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ORIGINAL ARTICLE / ÖZGÜN MAKALE

CURCUMIN, THE BIOACTIVE COMPOUND OF TURMERIC, MAY IMPROVE THE ANTI-MALIGNANT PROPERTY OF GEMCITABINE IN PROSTATE CANCER CELLS

ZERDEÇALIN BİYOAKTİF BİLEŞİĞİ KURKUMİN, GEMSİTABİNİN PROSTAT KANSERİ HÜCRELERİNDEKİ ANTİ-MALİGNANT ÖZELLİĞİNİ GELİŞTİREBİLİR

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ABSTRACT

Objective: *The aim of this study was to investigate the possible synergistic effect of curcumin on the anticancer features of gemcitabine on prostate cancer cells.*
Material and Method: *The human prostate adenocarcinoma cell line LNCaP was used in the studies. The effect of the co-administration of gemcitabine and curcumin on the viability of LNCaP cells was investigated by the WST-1 assay. Autophagy, ubiquitin-proteasome system (UPS), unfolded protein response (UPR) and cell death-associated proteins, androgenic signaling, proto-oncogenic, angiogenic and epithelial-mesenchymal transition (EMT) associated protein levels were investigated by immunoblotting studies.*
Result and Discussion: *Our results showed that curcumin potentiated the anticancer effects of gemcitabine on LNCaP cells. Co-administration of curcumin and gemcitabine strengthened the suppressive effect of gemcitabine on cell viability. Moreover, co-administration modulated the autophagy, more strongly stimulated UPS and UPR, suppressed androgenic signaling, led to the activation of cell death-related poly [ADP-ribose] polymerase 1 (PARP-1) and caspase-3 and strongly suppressed the expression levels of proto-oncogenic c-Myc and angiogenic vascular*

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endothelial growth factor-A (VEGF-A). In addition, it was determined that co-administration negatively regulated EMT by stimulating E-cadherin expression and suppressing N-cadherin level. These results suggest that the combined usage of gemcitabine and curcumin may offer a potent therapeutic approach for prostate cancer by enhancing the anticancer effects of gemcitabine.
Keywords: *Autophagy, curcumin, gemcitabine, unfolded protein response, prostate cancer*

ÖZ

Amaç: *Bu çalışmanın amacı kurkuminin gemsitabinin prostat kanseri hücreleri üzerindeki antikanser özelliklerine olan olası sinerjistik etkisinin araştırılmasıdır.*
Gereç ve Yöntem: *Çalışmalarda insan prostat adenokarsinoma hücre hattı LNCaP kullanıldı. Gemsitabin ve kurkuminin birlikte uygulanmasının LNCaP hücrelerinin canlılığı üzerindeki etkisi WST-1 yöntemiyle araştırıldı. Otofaji, ubikitin-proteazom sistemi (UPS), katlanmamış protein yanıtı (UPR) ve hücre ölümü ile ilişkili proteinler, androjenik sinyal, proto-onkojenik, anjiyojenik ve epitelyal-mezankimal geçiş (EMT) ile ilişkili protein düzeyleri immünoblotlama çalışmaları ile incelendi.*
Sonuç ve Tartışma: *Sonuçlarımız kurkuminin gemsitabinin LNCaP hücreleri üzerindeki anti kanser etkilerini güçlendirdiğini gösterdi. Kurkumin ve gemsitabinin eş uygulaması gemsitabinin hücre canlılığı üzerindeki baskılayıcı etkisini güçlendirdi. Bununla birlikte eş uygulamanın otofajiyi düzenlediği, UPS ve UPR'yi daha güçlü uyardığı, androjenik sinyali baskıladığı, hücre ölümü ile ilişkili PARP-1 ve kaspaz-3 aktivasyonuna yol açtığı, proto-onkojenik c-Myc, anjiyojenik VEGF-A ifade düzeylerini güçlü şekilde baskıladığını gösterdi. Ayrıca eş uygulamanın E-kaderin ifadesini uyararak ve N-kaderin düzeyini baskılayarak EMT'yi negatif düzenlediği belirlendi. Bu sonuçlar, gemsitabin ve kurkuminin birlikte kullanımının, gemsitabinin antikanser etkilerini geliştirerek prostat kanserine yönelik güçlü bir terapötik yaklaşım sunabileceğini düşündürmektedir.*
Anahtar Kelimeler: *Gemsitabin, katlanmamış protein yanıtı, kurkumin, otofaji, prostat kanseri*

INTRODUCTION

Cancer is a significant health problem and one of the main causative factors for mortality worldwide [1]. Among various cancer types, prostate cancer is the second most frequently diagnosed cancer type in men after skin cancer. According to American Cancer Society (ACS) statistics, 268.490 new cases and 34.500 deaths were recorded in 2022 [2]. Despite androgen deprivation therapy, immunotherapy, radiation and chemotherapy are the primary treatment approaches for prostate cancer, the side effects depending on long-term therapy or acquired resistance limit the efficacy of the treatment [3]. Therefore, to reduce these undesirable effects, natural products offer a good option for many years. The role of natural compounds on cancer progression and their combined application with existing chemotherapeutics, including doxorubicin, docetaxel, paclitaxel and gemcitabine, have been intensively studied around the world [4,5]. The discovery of natural bioactive products with high efficacy and safety and their advanced characterization are being extensively studied worldwide. Curcumin is one of the most studied natural products in terms of its biochemical effects *in vitro* and *in vivo*.

Curcumin (1,7-bis(4-hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5-dione) which is a component of turmeric (*Curcuma longa*) has numerous biological activities, including anti-inflammatory, antioxidant, anti-diabetic, anti-viral and anti-tumor [6-9]. Besides, it has been reported that curcumin has potent therapeutic effects on arthritis, wound healing, Alzheimer's disease and different cancer types, such as pancreatic, colon and prostate [10-12]. In addition, there are many ongoing and completed clinical trials on the use of curcumin in the treatment of various cancers, including breast, colon and prostate [13]. For this reason, it is very valuable to investigate the synergistic effects of curcumin with well-known chemotherapeutics such as gemcitabine which have been used in cancer treatments for many years.

Gemcitabine is a pyrimidine nucleoside analog and has been approved as a chemotherapeutic agent for treating numerous solid tumors, including non-small cell lung cancer (NSCLC), bladder cancer, pancreatic cancer and breast cancer. It exerts a cytotoxic effect on cancer cells by arresting the cells at S and late G1 phases [14-16]. Even though gemcitabine has low toxicity and may be a suitable alternative treatment option for elderly patients, the development of resistance causes limitations in its

clinical use [14]. Therefore, combining traditional chemotherapeutics with natural compounds could prevent developing resistance and reduce the side effects of existing treatments.

In the present study, we aimed to examine the potential booster anticancer effect of curcumin on a conventional chemotherapeutic drug, gemcitabine, in androgen-sensitive human prostate LNCaP cells. In this accordance, we first tested the cytotoxic effect of combining the administration of curcumin with gemcitabine. Moreover, we evaluated the effects of the co-treatment of gemcitabine with curcumin on autophagy, the ubiquitin-proteasome system (UPS), unfolded protein response (UPR) signaling, apoptosis, androgenic signaling, epithelial-mesenchymal transition (EMT), angiogenic and proto-oncogenic factors. Our data suggest that co-administration of gemcitabine and curcumin by determining appropriate dose-response ranges may offer a supportive therapeutic approach in prostate cancer by overcoming the systemic toxic effects of gemcitabine.

MATERIAL AND METHOD

Materials

Fetal bovine serum (FBS), tissue culture media and other supplements were obtained from Capricorn-Scientific (Ebsdorfergrund, Germany). Cell culture plastic materials were purchased from Sarsdeth (Sarsdeth, Ireland). Monoclonal rabbit anti-Hrd1 (#14773)(1:3000) and polyclonal rabbit anti-eIF2α (#9722)(1:2500), anti-phospho-eIF2α (Ser51) (#9721)(1:2500), anti-p62/SQSTM1 (#5114)(1:2000), anti-Beclin-1 (#3495)(1:1500), anti-LC3-I/II (#12741)(1:3000) and anti-caspase-3 (#9692)(1:1000) were provided from Cell Signaling Technology (Cell Signaling Technology Inc., Danvers, Massachusetts, USA). Polyclonal rabbit anti-PARP-1 (#13371-1-AP)(1:2000), anti-gp78 (#16675-1-AP)(1:3000), anti-XBP-1s (#24868-1-AP)(1:2000), anti-ubiquitin (#10201-2-AP)(1:1000), anti-PERK (#24390-1-AP)(1:3500), anti-IRE1α (#27528-1-AP)(1:3000), anti-E-cadherin (#20874-1-AP), anti-N-cadherin (#22018-1-AP), anti-AR (#22089-1-AP)(1:2500), anti-c-Myc (#10828-1-AP) and mouse monoclonal anti-PSA (#60338-1-Ig) were obtained from Proteintech (Proteintech Europe, Manchester, UK). Polyclonal rabbit anti-VEGF-A (#E-AB-53277) was purchased from Elabscience (Elabscience, Wuhan, China). Mouse monoclonal anti-beta-actin antibody (#A5316)(1:10000) was purchased from Sigma-Aldrich (Sigma-Aldrich, St. Louis, USA). HRP-conjugated goat anti-mouse (#31430)(1:5000) and goat anti-rabbit (#31460)(1:5000) IgG (H+L) were provided from Thermo Scientific (Thermo-Scientific Pierce, Fisher Scientific, Dublin, Ireland). Gemcitabine (G6423) and curcumin (C1386) were provided from Sigma-Aldrich (Sigma-Aldrich, St. Louis, USA). Staurosporine (#9953) and bafilomycin A1 (#54645) were obtained from Cell Signaling Technology (Cell Signaling Technology Inc., Danvers, Massachusetts, USA).

Cell Culture and Treatments

Human androgen-sensitive prostate adenocarcinoma cell line, LNCaP (CRL-1740^{TM}) was obtained from American Type Culture Collection (ATCC, Rockville, MD). Cells were cultured in Roswell Park Memorial Institute (RPMI) 1640 media enriched with 10% FBS, 5 mg ml^{-1} penicillin/streptomycin and 2 mM L-glutamine and were kept in a humidified atmosphere of 5% CO_2 and 95% air at a constant temperature of 37°C.

Gemcitabine and curcumin were dissolved in sterile cell culture grade H_2O and dimethyl sulfoxide (DMSO), respectively. Compound stocks were prepared at 1000x concentration. DMSO was applied as the vehicle and the final concentration of DMSO applied to the cells did not exceed 0.05%.

Morphological Examination

Morphological alterations were investigated using a phase-contrast inverted microscope (Sunny SopTop ICX41) and a digital camera system (OD400UHW). Cell photographs were taken with 10x or 20x magnifications.

Cell Viability Assay

WST-1 assay (TaKaRa, Mountain View, CA, USA) was performed according to the manufacturer's instructions. Cells were seeded into a 96-well plate (10000 cells/well) and 24 h later

treated with compounds for 48 h. After the incubation time, 10 µl of premixed WST-1 solution was added per well and then the cells were incubated for 2-4 h in conventional cell culture conditions. The absorbance was determined at 450nm with 600nm set as the reference wavelength by microplate spectrophotometer (BioTek, Epoch 2). Results were presented in the graph as a % cell-viability change.

Protein Isolation and Immunoblotting

Cells were seeded in a 6-well plate and incubated for 24 h for protein analysis studies ($2.5x10^5$ cells/well). Cells were then treated with compounds for 24 h and protein isolation studies were carried out. Cells were lysed within radioimmunoprecipitation assay (RIPA) buffer and then centrifugated at 14.000 x rpm for 20 min at 4°C. The supernatant was collected and total protein ingredients were defined by bicinchoninic acid (BCA) protein assay (TaKaRa, Mountain View, CA, USA). 20-30 µg protein content was used in immunoblotting studies. Samples were denatured in 4x Laemmli buffer at 95°C for 5 min and then samples were electrophoretically separated on hand-cast polyacrylamide gels and transferred to an Immobilon®-P polyvinylidene difluoride (PVDF) membrane (Bio-Rad, Hercules, CA, USA). The membrane was blocked with 5% nonfat dry milk in Phosphate-buffered saline (PBS) containing 0.1% Tween (PBS-Tween) for 1 h at room temperature and then incubated with primary and secondary antibodies, respectively. Interested proteins were visualized by enhanced chemiluminescence (ECL) solution in ChemiDoc XRS+ (Bio-Rad, Hercules, CA, USA). The densitometric analysis of protein bands was carried out by ImageJ software.

Statistical Analysis

Data were presented as means ± standard deviation (SD). The statistical significance of differences between groups was determined by a two-tailed equal variance Student's *t*-test. The statistical significance of multiple comparisons was analyzed by one-way ANOVA and Tukey's test using GraphPad Prism 7. $p<0.05$ was considered statistically significant.

RESULT AND DISCUSSION

Evaluation of the Co-administration of Curcumin and Gemcitabine on Viability of LNCaP Cells

To evaluate the enhancer effect of curcumin on gemcitabine in human prostate cancer LNCaP cells and WST-1 based cell viability test was carried out. Gemcitabine and curcumin doses used in the study were selected in line with the literature [17-20]. For this aim, we treated the LNCaP cells with 0.1µM gemcitabine and 5, 10 and 20µM curcumin or their combination for 48 h. In this assay system, 0.1µM staurosporine was used as a positive control of programmed cell death induction, which is a well-known potent cell-permeable alkaloid [21]. Microscopic evaluation revealed that as expected staurosporine treatment markedly induced blebbing and separating cellular forms (Figure 1a). Moreover, combined treatments of curcumin and gemcitabine remarkably enhanced the cell-death-related morphological alterations compared to curcumin or gemcitabine-treated alone groups (Figure 1a).

LNCaP cells were treated with 0.1µM gemcitabine and 5, 10 and 20µM curcumin or with their combination for 48 h. **(a)** A morphological examination was carried out by an inverted microscope and photographed. Scale bar: 5 µm. **(b)** Cell viability was analyzed by WST-1 assay. Three independent biological and three technical repeats per experiment were used. Statistical significance among the groups was analyzed by Student's *t*-test or one-way ANOVA and Tukey's test (*$p<0.05$, **$p<0.001$).

WST-1 assay data showed that co-administration of curcumin with gemcitabine more strongly decreased the viability of LNCaP cells compared to gemcitabine or curcumin-treated alone groups in a dose-dependent manner (Figure 1b). Furthermore, 0.1µM gemcitabine administration significantly reduced the viability of LNCaP cells compared to the control group (Figure 1b). Also, the administration of curcumin alone efficiently decreased the viability of LNCaP cells in a dose-dependent manner (Figure 1b). These data suggested that co-administration of gemcitabine with curcumin strongly enhanced the anticancer features of gemcitabine compared to gemcitabine or curcumin administration alone.

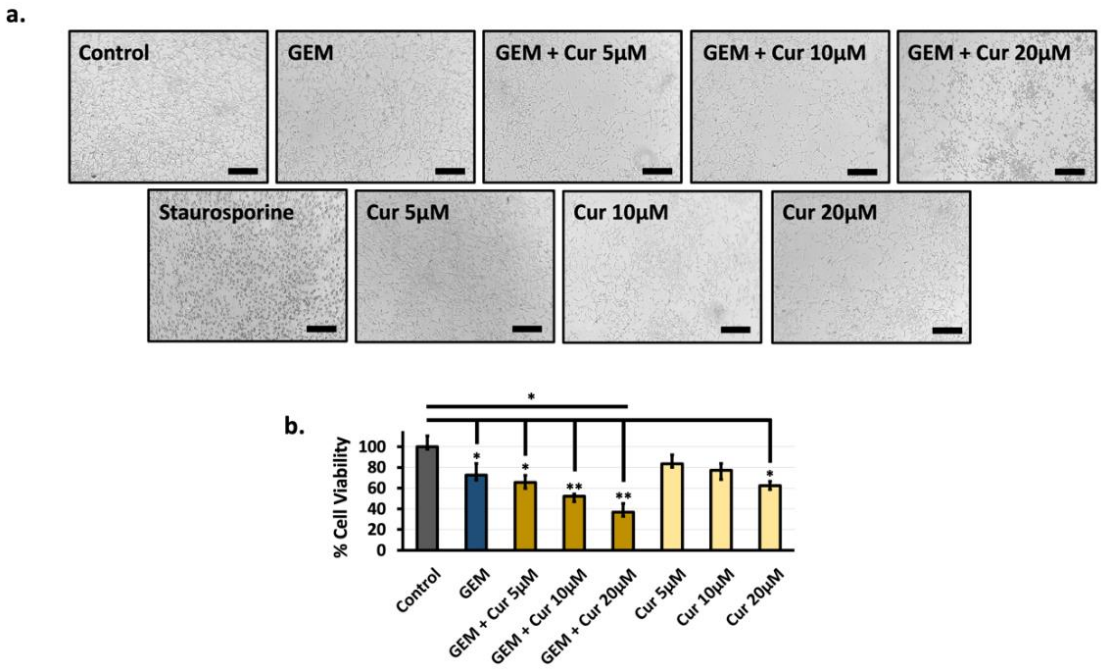

Figure 1. Evaluation of the effect of curcumin and gemcitabine on viability of LNCaP cells

Investigation of the Co-treatment of Curcumin and Gemcitabine on Autophagy

We examined the effect of curcumin, gemcitabine and a combination of curcumin and gemcitabine on changes in cell morphology. 0.1µM gemcitabine treatment did not affect the morphological characteristics of LNCaP cells. Curcumin administration markedly induced the vacuolar structures in cytoplasmic areas of LNCaP cells in a dose-dependent manner. Co-administration of curcumin and gemcitabine more strongly increased the progressively growing vacuolar structures in LNCaP cells. Also, combining the highest dose of curcumin with gemcitabine caused shrinkage and swelling in the morphology of LNCaP cells (Figure 3a). In this experimental system, bafilomycin A1 (BafA1) was used as an autophagic inhibitor and staurosporine was used as a positive control of apoptotic cell death [22]. Compared with the staurosporine and BafA1 treated groups, it was determined that the morphological changes on the LNCaP cells of co-administration were similar to the BafA1 group (Figure 3a).

Cells were treated with vehicle or 0.1µM gemcitabine and 5, 10 and 20µM curcumin or with their combination for 24 h. **(a)** Morphological alterations and vacuolar structures were investigated. BafA1 (1µM) was used as a positive control of autophagic inhibition. Staurosporine (0.1µM) was used as a positive control of apoptotic induction. Arrows are indicated vacuolar structures. Scale bar: 25 µm. **(b)** The expression levels of Beclin1, p62/SQSTM1 and LC3-I/II were analyzed by immunoblotting. Beta-actin was used as a loading control. The protein band density of the control group was set as a 1.

To understand the impacts of the combined treatment of curcumin and gemcitabine, we tested the levels of some autophagy-related proteins, including Beclin1, p62/SQSTM-1 (sequestosome-1) and microtubule-associated protein 1A/1B-light chain 3 (LC3-I/II) by immunoblotting assay. Our results indicated that curcumin treatment dose-dependently increased the p62/SQSTM1 levels, while decreasing Beclin1 levels (Figure 2b). Moreover, LC3-I levels were not affected by curcumin, whereas the level of LC3-II was increased in a dose-dependent manner. Gemcitabine alone treatment did not prominently affect the levels of Beclin1 and LC3-I or LC3-II, but the expression level of p62/SQSTM1 was markedly decreased by gemcitabine treatment compared to the control group (Figure 2b). Co-treatment of curcumin and gemcitabine caused a gradual decrease in the Beclin1 and p62/SQSTM1 levels and an increase in LC3-I levels (Figure 2b).

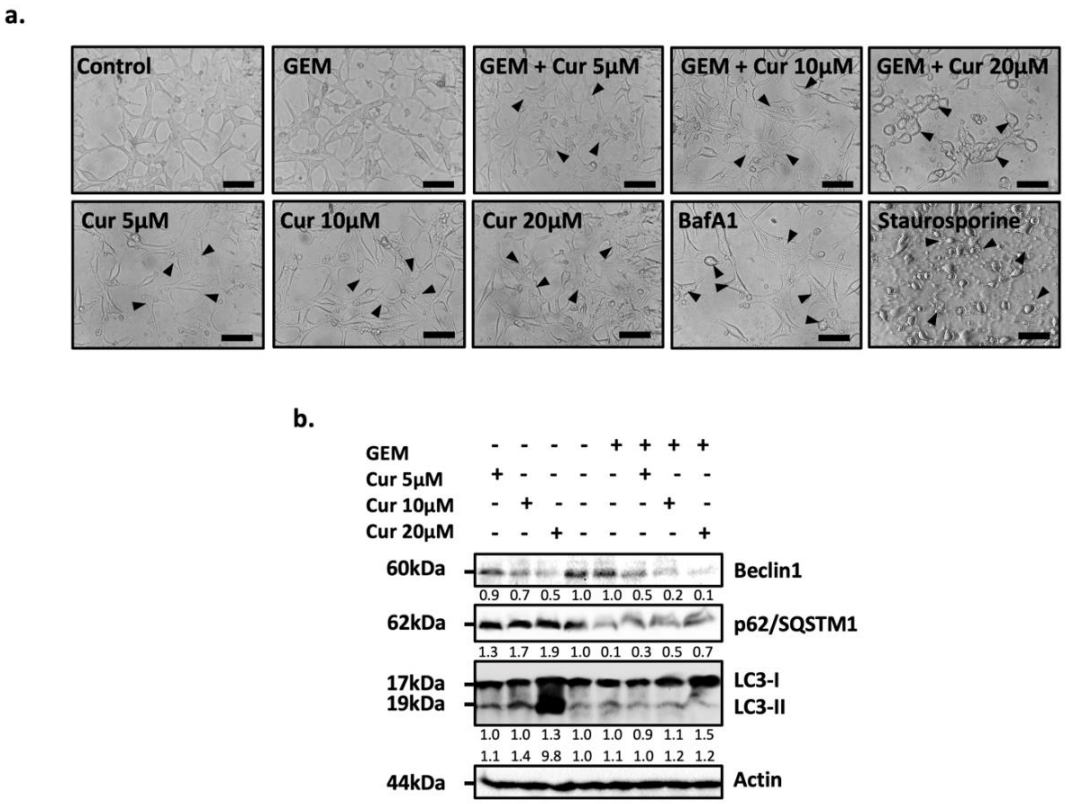

Figure 2. Evaluation of the effect of curcumin and gemcitabine on autophagy proteins in LNCaP cells

Evaluation of the Impacts of Co-treatment of Curcumin and Gemcitabine on UPS and UPR

We examined the effect of the combined treatment of curcumin and gemcitabine on UPS and UPR signaling. Our findings indicated that curcumin treatment increased the hydroxymethyl glutaryl-coenzyme A reductase degradation protein 1 (Hrd1), glycoprotein 78 (gp78) and poly-ubiquitin levels compared to the control group. Moreover, gemcitabine administration also slightly increased the levels of these proteins. Co-administration more strongly increased the gp78, Hrd1 and poly-ubiquitin levels compared to curcumin or gemcitabine alone in a dose-dependent manner (Figure 3a).

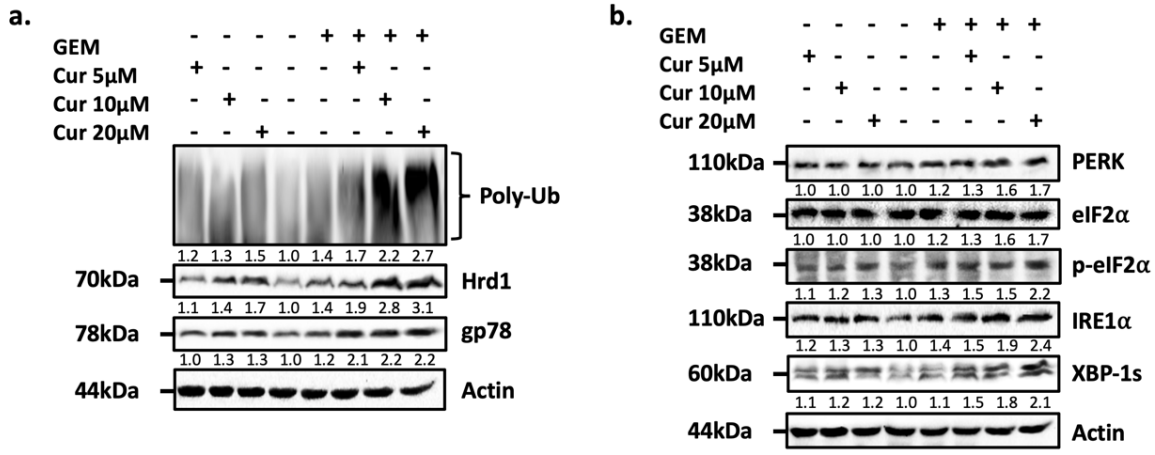

Figure 3. Assessment of the effect of curcumin and gemcitabine on UPS and UPR signaling-related proteins in LNCaP cells

Cells were treated with vehicle or 0.1μM gemcitabine and 5, 10 and 20μM curcumin or with their combination for 24 h. The expression level of **(a)** UPS components, including polyubiquitin, Hrd1 and gp78 levels and **(b)** UPR signaling-related eIF2α, p-eIF2α, PERK, IRE1α and XBP-1s proteins were analyzed by immunoblotting. Beta-actin was used as a loading control. The protein band density of the control group was set as a 1.

To evaluate the effect of curcumin and gemcitabine on UPR signaling, we examined the protein levels of protein kinase RNA-like ER kinase (PERK), eukaryotic initiation factor 2α (eIF2α), phosphorylated eIF2α at serine 51 position (p(Ser51)-eIF2α), inositol-requiring enzyme-1α (IRE1α) and X-box binding protein-1 spliced (XBP-1s) by immunoblotting studies. Our results revealed that curcumin treatment slightly increased the p(Ser51)-eIF2α, IRE1α and XBP-1s levels. Besides, gemcitabine administration also slightly increased the expression level of p(Ser51)-eIF2α, IRE1α and XBP-1s proteins (Figure 3b). Co-administration of curcumin and gemcitabine more strongly induced the protein levels of PERK, p(Ser51)-eIF2α, IRE1α and XBP-1s in a dose-dependent manner (Figure 3b). Collectively, these results indicated that the combined application of curcumin and gemcitabine remarkably augmented the levels of UPS and UPR signaling-related proteins compared to curcumin or gemcitabine alone.

Determination of the Effect of Co-administration of Curcumin and Gemcitabine on Apoptotic Protein Levels

We tested the possible improving effect of curcumin on gemcitabine-induced programmed cell death. Therefore, we examined the poly(ADP-ribose) polymerase 1 (PARP-1) and caspase-3 levels by immunoblotting. Our data revealed that curcumin treatment alone induced 89 kDa of PARP-1 and 17, 19 kDa of caspase-3 cleavage forms. Gemcitabine administration alone did not lead to the cleavage of PARP-1 or caspase-3 at 0.1μM dose. Co-treatment of gemcitabine and curcumin strongly induced the PARP-1 and caspase-3 fragments compared to curcumin or gemcitabine-treated alone (Figure 4).

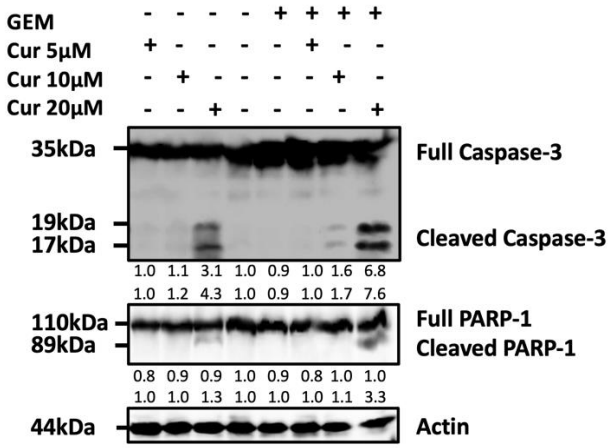

Figure 4. Investigation of the effect of curcumin and gemcitabine on cell death-associated proteins in LNCaP cells

Cells were treated with vehicle or 0.1μM gemcitabine and 5, 10 and 20μM curcumin or with their combination for 24 h. The protein level of full and cleaved caspase-3 and PARP-1 were analyzed by immunoblotting studies. Beta-actin was used as a loading control. The protein band density of the control group was set as a 1.

Evaluation of the Impacts of Co-treatment of Curcumin and Gemcitabine on the Levels of Several Tumorigenic Proteins in LNCaP Cells

To test the enhancer effect of co-administration of curcumin on gemcitabine in LNCaP cells, we tested the expression level of androgenic signal-related androgen receptor (AR) and prostate-specific

antigen (PSA) proteins, proto-oncogenic protein c-Myc, angiogenic factor vascular endothelial growth factor-A (VEGF-A) and EMT-associated N-cadherin and E-cadherin protein levels by immunoblotting. Our data revealed that gemcitabine treatment slightly reduced the protein levels of PSA, AR, c-Myc, VEGF-A and N-cadherin compared to the control group (Figure 5a). Similar to gemcitabine, alone curcumin treatment decreased the level of PSA, AR, c-Myc, VEGF-A and N-cadherin levels and increased the E-cadherin levels in a dose-dependent manner (Figure 5a, b). Combined treatment of gemcitabine with curcumin more strongly reduced PSA, AR, c-Myc, VEGF-A and N-cadherin levels and increased E-cadherin levels compared to gemcitabine or curcumin treatment alone in a dose-dependent manner (Figure 5a, b).

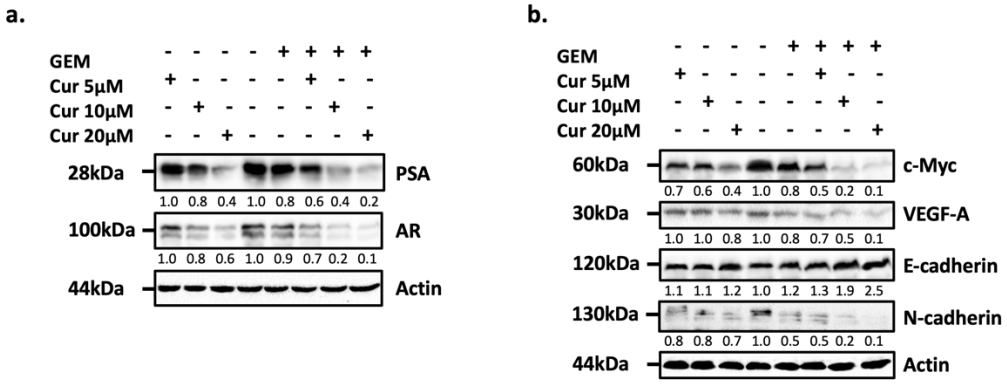

Figure 5. Testing the effect of curcumin and gemcitabine on prostate cancer tumorigenesis-associated protein levels in LNCaP cells

Cells were treated with vehicle or 0.1µM gemcitabine and 5, 10 and 20µM curcumin or with their combination for 24 h. The expression level of **(a)** androgenic signaling proteins PSA and AR and **(b)** tumorigenic proteins c-Myc, E-cadherin, N-cadherin and VEGF-A were analyzed by immunoblotting studies. Beta-actin was used as a loading control. The protein band density of the control group was set as a 1.

Today, potent anticancer drugs with a broad spectrum of action are approved for treatment and commonly used in clinics. Traditional chemotherapeutic agents, including gemcitabine, are still more widely used and are very valuable as it increases the survival rate of patients. However, their systemic side effects and acquired resistance as a result of long-term usage substantially restrict the efficacy of these therapeutics. Therefore, studies have focused on the combinatory administration of natural products and routinely used chemotherapeutics. Numerous *in vitro* and *in vivo* studies, as well as clinical trials, have shown that natural compounds have beneficial effects on the reduction of side effects of chemotherapeutics [15,16].

Research on curcumin, which is one of the most researched natural compounds and is a component of golden spice turmeric (*Curcuma longa*), has been ongoing over the past half-century [23]. Today, its safety and efficacy profile is proven by numerous preclinical and clinical studies. Also, it has been shown that it may be used in numerous diseases such as arthritis, cardiovascular diseases, diabetes and cancer by modulating multiple signaling pathways [24]. Moreover, it also demonstrated that curcumin exhibits potent anticancer properties by inhibiting growth, survival and invasion of cancer cells and inducing apoptotic signaling pathways in malignant cells [25,26]. Possible anti-malignant properties of curcumin have also been reported in prostate tumorigenesis, where abnormal cell proliferation, angiogenesis and metastasis are involved [27].

Herein, we aimed to investigate the booster effect of curcumin on the anti-cancer properties of gemcitabine, a pyrimidine nucleoside antimetabolite widely used in the treatment of various cancer types, including NSCLC, pancreatic cancer and breast cancer. We first tested whether curcumin enhances the anti-proliferative properties of gemcitabine by WST-1 assay. Gemcitabine and curcumin doses were preferred considering the studies performed on LNCaP cells [28,29]. 20µM curcumin and

0.1μM gemcitabine administration significantly decreased the proliferation of LNCaP cells. In contrast, administration of 5 and 10μM curcumin had no significant effect on the proliferation of LNCaP cells (Figure 1b). Similar findings were also observed in microscopic examinations (Figure 1a). Co-administration of curcumin with gemcitabine more strongly decreased the proliferation of LNCaP cells in a dose-dependent manner compared to alone gemcitabine or curcumin-treated groups (Figure 1a, b). These results confirmed the potent enhancer effect of curcumin on the anti-cancer features of gemcitabine.

It is known that many chemotherapeutics and bioactive natural products have inducing or suppressive properties on autophagy [30,31]. Autophagy is a highly conserved and regulated cellular degradation process in eukaryotic cells. Basically, autophagy plays a role in the removal of malformed proteins and damaged organelles through double-membrane vesicles-mediated lysosomal degradation. Complexes formed by many autophagy-related (Atg) proteins are involved in the coordination of autophagy [32]. There are used a variety of methodologies for monitoring autophagy in cells and the most commonly used are the investigation of changes in the levels of Atg proteins, the examination of p62/SQSTM1, which is an autophagic cargo protein and whose levels decrease with increasing autophagic activity and the detection of LC3-I to LC3-II conversion, which is known to be involved in autophagosomal membrane expansion [32]. Since autophagy is a dynamic and cascading mechanism, including induction, nucleation, expansion, fusion and degradation of cargo content through lysosome, many parameters must be examined simultaneously to evaluate the autophagic activity in cells [33].

Present data indicated that curcumin treatment markedly increased the p62/SQSTM1 and LC3-II levels whereas Beclin1, which is an essential mediator of autophagy, decreased in a dose-dependent manner. Additionally, gemcitabine strongly decreased the p62/SQSTM1 levels and had no significant effect on Beclin1 and LC3 levels (Figure 2b). These results showed that curcumin suppresses autophagic flux in prostate cancer cells, while gemcitabine enhances it. In co-administration studies, it was determined that Beclin1 levels decreased in a dose-dependent manner, while p62/SQSTM1 levels decreased due to gemcitabine administration and increased with curcumin administration. However, p62/SQSTM1 levels were still found to be lower compared to the control group. Furthermore, a dose-dependent increase was observed in LC3-I levels due to co-administration (Figure 2b). These results indicated that the increased autophagic activity induced by gemcitabine persisted in LNCaP cells due to the co-administration of gemcitabine and curcumin. In addition, in the results of the microscopic examination, it was observed that there was an increased vacuolization and a change in the cell form in LNCaP cells (Figure 2a). These results supported the immunoblotting findings.

It is known that UPS and autophagy, two main degradation systems in cells, work in interaction [34]. Thus, we examined Hrd1 and gp78 E3 ubiquitin ligase enzyme levels, which are responsible for ubiquitin conjugation to substrate molecules and steady-state level of polyubiquitination [35,36]. It was determined that the application of curcumin or gemcitabine alone increased Hrd1, gp78 and polyubiquitin levels. Also, we found that co-administration more strongly increased the levels of all tested proteins (Figure 3a). These results indicated that the co-administration of gemcitabine and curcumin more strongly induced the UPS. In addition, we examined UPR signaling, which is an essential signaling mechanism in prostate cancer cells and regulates the levels of UPS activity-associated proteins.

To respond to the cell's needs for increased protein synthesis and protein quality control, the UPR generates a series of signals mediated by the three transmembrane proteins IRE1α, PERK and ATF6, which are localized to the ER membrane. Moreover, it is known that cancer cells have increasing UPR activity, thereby UPR signaling plays an adaptive role against the changing metabolic conditions required by cancer cells. It is also well-known that prolonged or excessive UPR stimulation induces programmed cell death [37].

We found that curcumin or gemcitabine alone administration induced IRE1α and PERK branches of UPR signaling. It was determined that eIF2α protein was phosphorylated at the serine 51 position due to PERK activation and XBP-1s levels formed by alternative splicing with IRE1α activation increased. Co-administration of curcumin and gemcitabine more strongly increased the phospho-eIF2α and XBP-1s levels in a dose-dependent manner (Figure 3b). Considering all these results, it seems that the combination of curcumin with gemcitabine more potently stimulates the IRE1α and PERK arms of the UPR. In addition, these results suggest that the effect of the co-administration on the UPS occurs directly

and/or depending on the activation of the UPR.

It is known that caspase-3 and PARP-1, stimulator of programmed cell death, are activated due to the anticancer properties of many chemotherapeutics and natural products [38]. The 89kDa and 17/19kDa cleavage products of activated PARP-1 and caspase-3, respectively, are frequently studied for monitoring programmed cell death at the cellular level [38].

Our data indicated that curcumin administration dose-dependently stimulated caspase-3 and PARP-1 activation. Gemcitabine administration did not cause any response, whereas co-administration strongly activated caspase-3 and PARP-1 in a dose-dependent manner (Figure 4). These results strongly suggest that curcumin efficiently potentiates the anticancer effect of gemcitabine on LNCaP cells.

The androgenic signal and its coordinated oncogenic program as well as EMT are critical in the progression of prostate carcinogenesis [39,40]. It is known that the specialized transcriptional program regulated by the AR arranges numerous molecular signaling pathways [39]. Herein, we found that curcumin or gemcitabine administration reduced the AR levels and also suppressed PSA expression, which is controlled by the transcriptional program of AR. Co-treatment of curcumin and gemcitabine more robustly reduced the AR and PSA levels compared to curcumin or gemcitabine alone (Figure 5a). These results suggest that the co-administration of curcumin and gemcitabine offers a powerful approach to suppressing androgenic signal in prostate cancer cells.

Finally, we examined the levels of proto-oncogenic protein c-Myc, which is known to support tumor progression, angiogenic factor VEGF-A and two key proteins associated with EMT, E-cadherin and N-cadherin. The c-Myc protein is overexpressed in more than 70% of human tumors, including prostate and breast cancers. c-Myc, a transcription factor, has regulatory roles in processes such as cell growth, proliferation, metabolism and differentiation [41,42]. Our results showed that co-administration strongly suppressed c-Myc levels when compared with either curcumin or gemcitabine administration alone (Figure 5b). Additionally, our findings showed that VEGF-A levels, which support the development of tumor-associated blood vessels, provide the way for invasion and are associated with self-renewal and metastasis, were negatively regulated (Figure 5b) [43].

EMT, which is considered a key mechanism for invasion and metastasis, is a fundamental process of the development, progression and spreading of cancer cells. The decrease in E-cadherin levels and the increase in N-cadherin levels act as a stimulating mechanism for invasion and metastasis [44]. Our results showed that co-administration more strongly decreased N-cadherin levels and increased E-cadherin levels compared to curcumin or gemcitabine alone (Figure 5b). These results indicated that curcumin exhibited an enhancer impact on the anti-cancer properties of gemcitabine on prostate cancer cells.

Present data showed that curcumin potentiates the anti-tumorigenic capacity of gemcitabine by enhancing UPS, re-modulating autophagy, activating key regulators associated with programmed cell death, as well as negatively regulating androgenic signaling, suppressing proto-oncogenic and angiogenic protein levels and reducing EMT in prostate cancer cells.

These results indicated that using curcumin, in addition to gemcitabine, might enhance the anticancer properties of gemcitabine in prostate cancer. Present data suggest that curcumin, whose biochemical effects have been widely studied, can be used as a booster in prostate cancer treatments.

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AUTHOR CONTRIBUTIONS

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CONFLICT OF INTEREST

The authors declare that there is no real, potential, or perceived conflict of interest for this article.

ETHICS COMMITTEE APPROVAL

The authors declare that ethics committee approval is not required for this study.

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