitor, phenylbutyrate. Phenylbutyrate also partially reversed the inhibition of cell growth by clofoctol. These results suggested that anticancer mechanism of clofoctol can be explained by induction of ER stress, followed by inhibition of translation and G_1 cell cycle arrest. We also tested inhibitors of the known cell death pathways including apoptosis, autophagy, necroptosis and ferroptosis in the cells treated with clofoctol. None of those inhibitors could reverse clofoctol effect on the viability of PC3 cells, suggesting that inhibition of prostate cancer cell growth by clofoctol does not involve conventional cell death mechanisms, but is likely due to the inhibition of cell cycle progression.

Tunicamycin and thapsigargin are both well-known inducers of ER stress, although their mechanisms of induction of ER stress are quite distinct. They are both known to activate JNK through activation of IRE1 and to increase LC3 puncta following ER stress (Ogata *et al.*, 2006). Tunicamycin induces autophagy through IRE1-JNK pathway. In contrast, thapsigargin inhibits autophagy even though it activates JNK and increases LC3 puncta (Ganley *et al.*, 2011). Clofoctol acted very similarly to thapsigargin in prostate cancer cells. It induced ER stress and activated the JNK pathway, followed by an increase in LC3 puncta. However, neither the JNK inhibitor nor autophagy inhibitor rescued the growth inhibitory effect of clofoctol on prostate cancer cells. In addition, bafilomycin treatment did not further enhance LC3 puncta formation by clofoctol, suggesting that clofoctol inhibited autophagic flux. These observations imply that clofoctol, similar to thapsigargin, might inhibit either fusion between autophagosome and lysosome or lysosome-dependent degradation pathway. Further studies including identification of the molecular target of clofoctol in eukaryotic cells would be necessary to elucidate its underlying mechanism of induction of ER stress and inhibition of autophagy.

Clofoctol is a structurally unique drug, with a largely hydrophobic core containing a single polar phenol moiety. In a preliminary structure-activity relationship study, we synthesized a number of analogues and found that the phenolic oxygen can tolerate most chemical modifications without significant loss in antiproliferative activity (Supporting Information Table S1). Interestingly, conversion of the methylene bridge between the two phenyl rings into a polar carbonyl group (CFT-CO) led to inactivation of the compound, underscoring a critical role of the large hydrophobic core in the activity of clofoctol.

Recently, ER stress and the UPR pathway have been proposed as a promising target for developing drugs for several human diseases. A number of small molecules have long been known to induce ER stress and activate the UPR pathway, including oxidizing agents, thapsigargin, tunicamycin and brefeldin A among others (Ma and Hendershot, 2004; Kim *et al.*, 2008). The majority of those inducers, however, are too toxic to be developed into therapeutic agents. As clofoctol has been used as a drug in humans for a long time with tolerable side effects, it is ready to be evaluated in human clinical studies for the treatment of prostate cancer. Its unique accumulation in the lung suggests that it could also be effective for treating lung cancer. Moreover, activation of UPR pathway has been implicated in the clearance of protein aggregates in a number of neurodegenerative diseases (Kim *et al.*, 2008), our findings also suggest that clofoctol may have therapeutic effects on those diseases in addition to prostate and other cancers.

Acknowledgements

Author contributions

J. O. L., M. W., J. S. S. and R-J. L. designed the experiments; M. W., J. S. S., R-J. L., Y. D., Q. H. and M. D. performed the experiments; J. O. L., M. W., J. S. S., R-J. L. and M. D. analysed the results; J. O. L., J. S. S., M. W. and R-J. L. wrote the manuscript.

Conflict of interest

None.

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Supporting information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

http://dx.doi.org/10.1111/bph.12800

Figure S1 (A) Chemical structure of clofoctol. (B) Clofoctol induces massive vacuolization in PC3. PC3 cells were treated with 20 μM clofoctol for 24 h and the cell morphology was observed under a phase contrast microscope.

Figure S2 Clofoctol induces ATF6 nuclear translocation in PC3 cells. PC3 cells were treated with or without 10 μM clofoctol for 24 h. Endogenous ATF6 was labelled with an antibody specific for ATF6. Confocal microscopy demonstrated that clofoctol treatment induced the nuclear translocation of ATF6 in PC3 cells.

Figure S3 (A–E) PC3 cells were grown in 96-well plates and treated with 20 μM clofoctol and various inhibitors including 3-methyladenine (3-MA; 5 mM), zVAD-fmk (zVAD; 20 μM), SP600125 (SP; 10 μM), necrostatin-1 (Nec-1; 2 μM) and ferrostatin-1 (Fer-1; 2 μM) for 72 h. AlamarBlue assay was conducted to assess the cell viability. Data represent mean ± 95% confidence interval from three independent experiments. NS denotes not significant ($P > 0.01$).

Figure S4 Reversal effect of phenylbutyrate (2 mM) on inhibition of PC3 cell growth by clofoctol (15 μM). Data represent mean ± 95% confidence interval from three independent experiments (*$P < 0.01$).

Figure S5 (A and B) Coomassie brilliant blue (CBB) staining of total proteins used in Figure 5A and B is shown.

Table S1 IC_{50} Values for clofoctol and its analogues for inhibition of PC3.