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The PERK/Akt Pathway Mediates Apoptosis Resistance to ER $Ca2^+$ Stress in LNCaP Prostate Cancer Cells

Charlotte Dubois*, Dheeraj Kannancheri Puthooru, Fabien Vanden Abeele*

Abstract

Selective and targeted sarco-endoplasmic reticulum Ca2+-ATPase (SERCA) inhibitors represent a promising and new class of cancer chemotherapeutics that are under investigation in different clinical trials. However, resistance to cancer chemotherapeutics treatments is a common phenomenon and recent evidence suggests that SERCA inhibition could also lead to innate and/or acquired ability of cancer cells to evade cell death but the molecular mechanisms are not fully elucidated. In LNCaP prostate cancer cells, we discovered that activation of the PERK branch of the unfolded protein response (UPR) pathways is involved in therapeutic escape of the endoplasmic reticulum (ER)-Ca2+ stress inducer ASP-8ADT, the active compound of Mispsagargin. We show that the activation of the Akt pathway in response to UPR allows the survival of LNCaP cells to ASP-8ADT exposure. Interestingly, pharmacological inhibition of the PERK downstream factors GSK-3β or eIF2α by drugs currently used in clinic greatly sensitizes LNCaP cells to ASP-8ADT. In summary, our findings highlight additional strategies to increase clinical response to targeting-ER-Ca2+ stressor drugs such as Mipsagargin family.

Keywords: Prostate Cancer; Apoptosis resistance; ER homeostasis

Introduction

The ER is an organelle involved in multiple cellular processes. Studies have demonstrated that alteration of ER homeostasis and its appropriate functioning leads to a cascade of signaling events known as ER stress response (also called UPR). The UPR pathways involve three different sensors localize at the ER membranes: PERK (protein kinase RNA(PKR)-like ER kinase, IRE1 (inositol requiring enzyme1α) and ATF6 (activating transcription factor 6). The main purpose of the UPR is to maintain ER homeostasis but in case of a persistent ER stress, UPR cell death will be induced. Indeed, activated PERK phosphorylates eIF2α (eukaryotic Initiation Factor 2), which can selectively induce expression of proapoptotic transcription factors ATF4 and CHOP. However, in order to restore ER homeostasis and maintain cell fate, activated PERK (phosphorylated) also promotes global translation attenuation thanks to eIF2α activation. Besides, its pro-survival role, GSK-3β has been also identified as another important downstream target of activated PERK in condition of ER stress [1]. Finally, PERK also mediates regulation of the mTOR-PI3K-Akt pathway. Indeed, activated PERK contains lipid kinase activity and converts diacylglycerol to phosphatidic acid (PA). PA is instrumental in mammalian target of rapamycin (mTOR) complex formation. mTOR complex can activate Akt and downstream pathways via

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phosphorylation [2]. Based on this duality, PERK plays a central role in the control of cell death/survival balance and for this reason different therapeutic strategies have been developed to promote a "prolonged state of ER stress" as a potent apoptotic inducer in cancer cells.

In that context, a specific class of chemotherapy has been designed from the largely used ER stressor agent Thapsigargin (TG), known to induce Ca2+ homeostasis disruption leading to cell death [3]. Indeed, TG is an inhibitor of SERCA that pumps Ca2+ ions from the cytoplasm into the lumen of the endoplasmic. SERCA pumps are necessary for cellular viability since their inhibition will cause an increase in the cytoplasmic and mitochondrial Ca2+ levels while also depleting ER stores. Depletion of ER Ca2+ stores results in UPR activation while the increase in both cytoplasmic and mitochondrial Ca2+ levels largely result from Store operated Ca2+ entry (SOCE). Indeed, ER Ca2+ depletion mediated by TG is sensed by the Ca2+-binding luminal EF-hand domain of STIM1, a disruption of which leads to activation of STIM1. Following activation, STIM1 can recruit ORAI channels into clusters leading to SOCE [4]. The result will lead to apoptosis and cell death but the molecular mechanisms are still not fully elucidated [5-7]. Nonetheless, several analogues have been synthetized [8] and characterized leading to the identification of ASP-8ADT as a cytotoxic and targetable ER stressor compound [9]. ASP-8ADT has been used to develop a pro-drug approved by the Food and Drug Administration (FDA) and named Mipsagargin (also known as G-202). The Mipsagargin is successively cleaved by the Prostate Specific Membrane Antigen enzyme (PMSA) that is over-expressed by tumoral endothelial cells of several solid cancers among prostate, releasing the active compound (ASP-8ADT) within the tumor. Unfortunately, Mipsagargin treatment alone only induces a disease stabilization of patients with solid tumors [10]. Interestingly, we previously described that apoptosis induced by ASP-8ADT is very modest compared with the parental molecule TG. We deciphered in detail their distinct action mechanisms and pointed out that ASP-8ADT analog has lost its ability to block autophagy. In fact, this unblocking is used by cancer cells as a survival pathway to counteract apoptosis mediated by Ca2+homeostasis disruption as observed for TG. We have also uncovered in this previous study the crucial role of the PERK pathway in apoptosis induction following ER Ca2+ stress. Indeed, using siRNAs against PERK and CHOP we have observed a drastic decrease of apoptosis following TG treatment which was not related to a reduction of the Ca2+ homeostasis disruption. Here, in line with these findings we investigated the potential inhibition of PERK downstream factors GSK-3β and eIF2α by drugs currently used in clinic to increase toxicity associated with ER stress.

Methods

Cell culture

Prostate cancer cell lines LNCaP were purchased from the American Type Culture Collection (ATCC) and cultured in RPMI-1640 medium (Gibco-Life Technologies, Grand Island, NY, USA) supplemented with 5 mM L-glutamine (Gibco-Life Technologies) and 10% fetal bovine serum (Sigma- Aldrich).

Western Blot

Protein samples (50 µg) were subjected to SDS-PAGE and transferred to a nitrocellulose membrane by semi-dry western blotting (Bio-Rad). The membrane was blocked in TNT buffer (Tris-HCl, pH 7.5, 140 mM NaCl, and 0.05% Tween 20) containing 5% milk for 45 min at room temperature. Then membranes were gently incubated overnight at 4°C with specific primary antibodies. Rabbit polyclonal anti-p-Akt (4058S), rabbit polyclonal anti-Akt (9272S) and rabbit polyclonal anti-PARP (9542) were from Cell Signaling (Danvers, MA, USA). Polyclonal rabbit anti-GRP78 (H-129), were from Santa Cruz Biotechnology (Heidelberg, Germany). After three 5 min washes in TNT buffer, membranes were transferred into anti-rabbit IgG horseradish peroxidase-linked secondary antibodies (Chemicon, 1/50,000) or anti-mouse horseradish peroxidaselinked secondary antibodies (Chemicon, 1/10,000) for 1h at room temperature. After three 15 min washes in TNT buffer, the membrane was processed for chemiluminescent detection using Supersignal West Dura chemiluminescent substrate (Pierce), according to the manufacturer's instructions. Quantifications have been done using FIJI software. First, each relative density of band is normalized to its standard (b-actin or calnexin) to take into account loading variation.

Quantitative real-time PCR

Total RNA was extracted using TRI reagent (Sigma) and treated with DNAse (Ambion). cDNA was synthesized by reverse transcription. qRT-PCR was performed in a realtime thermal cycler Cfx C1000 (Biorad) using EvaGreen Supermix (Biorad). List of primers used for QPCR assays No. Name Forward (5′- ...-3′); Backward (5′-...-3′). hDDIT3 GAA-CCA-GGA-AAC-GGA-AACAGA; TCT-CCT-TCA-TGC-GCT-GCT-T. hGAPDH TTCGTCATGGCTGTGAACCA; CAGTGATGCGCATGGACTGT

Apoptosis assay

Cells were seeded in six-well plates (BD Falcon, VWR, Herlev, Denmark) in RPMI 1640 medium containing 10% fetal bovine serum and 5 mM glutamine. After 24 h cells were treated with TG or ASP-8ADT analogue (1 µM), salubrinal (25 µM) BIO (5 µM) for 48 h. Cells from the supernatant were collected with cells from the slide by treatment with trypsin (0.05%), then subjected to a centrifuge spin, and resuspended

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in PBS. From this suspension, aliquots were deposited on slides with a cytospin centrifuge. For morphological analysis, cells were fixed with ice-cold methanol for 10 min and washed twice with PBS. Cells were then stained with 5 µg·mL−1 Hoechst 33258 for 10 min at room temperature and mounted in glycergel (DAKO, Glostrup, Denmark). Nuclear morphology was displayed on an Olympus BH-2 fluorescence microscope (405-435 nm). The percentage of apoptotic cells was determined by counting at least 500 cells in random fields in triplicate for each condition.

Calcium Imaging

Cells were plated onto 30-mm glass coverslips and grown for 3 days. Cells were loaded with 4 µM Fura-2 AM in the incubator at 37°C for 30 min in the growth medium. Ca2+ recordings were performed at 37°C by using a temperature controller associated with the imaging platform (incubator box combined with a precision air heater: LIFE IMAGING SERVICES; Efringerstrasse 79; CH- 4057 Basel; Switzerland). Recordings were performed in HBSS containing (in mM): 140 NaCl, 5 KCl, 1 MgCl2, 2 CaCl2, 0.3 Na2HPO3, 0.4 KH2PO4, 4 NaHCO3, 5 glucose and 10 HEPES adjusted to pH 7.4 with NaOH. The cells were then washed three times in HBSS. The fluorescent intensity of Fura-2 in each cell was monitored and recorded at 340 and 380 nm. To represent the variation in the intracellular free calcium concentration, the fluorescence intensity ratio represented by F340/F380 was used as an indicator of changes in cytosolic Ca2+ concentrations. Fluorescence was excited using an illumination DG4 system (Sutter) fitted with a xenon lamp (300 W). All recordings of Calcium fluorescence were acquired using objective 20. in the Superfluor Nikon Eclipse Ti- series inverted microscope coupled to an EMCCD camera Rolera EM-C2 (Qimaging) and processed using Metafluor 7.7.5.0 software. Typically, we measured individually between 60-80 cells per experiment and we repeated this at least 3 or 4 times and a representative figure is presented. Background fluorescence was selected as an area containing no cells and subtracted from each data point obtained during experiment. To evaluate Mitochondrial Ca2+ content ([Ca2+]m), cells were exposed to FCCP (a potent uncoupler of mitochondrial oxidative phosphorylation) in a Ca2+-free medium.

Results

Exposure to ER stress induced by ASP-8ADT stimulates Akt pro-survival pathway in LNCaP prostate cancer cells.

We have previously demonstrated that ASP-8ADT as others derivative of TG are all able to induce an influx of Ca2+ across the plasma membrane via the activation of SOCE in response to ER Ca2+ store depletion triggered by SERCA inhibition. Following ER Ca2+ depletion, we also observed after 24 h treatment with TG and ASP-8ADT (1 µM) an increase of GRP78 a marker of the UPR (Figure 1A, 1B) confirming that both compounds are able to activate UPR at the same extent. Considering the dual role of UPR in cell fate with the promotion of apoptosis or cell survival, we undertook a screening of survival cellular pathways that could be differentially activated or inhibited in response to TG or ASP-8ADT. Phosphorylation of Akt at Ser473 is a major survival cellular pathway in prostate cancer [11,12] and in this study, we have used LNCaP cells known to rely on phosphorylation of Akt at Ser473 in basal condition of proliferation [11]. Beside, phosphorylation of Akt at Ser473 is also considered as an excellent predictor of poor clinical outcome [13] we have observed an increase of the phosphorylation of Akt following ASP-8ADT treatment (Figure 1C, 1D). Thus, ASP-8ADT stimulates the pro-survival pathway by phosphorylating Akt, such promotion could be involved in the limited cytotoxicity observed in response to ASP-8ADT. On the contrary, TG treatment decreases the phosphorylation of Akt. These results open up the possibility for alternative combined strategies using ASP-8ADT.

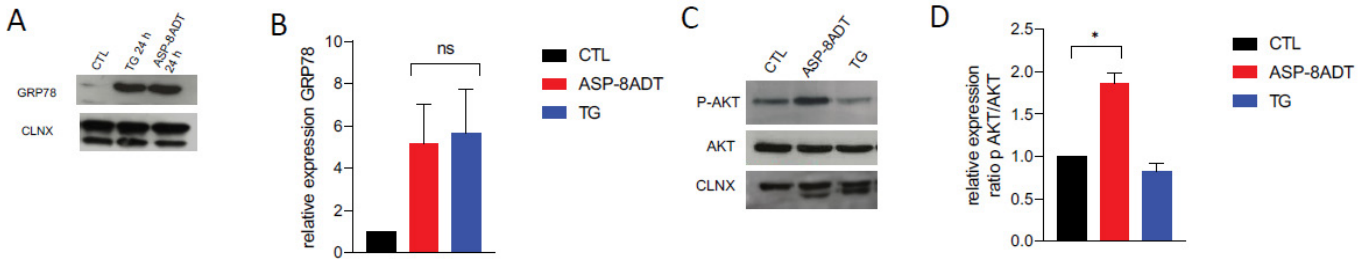

Figure 1: Exposure to ER stress induced by ASP-8ADT stimulates Akt pro-survival pathway.

(A) Representative western blot of the ER stress marker GRP78 and calnexin (CLNX) as a loading control in LNCaP cells treated with ASP-8ADT or TG (1 µM 24 h). (B) quantifications relative to western bot presented in (A). (C) Representative western blot of p-Akt, Akt and calnexin treated as in (A). (D) quantifications relative to western blot presented in (C). Experiments have been performed in triplicate; quantifications have been performed using FIJI software. Bars represent mean ± SEM. ∗p <0.05, one sample T test and Wilcoxon test using PRISM Graph Pad 9.

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Inhibition of PERK downstream pathways potentiates apoptosis induced by ASP-8ADT via the CHOP axis

To counteract the pro-survival role of Akt in the UPR mediated by ASP-8ADT we have selected from the literature two compounds: salubrinal and BIO. We used the salubrinal compound which is a selective inhibitor of eIF2α dephosphorylation [14]. It has been described that treatment of hepatoma cells with salubrinal, enhances TRAIL-induced eIF2α phosphorylation, CCAAT/enhancer-binding protein homologous protein (CHOP) expression and finally caspase activation [15]. In an ovarian cancer model, salubrinal synergically increases the potency of Valosin-containing protein to induce apoptosis by increasing the expression of CHOP [16]. Moreover, it has been shown that ER stress also modulates cell fate by inhibiting mTORC2 and Akt signaling through GSK-3β-mediated phosphorylation of rictor [11]. In that study, authors used TG as an ER stressor and demonstrated that TG inhibits Akt signaling. Our results confirm the ability of TG to inhibit Akt phosphorylation (Figure 1C). On the contrary, ASP-8ADT was unable to inhibit Akt phosphorylation and we have even noted an important increase in Akt phosphorylation (Figure 1C). This promotion of Akt signaling pathway in response to ASP-8ADT may be involved in the fact that apoptosis induced by ASP-8ADT is very modest compared with the parental molecule TG. Thus, we decided to use the BIO compound which is known to inhibit GSK-3β-mediated phosphorylation of rictor [11]. To evaluate the ability of salubrinal and BIO compounds to increase cell death induced by ASP-8ADT we have performed combined treatment and evaluate the number of apoptotic cells after 48 h treatment on LNCaP cells (Figures 2A, 2C). We also evaluated the cleavage of PARP which is a master regulator of caspase mediated cell death (Figures 2B, 2D). We observed that both salubrinal and BIO were able to greatly enhance apoptosis induced by ASP-ADT and also by TG. Both compounds are non-toxic when used as monotherapy. Apoptosis induced by salubrinal has been described as mediated by CHOP. Thus, we have performed qPCR for CHOP and we observed an increase of CHOP at mRNA level under ER stress condition (ASP-8ADT and TG) but level is drastically increased when ASP-8ADT is combined to salubrinal or with BIO (Figure 2E).

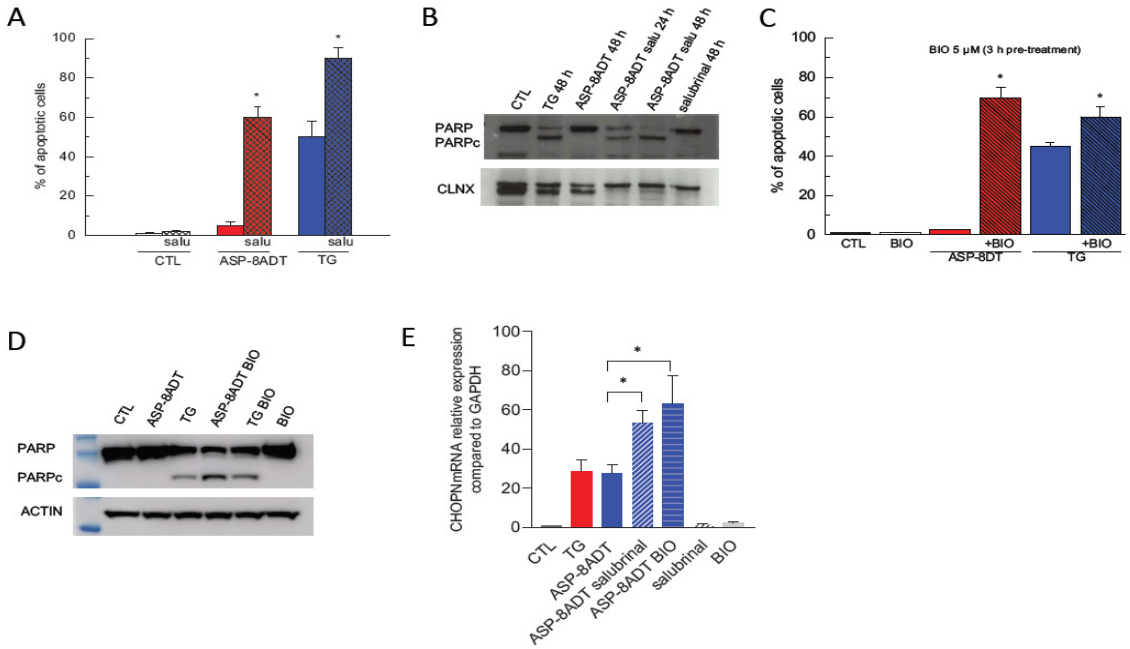

Figure 2: Inhibition of PERK downstream pathways potentiates apoptosis induced by ASP-8ADT via the CHOP axis.

(A) Quantification of apoptotic cells, as determined by manual counting of condensed nuclei following Hoechst staining, 48 h after treatment with control, TG alone and ASP-8ADT alone, or in combination with salubrinal (25 µM 24 h and 48 h). (B) Representative western blot of the apoptotic marker PARP which is cleaved PARPc by caspases and calnexin (CLNX) as a loading control in LNCaP cells treated with ASP-8ADT or TG (1 µM/24 h) combined with salubrinal (25 µM/24 h). (C) Quantification of apoptotic cells, as determined by manual counting of condensed nuclei following Hoechst staining, 48 h after treatment with control, TG and ASP-8ADT alone, or in combination with BIO (5 µM/48 h). (D) Representative western blot of the apoptotic marker PARP which is cleaved by caspases (PARPc) and calnexin (CLNX) as a loading control in LNCaP cells treated with ASP-8ADT or TG (1 µM/24 h) combined with BIO (5 µM/24 h). Experiments have been performed in triplicate. (E) Quantification of the level of CHOP expression at mRNA level obtained by qPCR in LNCaP control cells, treated with TG or ASP-8ADT alone (1 µM/24 h), ASP-8ADT in combination with salubrinal (25 µM) or BIO (5 µM) and salubrinal and BIO alone. Experiments have been performed in triplicate. Bars represent mean ± SEM. $*p < 0.05$, one-way ANOVA with Dunnett multiple-comparison correction for (A and C) and one sample T test for (E).

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Effects of salubrinal and BIO on cytosolic and mitochondrial Ca2+ remodeling induced by ASP-8ADT

We have previously described that ASP-8ADT is able to induce complete ER Ca2+ store depletion leading to SOCE activation and mitochondrial Ca2+ overload but without achieving proper cell death induction. As combined treatments with salubrinal and BIO lead to increased cell death mediated by ASP-8ADT we have evaluated their ability to affect or not the Ca2+ homeostasis over a longer period of time (18 h), as it has been associated with apoptosis induction [17,18]. We observed that the combination of ASP-8ADT with either salubrinal (25 µM/18 h) or BIO (5 µM/18 h) did not drastically modify the cytosolic Ca2+ concentration compared to ASP-8ADT alone (Figure 3A, B). Moreover, it is also largely accepted that such sustained cytosolic Ca2+ concentration mediated by SOCE is a major factor in the process of mitochondrial Ca2+ overload [19-22]. We then evaluated the extent of mitochondrial Ca2+ overload following the same setting of treatment. Interestingly, we observed an intermediate state of the mitochondrial Ca2+ content for ASP-8ADT in combination with salubrinal between control cells (untreated) and cells exposed to ASP-8ADT alone (Figure 3A, C). In contrast, we observed a drastic increase in the mitochondrial Ca2+ content under co-treatment with BIO. These results confirmed the fact that mitochondrial Ca2+ overload *per se* is not enough to induce apoptosis as described by our group and others. This lack of significant effect of salubrinal on both the cytosolic and mitochondrial Ca2+ content in ASP-8ADT treated cells suggests that the drastic increase observed in apoptosis is not mediated by a Ca2+-dependent mechanism. These results highlight that salubrinal or BIO should be used to increase cancer cell sensitivity to ER-Ca2+ stressor drugs such as Mipsagargin family.

Discussion

Though localized PCa is usually treated by radical prostatectomy, androgen deprivation therapy is preferred in locally advanced disease in combination with chemotherapy. For this reason, we used in this study LNCaP cells which are androgen receptor (AR) positive and exhibits androgen-sensitive growth. Besides, phosphorylation of Akt at Ser473 is a major survival cellular pathway in prostate [11,12] and LNCaP cells are known to rely on phosphorylation of Akt at Ser473 in basal condition of proliferation [11]. In fact, resistance to cancer chemotherapeutics treatments is a common phenomenon especially in progressive disease such as prostate cancer. However, prodrug chemotherapy is considered as a promising approach in which an inactive non-toxic agent is administered to the patient and gets activated within the body at specific locations, resulting in a higher concentration of the cytotoxic form at a tumor location while avoiding general side effects. ASP-8ADT used in this study is the active ingredient of mipsagargin which is an example of a recent prodrug chemotherapy approved by the FDA. It is activated by PSMA, which is expressed by some cancer cells and in the blood vessels of most solid tumors, but not by normal cells or blood vessels in normal tissue. It is believed that activation of the prodrug G-202 will allow the drug to kill cancer cells. However, in previous studies [5,7], we uncovered the crucial role of autophagy inhibition to induce potent cell death in response to Ca2+ homeostasis perturbations induced by ASP-8ADT in various cancer cells types. This finding revealed a novel and promising priming strategy to improve clinical outcomes for patients with solid cancers. Interestingly, we also identify in this study that PERK siRNA knockdown impedes apoptosis mediated by TG or ASP-8ADT without affecting cytosolic or mitochondrial Ca2+ overload. This result suggests that cytosolic or mitochondrial Ca2+ overload is not sufficient by itself to induce cell death

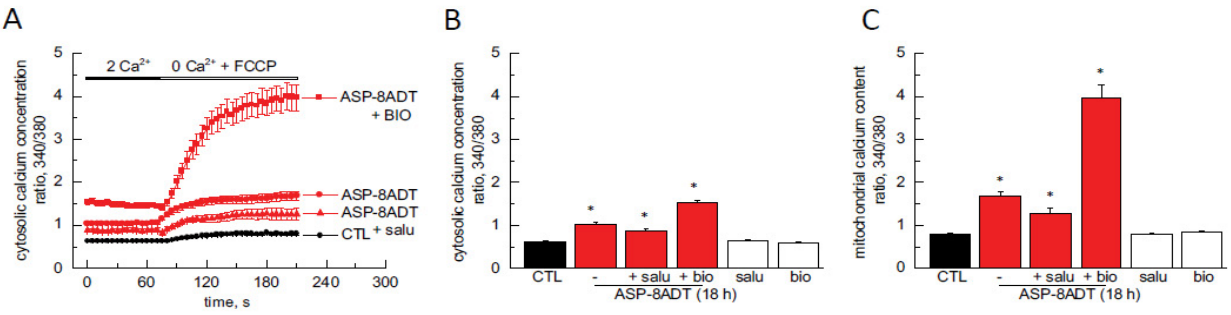

Figure 3: Cytosolic and mitochondrial Ca2+ remodeling.

(A) Representative recordings of [Ca2+]c in LNCaP cells subjected or not (CTL) to SERCA pump inhibitor treatment (18 h) alone (ASP-8ADT) or combined with salubrinal (25 µM) or BIO (5 µM). Mitochondrial Ca2+ content was estimated using the proton ionophore FCCP in a Ca2+-free solution (0 Ca2+). (B) Quantification of [Ca2+]c in LNCaP cells subjected or not (CTL) to cytosolic Ca2+ overload 18 h after SERCA pump inhibitor treatment alone (ASP-8ADT) or combined with salubrinal (25 µM) or BIO (5 µM). (C) Quantification of FCCP-induced [Ca2+]c elevation in LNCaP cells subjected or not (CTL) to mitochondrial Ca2+ overload 18 h after SERCA pump inhibitor treatment alone (ASP-8ADT) or combined with salubrinal (25 µM) or BIO (5 µM). Experiments performed in triplicate. Bars represent mean ± SEM. ∗$p < 0.05$, one-way ANOVA with Dunnett multiple-comparison

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and that other signaling pathways such as PERK are required. However, the potential role of PERK pathway as a priming mechanism in response to ASP-8ADT was not investigated at that time. Here, we decided to target eIF2α by using a drug currently used in clinic, salubrinal. Indeed, several studies have demonstrated that the specific inhibitor of eIF2α phosphatase, salubrinal, can increase the efficiency of different chemotherapeutic agents in several models of cancer such as hepatocarcinoma, breast carcinoma and cholangiocarcinoma [23-25]. In our model, salubrinal was able to greatly enhance apoptosis induced by ASP-ADT confirming a strong interplay between the apoptotic stimuli referred as cytosolic or mitochondrial Ca2+ overload and PERK/eIF2α pathway. Thus, inhibition of PERK/eIF2α pathway should be used to increase the susceptibility of tumor cells to death in response to Ca2+ homeostasis perturbations induced by ASP-8ADT. Further studies are needed to confirm the potential use of such strategy in other prostate cancer cell lines and different types of cancers. This combined therapy should be a relevant approach since Mipsagargin treatment alone only induced a disease stabilization of patients with solid tumors [10]. Besides, our data clearly showed that salubrinal combined treatment did not alter Ca2+ homeostasis suggesting that the drastic increase observed in apoptosis was not mediated by a Ca2+-dependent mechanism. Indeed, it is well known that TG-induced death requires changes in the cytoplasmic Ca2+ concentration initiating a calmodulin/calcineurin/calpain-dependent signaling cascade that involves BAD-dependent opening of the mitochondrial permeability transition pore [26]. Here, we show that inhibition of PERK/eIF2α pathway constitutes a promising strategy to sensitize cancer cells in response to Ca2+ homeostasis perturbations.

A second survival pathway has drawn our attention since it was well accepted that PERK and Akt signaling pathways share several regulatory functions and have the capacity to determine cell outcome under specific conditions. Indeed, the UPR has been reported to activate [27-30] or inhibit [31-33] the Akt pathway depending on the nature and severity of the ER insult. It was recently proposed that Akt phosphorylates and inhibits PERK [34], although the role of the Akt pathway in regulating the UPR remains poorly understood. Here we show that the pro-survival Akt pathway is also activated following ASP-8ADT. In fact, phosphorylation of Akt at Ser 473 is considered as an excellent predictor of poor clinical outcome in prostate cancer [13]. This activation of the pro-survival Akt pathway by ASP-8ADT may explain why Mipsagargin treatment alone only induces a disease stabilization of patients with solid tumors [10]. In an attempt to bypass the role of Akt in cell death resistance to ASP-8ADT, we have selected the GSK-3β inhibitor BIO as an alternative strategy that should be evaluated in the future in clinical trials. Indeed, this compound successfully demonstrated its potential to increase chemotherapy efficiency for several models of cancer. Especially, BIO can increase the efficiency of anti-androgen therapy treatment via decreasing the Androgen Receptor (AR) splice variant AR-V7 which is important in prostate cancer drug resistance and cancer progression [35]. In our study, we were able to drastically increase ASP-8ADT toxicity with a 15-fold increase. At the molecular level, these results demonstrate that under UPR activation and Ca2+ homeostasis disruption, the pro-apoptotic role of Ca2+ overload is in balance with the pro-survival Akt pathway, thereby providing new insights into clinical application of Mipsagargin.

Currently new engineering studies are ongoing to improve TG-based prodrugs delivery options and to determine how optimally these should be used in combination with other drugs [36]. Several clinical trials have been conducted over the last decade for hepatocellular carcinoma (NCT01777594), glioblastoma (NCT02876003, NCT02067156), clear cell renal cell carcinoma (NCT02607553) but they did not achieved phase 3. However, pre-clinical studies are highlighting the potential of combined strategies for several solid cancers among prostate and breast cancers [5,37]. Further studies will be needed to better understand ER Ca2+ stress inducing cell death in solid tumor to personalize the combined strategy and ensure higher efficiency of chemotherapeutics from the Mipsagargin family.

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Authors Contributions

C.D. designed research studies, performed most of the experiments, conceived the experimental designs, analyzed the data, and drafted the manuscript. D.K.P performed experiments. F.V.A. directed the study, designed research studies, performed the experiments, conceived the experimental designs, analyzed the data, and drafted the manuscript.

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