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# Inhibition of the Inositol Requiring Protein 1α- X-Box Binding Protein-1 Pathway as a Promising Therapeutic Target for Human Prostate Cancer

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**Abstract** Prostate cancer (PCa) has been associated with endoplasmic reticulum stress (ERS) which activates the inositol requiring protein 1α- X-box binding protein-1 (IREα-XBP-1) pathway. The aim of the study was to investigate the role of this pathway in three human PCa cell lines (LNCaP, PC-3, and DU-145) by evaluating the expression of XBP-1 and glucose-regulated protein 78 (GRP78) genes. The effect of two ERS inducers (Thapsigargin, Tg and tunicamycin, Tm) alone and in combination with an inhibitor of the IRE1α RNase inhibitor (STF-083010) on expression profiling was followed using Quantitative-PCR. In vitro treatment of PCa cells with ERS inducers upregulated expression of XBP-1 gene. STF-083010 inhibited IRE1α-induced splicing of the gene and increased cytotoxicity. Inhibition of IRE1α RNase activity significantly decreased expression of chaperon protein GRP78. The results confirm and extend the concept that selective targeting of IRE1α-XBP-1 pathway might be a novel therapeutic approach that curbs PCa cell progression.

***Keywords:*** *ER stress, GRP78, IRE1 α-XBP-1 pathway, prostate cancer, STF-083010, unfolded protein response*

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## 1. Introduction

Prostate cancer (PCa) is one of the most common non-cutaneous tumors and the third leading cause of cancer death among males after lung and colorectal cancers world-wide [1]. The current therapeutic strategies; anti-androgen therapies, radical prostatectomy as well as radiotherapy/brachytherapy have alleviated symptoms and improved survival but the outcomes and patient compliance have not improved significantly over the last decade [2]. Thus, finding novel prognostic tools and new treatment options for therapy-resistant PCa has been not only highly desirable, but also a critical challenge.

Previous research has revealed that both ERS and the unfolded protein response (UPR) activation are implicated in tumorigenesis [3,4]. UPR involves three main signaling pathways: protein kinase RNA-like ER kinase (PERK), inositol requiring kinase1α (IRE1α), and activating transcription factor 6 (ATF6) [3,4]. Under normal conditions, the luminal domain of each of the three integral proteins is kept in an inactive state through the association with a well-known ER chaperone, called binding immunoglobulin protein (BiP; also known as GRP78). GRP78 is one major chaperone system in the ERS recovery and is also considered to be a master regulator of the UPR [5], since it leads to activation of the three sensors. Activated IRE1α sensor is responsible for the non-conventional splicing of unspliced XBP-1 (XBP-1u) mRNA to the active form spliced XBP1 (XBP-1s) through its endoribonuclase activity [6]. XBP-1s enters the nucleus and induces transcription of genes correlated with protein-folding capacity and R-associated degradation. XBP-1s is a central UPR effector and previous studies indicates that up- regulation of XBP-1s promotes cell proliferation and invasion of cancerous cells [7,8].

Manipulation of the key branches of the UPR has been suggested as potential anticancer therapies [7,9,10,11]. Due to lack of biochemical and structural understanding of ERS sensors' interaction with GRP78 the exact role of this chaperon in regulating their activity and in metastasis remains largely unknown [12]. However, it is believed that it induces a signaling cascade to mediate tumorigenesis and is linked with increased risk of various types of cancers and with lower survival rate [13]. Here, we have investigated the role of IREα-XBP-1 signaling pathway in PCa cell lines (PC3, DU145 and LNCaP) by evaluating the expression of XBP-1 and GRP78 genes using Quantitative PCR.

# 2. Materials and Methods

## 2.1. Cell Lines and Cell Culture

Human PCa cell lines (DU145, PC3 and LNCaP) were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). The cells were recovered in Dulbecco's Modified Eagle Medium/ Nutrient Mixture F-12 (DMEM/F12, Euro Clone, Italy) supplemented with 15% heat inactivated fetal bovine serum (FBS), 100 units/ml of penicillin, 100 µg/ml of streptomycin and 1% amphotericin B (25µg/ml, Caisson). The cells were incubated in a humidified atmosphere of 5% $CO_2$ at 37℃.

## 2.2. MTT Assays

The PCa cells were plated at a density of $1 \times 10^4$ in 200µl of the culture medium in a 96-well plate and left overnight to adhere. They were cultured for 72h in normal medium and then either left in untreated medium (as control) or medium containing the ERS inducers; thapsigargin (Tg, Santa Cruse, USA) 0.1µM, 0.3µM, 0.5µM, and 1µM or tunicamycin (Tm, Santa Cruse) 1µM, 4µM, 5µM, and 10µm. The plates were read with EPOCH™ Spectrophotometer system (Biotek Instruments, VT, USA) at 590nm absorbance with reference at 630nm. Percentage survival values as compared to the untreated control values against drug concentration were plotted and the approximate 50% inhibitory concentration (IC50) with each PCa cell line was established.

## 2.3. Evaluation of the IRE1α-XBP-1 Pathway

The dose-gene expression response experiments were initiated when cells reached near-confluence state. Based on data obtained from cytotoxicity, survival and pilot experiments, cells were exposed to 0.3µM Tg or 5µM Tm, individually or in combination with of one of the three following concentrations of the IRE1α RNase inhibitor (STF-083010); 30µM, 60µM or 90µM for various time periods of stimulation: 0, 0.5h, 2h, 3h, 6h, 9h or 24h. DMSO served as a solvent control in all experiments.

## 2.4. RNA Isolation and Reverse-transcription

Extraction of total RNA from cells cultured at different conditions was performed using ReliaPrep™ RNA Cell Miniprep System (Promega, Madison, WI, USA). Total RNA concentration was measured by EPOCH™ Spectrophotometer system (Biotek). The RNA was reverse transcribed with a PrimeScript™ RT Reagent Kit (Takara, Japan). The produced cDNA was diluted with nuclease free water (NFW) to get 10 ng for each sample.

## 2.5. Quantitative PCR

Amplification and quantification of cDNA were performed by KAPA SYBR® Fast Universal qPCR Kit (Kapa, Korea). Primers were designed to differentiate between XBP-1 s and XBP-1u, the following PCR primers were used: XBP-1s (forward 5'TGCTGAGTCCGCAGCAGGT'3 and reverse 5'GCTGGCAGGCTCTGGGGAA'3) and XBP-1u (forward 5'TGCTGAGTCCGCAGCACTC'3 and reverse 5'GCTGGCAGGCTCTGGGGAA'3). The specific primers used for GRP78 were as follows: (forward 5'CGTCCTAT GTCGCCTTCACT '3 and reverse 5'AATGTCTTTGT TTGCCCACC'3). Human GAPDH (housekeeping gene) primers (forward 5'GGAAACTGTGGCGTGATGG'3 and reverse 5'GTCCACCACTGACACGTTG'3). Quantitative PCR was carried out in triplicate, first at 95℃ for 3 min, 40 cycles of 95℃ for 3 sec, annealing/extension at 60℃ for 40 sec using by iQ5 (Biorade™ ) Real-Time detection system. Gene expression levels were normalized to GAPDH, calculated using comparative Ct ($2^{-\Delta\Delta CT}$) analyzing methods of relative quantification.

## 2.6. Statistical Analysis

All statistical analyses were performed using SPSS Version 21. Data are expressed as means with standard deviations (SD). ANOVA was used to compare the experimental groups with the control. A $P \leq 0.05$ vale was considered statistically significant.

# 3. Results

## 3.1. Drug Tolerance and Cell Survival

To estimate cell viability in vitro, the MTT assay was used to assess the inhibitory effect of Tm and Tg on human PCa cells. When compared to DMSO-treated and untreated controls cells, the 72h-treatment of both drugs resulted in concentration-dependent and statistically significant ($p \leq 0.05$) reductions in cell survival in all types of PCa cells. In addition, Tm and Tg differentially inhibited the growth of PCa cells; the DU145 cells showed the highest tolerance to Tg, while PC3 cells were the most sensitive. In contrast, LNCaP cells were the most Tm-resistant and DU145 the most sensitive to this drug. On the bases of cytotoxicity and cell survival data, 0.3µM and 5µM of Tg and Tm, respectively, which left about 50% of cells alive, were chosen for further experiments.

## 3.2. Expression of XBP-1

Expression of XBP-1s and XBP-1u was characterized in the three PCa cell lines by quantitative PCR analysis. The results showed upregulation of XBP-1s after treatment of the three PCa cells with Tg at 0.3µM or with Tm at 5µM at different intervals (Figure 1). Compared with the control, DU145 cells were the fastest (after 2h) and LNCaP cells the slowest (after 9h) to express XBP-1s mRNA; 2h and 9h, respectively. In PC3 cells, the highest level of XBP-1s gene was recorded at 6h and at 2h with Tg and Tm, respectively. All the three cell lines exhibited disparity in the expression level of XBP-1s; highest level of expression seen in DU145, while the lowest levels observed in PC3 cells. The XBP-1u expression was significantly lower than the spliced isoform and showed no specific pattern of expression in different cell lines. Maximum levels of expression of this isoform were noted in Tm-exposed LNCaP cells.

### 3.3. STF-083010 Inhibits the IRE1α- XBP-1 Pathway

Figure 2 depicts inhibition of IRE1α- XBP-1 pathway by STF-083010. The PCa cells co-treated with an ERS inducer (Tm or Tg) and an IRE1α suppressor (STF-083010) responded to these conditions by downregulation of XBP-1s to varying degrees depending on cell type and concentration of STF-083010. Relative to the control, STF-083010 greatly attenuated XBP-1s, even after Tm or Tg treatment in all PCa cells. PC3 cells were the most sensitive to STF-083010 inhibition. XBP-1expression DU145 was affected to a lesser extent by concurrent treatment with Tm and STF-083010 than with Tg and STF-083010. Conversely, LNCaP cells showed a reverse pattern; they were more resistant to STF-083010-Tg effect than to STF-083010- Tm.

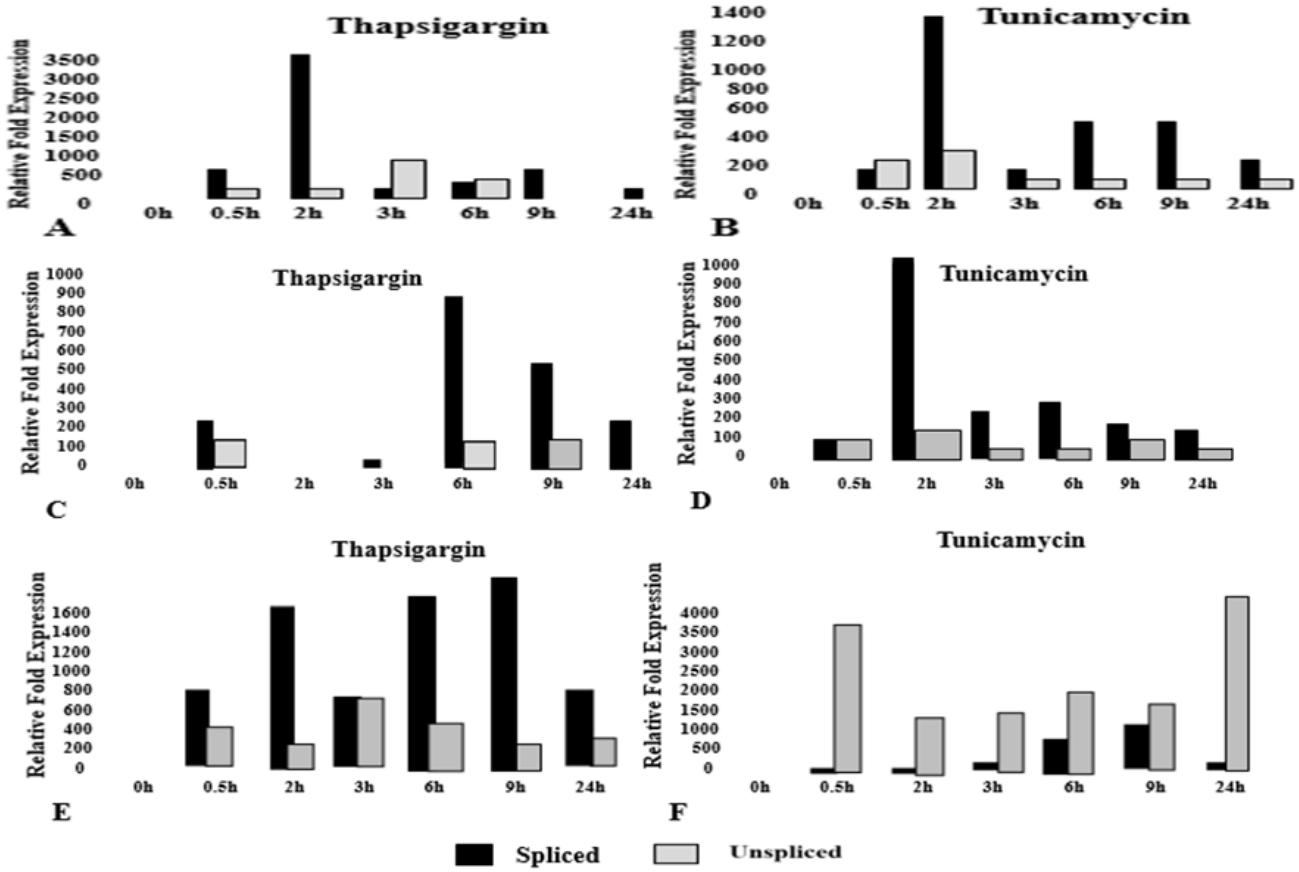


**Figure 1.** XBP-1 gene expression (drug treatment vs control) at different times of treatments. (A&B): DU145 cells, (C&D): PC3 cells; (E&F): LNCaP cells

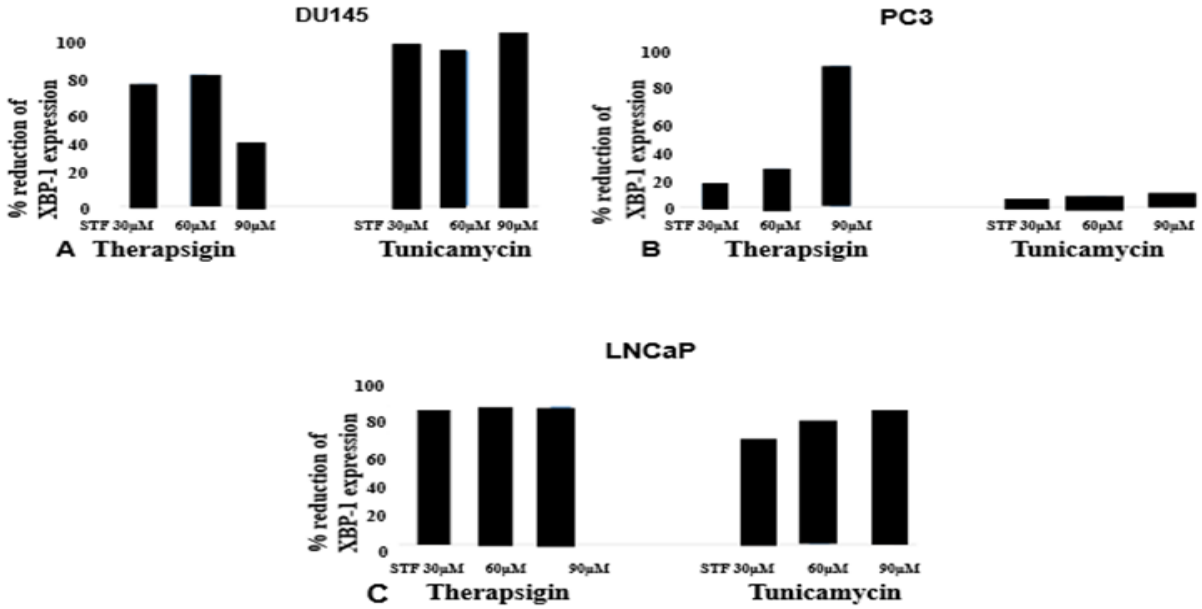


**Figure 2.** Effects of co-treatment of PCa cells with the IRE1 α –XBP-1 inhibitor (STF-083010) and ERS inducers (Tg; 0.3μM or Tm; 5μM) on expression of XBP-1s

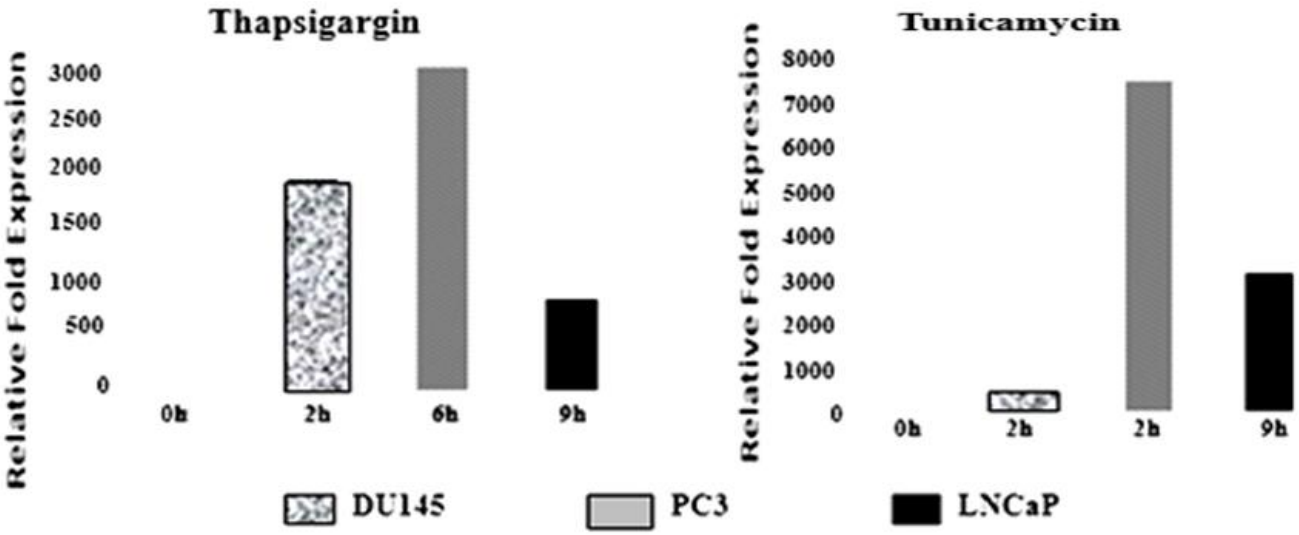


**Figure 3.** Effect of exposure of PCa cells (PC3, DU145 and LNCAP) to Tg or Tm for different time periods on GRP78 gene expression (drug treatment vs control)

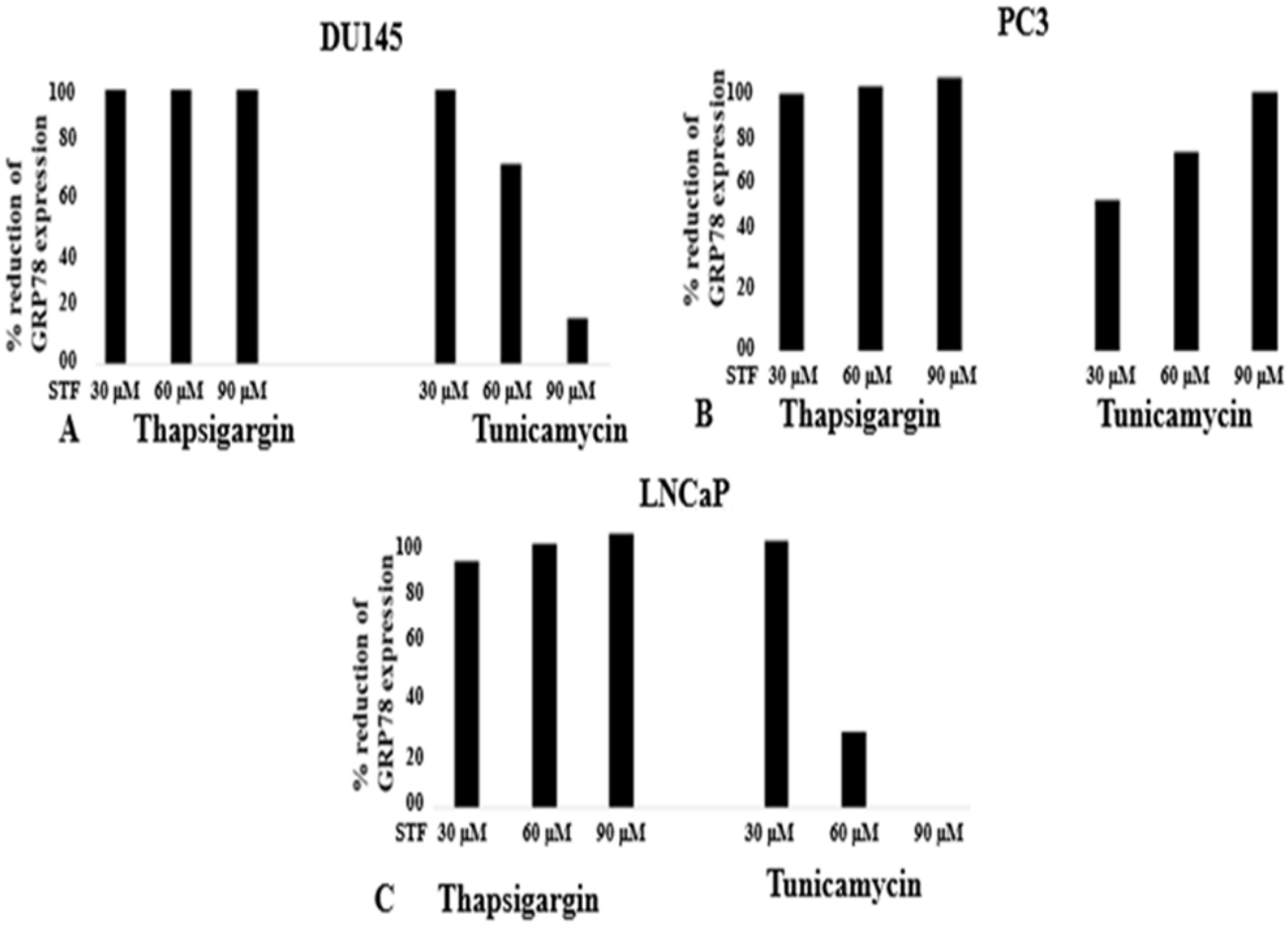


**Figure 4.** Effects of co-treatment of PCa cells with the IRE1 α –XBP-1 inhibitor (STF-083010) and ER stress inducers (Tg; 0.3µM or Tm; 5µM) on expression of GRP78 gene

### 3.4. XPB-1 Induces GRP78 Expression

To test if XBP-1 was responsible for induction of GRP78, the levels of GRP78 expression were examined in ERS- exposed PCa cells before (Figure 3) and after (Figure 4) inhibition of IRE1α-XBP-1 pathway using STF-083010. The results confirmed that GRP78 expression was downregulated following co-treatment of cells either with Tm or Tg and STF-083010.

## 4. Discussion

XBP-1 is a transcription factor that activates chaperone proteins to enhance protein folding and degradation of misfolded proteins. Several researchers reported that overexpression of active form of XBP-1, XBP1-s, correlates with angiogenesis in colorectal and pancreatic cancers [14], enhances breast cancer progression [7,15], promotes pathogenesis of multiple myeloma [16], colorectal

adenoma [8], melanoma [17] and glioma [18]. In contrast, in other previous finding [19]. XBP-1was down regulated in PCa in vivo and in vitro. The divergence in the results of XBP-1 expression may be partly related to cell type as well as to potentials for accommodation with oxygen and nutrients shortages through various tumor tissues.

Previous studies indicated that tumor invasion is regulated by ATF6 [20], PERK [21], and IRE1α [18,22,23] pathways of the UPR. The signaling pathway by which sensors of UPR regulates apoptosis is not completely understood. Modulation of IRE1α-XBP-1 signaling pathway with small molecules such as STF-083010 has been demonstrated as a promising way for cancer therapy [14]. Recently, blocked XBP-1s in glioma cells demonstrated a decreased ability of tumor formation [23]. Furthermore, several research groups reported that inhibition of XBP-1 expression enhances impairment tumor progression [8,16,18]. Our results showed that STF-083010 greatly attenuated the XBP-1s expression in PCa cells, even after Tm or Tg treatment. One of the most important chaperone proteins in UPR is GRP78 and there are several reports which correlate between upregulation of GRP78 and protection of PCa [24,25] and other cancer cells [13,20,23,26,27,28] from apoptosis. Moreover, different PCa cells exhibited different behavior in gene expression depending on the features of the cell line. It is known that LNCaP cells are androgen-sensitive, while DU145 and PC3 are not [29,30], which makes the latter type cells more aggressive and have a higher metastatic potential than LNCaP.

## 5. Conclusion

This study is the first to show the correlation between expression of XBP-1 and GRP78 genes in PCa cells. Co-treatment of PCa cells with an ERS inducer and an IRE1α suppressor (STF-083010) suppressed gene activity. Therefore, inhibition of IRE1α-XBP-1 pathway may be considered a useful approach for cancer chemotherapeutic.

## List of Abbreviations

ATF6, activating transcription factor 6; DMEM/F12, Dulbecco's Modified Eagle Medium/ Nutrient Mixture F-12; ER, endoplasmic reticulum; ERS, endoplasmic reticulum stress; FBS, fetal bovine serum; GRP78, glucose-regulated protein 78; IREα, inositol requiring protein 1α; NFW, nuclease free water; PCa, prostate cancer; PERK; protein kinase RNA-like ER kinase; Tg, thapsigargin; Tm, tunicamycin; XBP-1, UPR, unfolded protein response; X-Box Binding Protein-1; XBP-1s, spliced XBP-1; XBP-1u, unspliced XBP-1

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## Statement of Competing Interests

The authors have no competing interests.

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