Blocking Androgen Receptor/Androgen Receptor Variant7 Decreases Metastasis of Estrogen Receptor Positive Breast Cancer Cells Through Modulating Epithelial to Mesenchymal Transition

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Research Article

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Abstract

Purpose: Androgen receptor (AR) is often expressed in breast cancer, but its role in estrogen receptor positive (ER+) type is controversial. Although AR and its splicing variant 7 (ARV7) play a role in the pathobiology of breast cancer, the precise mechanisms are not fully understood. Therefore, the aim of the current study is to determine the influence of the blockers of AR (Enzalutamide) and ARV7 (EPI-001) on metastasis and epithelial to mesenchymal transition (EMT) in T47D, an ER+ breast cancer cell line.

Methods: T47D cells were cultured/treated with Enzalutamide or EPI-001; cytotoxicity and cell cycle analysis were determined using Sulphorhodamine-B (SRB) assay and Flowcytometry analysis, respectively, whereas metastasis was assessed by the Scratch wound healing assay. Enzyme-linked immunosorbent assay (ELISA) was used to determine the levels of AR, ARV7, C-myc, N-Cadherin, E-Cadherin, NF-κB, ROCK1 and 2 and Western blot was used for evaluating the protein expression of cyclin dependent kinases (CDK4, CDK6, Cyclin E), Fibronectin, vascular endothelial growth factor (VEGF) and matrix metalloproteinases (MMP2 and MMP9).

Results: Our results indicated that treatment with Enzalutamide or EPI-001 didn't significantly affect cell proliferation of T47D. However, cells were arrested at S-phase accompanied by a decline in CDK4, CDK6, Cyclin E and metastasis. Fibronectin, VEGF, MMP2, MMP9, ROCK1 and ROCK2 proteins were downregulated. C-myc and NF-κB levels were declined by EPI-001, but not Enzalutamide.

Conclusion: Blocking AR/ARV7 has no effect on cell proliferation, but decreases metastasis by regulating key markers and processes involved in EMT in ER+ breast cancer cells.

Introduction

Androgen receptor (AR) is evolving as a vital element in the pathogenesis of breast cancer, the most common malignancy among women worldwide (Aristomenis et al. 2020). AR is expressed in more than 60% of breast cancers and up to 90% of ER + tumors, signifying a possible androgen responsiveness [Li et al. 2016]. The signaling outcome of AR differs across breast cancer subtypes and of particular importance is its interplay with estrogen receptor signaling (KeeMing et al. 2015). The role of AR in ER + breast cancer is somehow controversial and restraining application of AR directed therapies. A recent research has reported that AR performs a tumor suppressor role in ER + breast cancer (Theresa et al. 2021). AR can bind to a group of estrogen response elements, thereby preventing the activation of target genes that facilitate the stimulatory effects of 17-beta-estradiol on breast cancer cells (Amelia et al. 2009). However, there is also indications suggesting that AR may be oncogenic and may contribute to acquired resistance against ER + treatments for breast cancer (De Amicis et al. 2010; Hu et al. 2011). Additionally, it was demonstrated that blocking of AR stopped the estradiol-induced proliferation of an ER+/AR + cell line model (Cochrane et al. 2014) and suppressed cell proliferation of `MCF7 (Shuyuan et al. 2003). Also, constitutively active AR sustained the metastasis of endocrine-resistant hormone receptor positive breast cancer (Bahnassy et al. 2020). Consequently, understanding different AR signaling effects

can help to harness the clinical availability of new potent AR-antagonists to hopefully improve current endocrine therapies for ER + breast cancers.

The AR variants arise owing to alternative splicing and/or structural rearrangements of the AR gene; the variants have variable structures, but each lacks all or a part of the ligand binding domain (LBD) (Dehm et al. 2011). This may produce constitutively active, ligand independent, transcription factors or variants resistant to drugs that reduce androgen production and biosynthesis (Li et al. 2013). Androgen receptor variant 7 (ARV7) is one of the most noticeable splice variants in prompting AR mediated gene transcription even under conditions of androgen deprivation and in driving cancer progression in prostate cancer (Bin et al. 2020). Moreover, their role in breast cancer has been recognized and is considered an area of active research (Dong et al. 2014, Theresa et al. 2015).

Several studies have pointed that the aberrant expression of AR/ARV7 is a chief factor that contributes to progression in many cancers, including breast (Macedo 2006), prostate (Adam et al., 2019), hepatocellular (Yeh and Chen 2010), bladder (Wagih and Kamel 2020), oral squamous cell carcinoma (Wu et al. 2015) and gastric cancer (Tian et al. 2013) by endorsing tumor cell growth. However, few studies have focused on metastasis, which is a complex procedure comprising remodeling of the extracellular matrix and leading to epithelial to mesenchymal transition (EMT). This transition is implicated in carcinogenesis and provides metastatic properties to cancer cells by enhancing mobility, invasion, and resistance to apoptosis (Antonella et al. 2017). Furthermore, EMT derived tumor cells attain stem cell characteristics and display a noticeable therapeutic resistance (Vivek 2018). Several biomarkers and pathways play a role in EMT and accordingly affect metastatic potential of cancer cells (Jing et al. 2020).

Therefore, the aim of this study was to determine the effect of blocking AR and ARV7 by Enzalutamide and EPI-001respectively on EMT and metastasis in T47D, an ER + breast cancer cell line. Thereby, several biomarkers were chosen to cover key processes occurring during EMT.

Materials And Methods

Cell culture

T47D cells were obtained from the American Type Culture Collection (ATCC, Minnesota USA) and were maintained and routinely checked at the Egyptian National Cancer Institute, Cairo, Egypt. They were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum (FCS), 2 mM L-glutamine, 1.5 g/l sodium bicarbonate and 1% penicillin/streptomycin and kept in a humidified atmosphere with 5% CO_2 at 37°C.

Drug, chemicals & reagents

Enzalutamide (catalogue #: A3003) and EPI-001 (catalog #: B6041) were purchased from ApexBio (HTX, US). Both inhibitors were dissolved in dimethylsulfoxide (DMSO) to prepare a stock solution of 1 mM. For

the cytotoxicity assay, different concentrations were used (6.25, 12.5, 25, 50, 100 and 200 uM). For the other experiments, the two inhibitors were added to the medium at a final concentration of 10 μM and 50 μM for Enzalutamide and EPI-001, respectively based on previous reports (Dawn et al. 2014; Brand et al. 2014).

For the Western blot technique, the primary antibodies obtained from Santa Cruz Biotechnology, (TX, US) were used against AR (catalog #: sc-7305), CDK6 (catalog #: sc-7961), CDK4 (catalog #: sc-23896), cyclin E (catalog #: sc-377100), Fibronectin (catalog #: sc-8422), MMP2 (catalog no: sc-13595), MMP9 (catalog no: sc-393859) and VEGF (catalog #: sc-7269). The primary antibody against ARV7 (catalog #: AG10008) was purchased from A&G Precision Antibody (MD, US).

The human ELISA kits for AR (catalog #: In-Hu4116), ARV7 (catalog #: In-Hu4117), C-myc (catalog #: In-Hu1853), NF-κB (catalog #: In-Hu2637), N-Cadherin (catalog #: In-Hu4118), and E-Cadherin (catalog #: In-Hu1892) were purchased from Innova Biotectech (BJ, China). Additionally, the human ELISA Kits for ROCK1 (Catalog #: OKEH06554) and ROCK2 (catalog #: LS-F22011) were procured from Aviva Systems Biology (LA, US) and LifeSpan BioSciences (WA, US).

Sulphorhodamine-B (SRB) assay

The antitumor activity of Enzalutamide and EPI-001 on T47D cells were evaluated by the SRB assay (Skehan et al. 1990). In brief, cells were seeded for 24 hr in 96-well plate at a density of 3×10^3 cells per well. They were treated with different concentrations (6.25, 12.5, 25, 50, 100 and 200 μM) of Enzalutamide or EpI-001. For each concentration two wells were used, then, they were incubated for 48 hr and fixed by 20% trichloroacetic acid and stained with 0.4% SRB dye. Finally, the optical density (O.D.) of each well was measured at 570 nm spectrophotometrically using ELISA microplate reader (TECAN sunrise, Germany). The cell survival fraction was calculated by dividing O.D. of treated cells over that of control cells.

Flowcytometry

Treated and control cells were collected after 24 hr and the cells were then fixed with 70% ice cold ethanol, washed and the pellets were resuspended in trypsin buffer and left for 10 min. After that, trypsin inhibitor and 1% RNase buffer were added and incubated for 10 min. followed by the addition of propidium iodide (100 μg/ml). Samples were then incubated in dark for 30 min. at 4°C and the distribution of each cell cycle phase was then determined using a EPICS® C Flow cytometer (FLA, US).

Scratch wound healing assay

T47D cells were seeded in six wells plate and incubated for 24 hr to allow for adherence. The formed monolayer was then scratched slowly with 1 ml pipette tip through each wheel center in one direction. After that, the wells were washed twice with medium in order to remove any detached cells. The cells were then treated with Enzalutamide or EPI-001 for 24 hr, washed twice with PBS then fixed with 3.7% paraformaldehyde for 30 min. and finally, the cells were stained with 1% crystal violet for 30 min. Photos

were taken for the stained monolayer and the reduction in gap area was measured using ImageJ software (MD, US).

Immunocytostaining of ARV7

T47D cells were collected and washed with PBS, resuspended in DMEM and placed on glass slide incubated in H_2O_2 for 15 min. then washed again using PBS to be incubated with monoclonal antibody against ARV7 for an hr and then washed three times using PBS. Biotinylated secondary antibody was added and incubated at room temperature for 10 min. and washed with PBS. After that, 3,3'-Diaminobenzidine (DAB) was dropped and incubated for 5 min. followed by counterstaining the slides with hematoxylin. The expression of ARV7 was displayed by brown nuclear color.

Enzyme-linked immunosorbent assay (ELISA)

Treated and control cells were collected and centrifuged for 5 min. at 10,000 rpm at 4°C. The supernatant was then aspirated and the cell pellet was resuspended in phosphate buffer saline (PBS) and the freeze/thaw process was conducted three times in order to lyse the cells. The freeze/thaw process consisted of freezing the samples at − 70°C then immediately thawing them at 37°C. The samples were centrifuged for 5 min. and the supernatant was collected for ELISA quantification in accordance with the manufacturer's instructions. Protein levels were determined using Bradford assay and their levels were calculated from the constructed calibration curve using second order polynomial curve-fitting models.

Western blot analysis

Treated and control cell were collected as pellets. The Ready Prep™ protein extraction kit (Catalog #:163–2086, Bio-Rad Inc., CA, US) was used for protein extraction. The quantitative protein analysis was conducted by Bradford protein assay kit (Catalog #: SK3041, Bio basic Inc. ON, Canada). After that, the extracted proteins were separated by SDS-PAGE using TGX Stain-Free™ FastCast™ (Catalog #: 161–0181, Bio-Rad Inc., CA, US) and blotted onto PVDF membranes. The membranes were then blocked with 5% non-fat dry milk and incubated at 4°C with primary antibodies overnight followed by incubation with peroxidase-conjugated secondary antibodies. In addition, β-actin antibody was probed in membrane for normalization. The intensities of the bands of the target proteins were normalized against β-actin and were analyzed on the ChemiDoc MP imager.

Statistical analysis

The data were presented as mean ± S.E.M. Multiple comparisons were performed utilizing one-way analysis of variance (ANOVA) followed by Tukey's as a post hoc test. F values were expressed to indicate the ratio between and within group variances. Significance was established when $p \leq 0.05$. All statistical analyses were performed using IBM SPSS Statistics for Windows, Version 26.0 (NY, US). Graphs were sketched using GraphPad InStat, version 5.0 (LA, US) (Supplemental data).

Results

Effect of EPI-001 and Enzalutamide on the proliferation of T47D

As depicted in Fig. 1 no significant increase in the proliferation of T47D was observed following treatment by EPI-001 or Enzalutamide as measured by SRB assay. For the two blocker no IC50 value was detected.

EPI-001 and Enzalutamide induced S-phase cell cycle arrest and downregulated cell cycle regulatory proteins

Treated T47D groups with EPI-001 and Enzalutamide demonstrated an S-phase cell cycle arrest with 50.38% and 14.01% of cell population retained in S-phase compared to 0.51% in untreated control group (Fig. 2A & 2B). These results were further confirmed by the qualitative evaluation of Cycline E, CDK4 and CDK6 protein expression levels by measuring the optical densities of their Western blot bands (2C). EPI-001 reduced Cycline E, CDK4 and CDK6 to 43.71%, 35.73% and 20.5% of the corresponding control group optical densities, respectively (Fig. 2D). Likewise, Enzalutamide showed a comparable reduction of Cycline E, CDK4 and CDK6 band densities to 38.82%, 33.18% and 19.94 respectively compared to control group (Fig. 2D).

EPI-001 and Enzalutamide inhibited cell migration and motility in T47D

The implication of both inhibitors on cell migration & motility was evaluated using Scratch wound healing assay. The wound area was measured using image j software after 24 hr of inhibitors incubation with T47D cells (Fig. 3A). The scratch area exhibited a reduction of 60.97% in control group after 24 hr. On the other hand, the scratch area reduction percent in EPI-001 and Enzalutamide treated groups were 33.67% and 34.54%, respectively (Fig, 3B); which is significantly different from control group [$F(2,17) = 44.358$, $P \leq 0.05$].

Expression of AR/ARV7 in T47D and the effects of EPI-001 and Enzalutamide on their expression

Immunocytochemistry, Western blot and ELISA techniques confirmed the expression of AR and ARV7 proteins inT47D (Fig. 4A, B). Moreover, the expression levels of AR and ARV7 were not significantly affected following treatment with EPI-001 or Enzalutamide (Fig. 4C) but their effects on cellular functions were further evaluated.

EPI-001 downregulated NF-κB and C-myc protein levels in T47D

The effect of EPI-001 and Enzalutamide on the expression levels of NF-κB and C-myc were measured by ELISA technique and illustrated in Fig. 5. EPI-001 significantly reduced both NF-κB to 50.55% [$F(2,5) =$

20.58, P ≤ 0.05] and C-myc to 73.88% [F(2,5) = 98.36, P ≤ 0.05] compared to control group while Enzalutamide had no significant effect on their protein expression.

Effect of EPI-001 and Enzalutamide on E-Cadherin and N-Cadherin

The effects of EPI-001 and Enzalutamide on the protein levels of the epithelial marker (E-Cadherin) and mesenchymal marker (N-Cadherin) were elucidated utilizing ELISA technique (Fig. 6). Both EPI-001and Enzalutamide significantly elevated the levels of E-Cadherin by 10.77 and 12.76 folds respectively compared to control group, [F(2,5) = 10155.723, P ≤ 0.05] but no significant effects were observed on N-cadherein levels.

EPI-001 and Enzalutamide downregulated Fibronectin

Alongside the demonstrated effect of EPI-001 and Enzalutamide on E-Cadherin; the Fibronectin protein level was reduced as measured by Western blot by 59.44% and 62.46% for both inhibitors, respectively (Fig. 7).

EPI-001 and Enzalutamide reduced angiogenesis

The possible anti-angiogenic potential of both inhibitors was measured through qualitative measurement of VEGF level (Fig. 8). Indeed, EPI-001 and Enzalutamide markedly reduced VEGF band densities to 51.25% and 42.2% compared to control group, respectively.

EPI-001 and Enzalutamide downregulated protein levels of matrix metalloproteinases (MMPs): MMP2 and MMP9

The quantification of MMP2 and 9 via Western blot analysis in different groups (Fig. 9A) showed a marked reduction of their levels after treatment with both inhibitors. Indeed, both MMP**s** 2 and 9 were reduced by EPI-001 to 41.16% and 35.79% respectively as compared to the control value. Along side, Enzalutamide reduced the band density to 40.39% and 32.53%, respectively (Fig. 9B).

EPI-001 and Enzalutamide downregulated ROCK1 and ROCK2 levels

The impact of AR and ARV7 on activation of Rho kinase 1and 2 (ROCK1,2) was evaluated through quantification of ROCK1,2 after treatment with EPI-001 and Enzalutamide. As illustrated in Fig. 10; EPI-001 reduced ROCK1 level to 30.18% of control group. Such a reduction was significantly different from either control or Enzalutamide treatment group [F(2,5) = 1181.889, P ≤ 0.05]. Meanwhile, Enzalutamide significantly reduced ROCK1 level to 40.48% of control group. On the other hand, both EPI-001 and Enzalutamide significantly reduced ROCK 2 activity to 23.42% and 28.94% of control value, respectively [F(2,5) = 438.537, P ≤ 0.05]

Discussion

The expression of AR in breast cancer makes it an attractive therapeutic target. However, its role in breast cancer development is still controversial as it can either inhibit or promote breast tumor growth and metastasis (Pia et al. 2018; Mengyao et al. 2020). Among AR variants, ARV7 is drawing attention as a prospective marker for one of the resistance mechanisms to anti AR therapy of castration resistant prostate cancer (CRPC) (Ye and Tian, 2021) and has also been detected in breast cancer subtypes (Dong et al. 2014). Yet, the role of both AR and ARV7 in ER + breast cancer needs further clarification. In the current investigation, the influence of AR/ARV7 on ER + breast cancer has been studied through blocking AR/ARV7 with Enzalutamide or EPI-001. It was noticed that there was no significant effect in the proliferation of the ER + cell line T47D following treatment by the two blockers. Nevertheless, there was a decrease in the protein expression of CDK4, CDK6 and Cyclin E which are known to play important roles in breast cancer (Khandan et al. 2002; Mara et al. 2019). This was accompanied by a decrease in number of cells in G1 phase and cell cycle arrest in S-phase. In harmony with our results, a previous study indicated that blockade of AR signaling decreased the G1-S cyclins in a resistant breast cancer cell line (Wenfei et al. 2019). This effect of the cell cycle which was not translated into decrease in cell proliferation can be explained by the existence of equilibrium between cell cycle arrest and proliferation governed by several other pathways and growth factors in the tumor microenvironment and extracellular matrix (ECM). Whether the balance is tilted towards cell cycle arrest or cell proliferation is influenced by how the cell incorporates the various signals, internal or external to the CDK network that stimulates or delays progression in the cell cycle (Claude and Albert 2014, 2016).

Breast cancer exhibits metastatic heterogeneity with distinctive precedence to many organs as bone, lung, liver and brain. Only few studies have been performed to evaluate AR expression in primary tumors and metastatic samples. It was demonstrated that AR persisted in most of metastatic samples from AR + triple negative breast cancers (TNBC) (Yasuhiro et al. 2015) as well as breast cancer bone metastasis (Nicola et al. 2018). Moreover, androgens activated myosin, a cytoskeletal protein having a role in cell motility and invasion in T47D cells (Maria et al. 2016). Similar to AR, not many studies have explored the relation between ARV7 and metastasis in breast cancer. However, in prostate cancer, it was revealed that ARV7 positive metastatic CRPC patients may have worse prognosis (Jiatong and Ranlu 2020). These studies and others stimulated our interest to investigate the effect of AR/ARV7 mediated metastasis in ER + breast cancer cell line by blocking AR/ARV7 with Enzalutamide or EPI-001. Our results indicated that these blockers inhibited metastasis in T47D as indicated by Scratch wound healing assay. A previous study reported that Enzalutamide inhibited cell migration and invasion in TNBC cell line in an AR dependent manner (Francesco et al. 2016). Also, targeting ARV7 axis resulted in altering the prostatic cancer cells progression and resistance to androgen deprivation therapy with Enzalutamide (Ronghaoet al. 2017).

The process whereby epithelial cells are transformed into mesenchymal cells (EMT) can impact cancer growth and dissemination whereby epithelial cells change their adhesion profiles from cell/cell contacts to cell/matrix interactions, leading to metastasis and therapy resistance EMT is a dynamic procedure demanding the interaction of main processes and signals in tumorigenesis; angiogenesis, inflammation, immunomodulation, matrix degradation, etc (Erik et al. 2020). In prostate cancer, both AR and AR splice

variants contribute to prostate cancer aggressiveness through induction of EMT (Campbell et al. 2013; Dejuan al., 2015). Breast cancer cells also undergo EMT modifications, enhancing tumor progression and metastais (Chiara et al. 2012; Renata et al. 2020). Therefore, In the present investigation, it was of interest to study the effect of AR/ARV7 inhibitors, Enzalutamide and EPI-001, on EMT in T47D to explain their observed antimetastatic effect. To explore this hypothesis, several markers which may impact EMT were studied.

One of the investigated markers is the inflammatory mediaor NF-κB which has a significant role in promoting aggressive phenotypes of ER + breast cancer. Both ER and NF-κB can suppress and activate one another and each of these mechanisms have the potential to contribute to more aggressive ER + breast cancer phenotype and metastasis (Emily et al. 2020). Several reports stated that NF-κB modulates EMT in breast cancer (Bruno et al. 2017; Margit et al. 2004; Li et al. 2012). Consequently, these reports and others support the need to reconsider targeting the NF-κB pathway in ER + breast cancer as an approach to prevent disease progression and relapse. The promoter region of the AR gene possesses NF-κB response elements and stimulation of NF-κB activity was found to elevate AR levels in prostate cancer cells (Liying et al. 2009). Therefore, NF-κB may represent a target for breast cancer therapy through regulation of AR. As displayed by our data, NF-κB protein levels declined following treatment by EPI-001 in T47D while no significant effect was observed by Enzalutamide. Since studies suggest an association between high estrogen receptor expression, invasion, metastasis and NF-κB (Xian-Long et al. 2014), thus the potential effect of EPI-001 as downregulator of NF-κB may be implicated, at least in part, in decreasing metastasis in ER + breast cancer. It's noteworthy that NF-κB controls a number of markers involved in cell cycle progression, inhibition of apoptosis, immunoinflammatory responses and cell adhesion. Some of these markers are cell adhesion molecules, C-myc, VEGF and MMPs (Takashi et al. 2007). Similarly, a pathological increase in cellular motility, leading to malignant transformation, was linked with RhoA–ROCK-mediated activation of NF-κB (Catherine et al. 2009).

E-Cadherin, N-Cadherin and Fibronectin are vital EMT markers. E-Cadherin is an epithelial marker and its decreased level correlates with increased invasiveness and metastasis of tumors. On the other hand, the mesenchymal N-Cadherin is regarded as an oncoprotein where it stimulates invasion, proliferation, angiogenesis and metastasis (Mayra et al. 2019). Similar to N-cadherin, Fibronectin glycoprotein is associated with metastatic tumors and poor prognosis in many cancers including breast cancer (Georgios et al. 2020). Yan-Nian et al. (2008) suggested that activated AR can downregulate E-Cadherin expression and promote activation of EMT and metastasis in breast cancer patients through binding to E-Cadherin regulatory sequences. Additionally, constitutively active AR variants upregulate the expression of N-Cadherin in prostate cancer cells (Félicie et al. 2013). Dibash et al. (2016) identified a novel pathway in prostate cancer whereby miR-1207-3p regulates the AR via a pathway involving Fibronectin. Consequently, in the present study, the effect of AR/ARV7 blockers Enzalutamide and EPI-001 on the expression levels of E-Cadherin, N-Cadherin and Fibronectin was investigated. Both blockers significantly elevated the levels of E-Cadherin but didn't induce significant change in N-Cadherin. Also, the two blockers reduced the levels of Fibronectin by 39.15% and 41.02% respectively.

C-myc is another driver of EMT in mammary epithelial cells (Kyoung et al. 2010) and TNBC (Shuping et al. 2017). It also plays a proliferative role and imparts resistance to chemotherapeutic agents in ER + breast cancer cell lines (Yassi et al. 2017). Therefore, targeting C-myc in combination with inhibitors of other oncogenic pathways may provide a therapeutic strategy for breast cancer. C-myc has a critical role in regulating the coordinated expression of AR and AR variants in CRPC (Shanshan et al. 2019) and AR promotes ligand independent prostate cancer progression through C-myc upregulation (Lina et al. 2013). Likewise, in molecular apocrine breast cancer, the androgen signaling pathway was found to upregulate the activity of myc (Keely et al. 2014). In harmony with our NF-κB data there was a significant downregulation of C-myc protein following treatment with EPI-001 but not Enzalutamide. This indicates that EPI-001 may regulate EMT, partly through inhibition of NF-κB /C-myc pathway.

Our data also indicated a marked decline in the angiogenic marker VEGF following treatment by Enzalutamide and EPI-001. Angiogenic cytokines such as VEGF have been identified in prostatic tumors and the AR is significantly associated with vascular endothelial growth through processes involving SP-1 and HIF-a (Jane et al. 2005; Kurtis et al. 2017). The stimulating effect of androgen on VEGF is significant and there is convincing evidence that part of the antitumor effect of antiandrogen therapy is mediated by its downregulatory effect on VEGF (Woodward et al. 2005). Moreover, it has been suggested that alterations in cellular pathways related to both endocrine and VEGF may contribute to breast cancer progression through EMT (Qian et al. 2013; Minna et al. 2016). Therefore, in the present study, it's possible that the tested blockers modulated EMT by reducing VEGF levels.

MMPs are proteolytic enzymes that degrade the ECM (Masoud et al. 2019) and are implicated in angiogenesis (Saray et al., 2019), invasion and metastasis in various tumors including breast cancer (Duffy et al. 2000). Moreover, elevated levels of MMPs in ECM significantly increase EMT (Cristian et al. 2019). Therefore, MMPs represent targets for cancer therapy (Arthur et al. 2018). It was demonstrated that expression of MMPs is associated with the presence of AR in epithelial ovarian tumors, hepatocellular carcinoma and prostate cancer. The presence of AR in these tumor types was a risk factor for overall survival or predictive of invasion and metastasis. Also, the regulation of MMPs in prostate cancer was abolished by androgen inhibitor Bicalutamide (Flavia et al. 2020; Yan et al. 2015; See-Tong et al. 2004). Moreover, AR has been correlated with the expression of some MMPs and TIMP-2 (Luis et al. 2008). Indeed, in the current study, there was a marked reduction of the levels of MMP2 and MMP9 in T47D cells exhibited by the two blockers Enzalutamide and EPI-001.

The Rho/ROCK are AGC family serine threonine kinases. Previous data indicate that Rho/ROCK signaling, which leads to cytoskeletal rearrangement, plays a central role in breast cancer cells and their disrupted tissue architecture. Therapy targeting the ROCK signaling cascade may provide a therapeutic opportunity for breast cancer (Masahiro and Mina 2016). ROCK pathway has been involved in regulating EMT in renal cell carcinoma by affecting the cytoskeleton during EMT reversal to stabilize the epithelial structure (Shreyas et al. 2009). Also, SHROOM, which is a key mediator of ROCK pathway, was found to be a potent antagonist for EMT in nasopharyngeal carcinoma cells (Jing et al. 2019). Links have been reported between androgens, AR and ROCK pathways: Expression of ROCK1 and ROCK2 are linked to AR

expression, androgens induce activation of RhoA and its translocation into the plasma membrane, RhoA acts as a direct AR targeted gene, and Rho signaling has been implicated in functional activation of AR (Wen et al. 2013; Kroiss et al. 2015). It was reported that androgens alter the architecture of cytoskeleton in T47D breast cancer

cell and promote cell migration and invasion through modulation of meosin (Maria et al. 2016). Consequently, in the present work, the effect of AR inhibitors Enzalutamide and EPI-001 on the protein levels of ROCK1 and ROCK2 in T47D cells were investigated. The results showed that both inhibitors reduced the protein expression of the two markers which may be reflected on invasion and metastasis of the malignant cells.

To sum up, our study indicated that blocking AR/ARV7 by Enzalutamide and/or EPI-001 impacted the cell cycle by downregulating cell cycle regulatory genes CDK4, CDK6, Cyclin E and inducing S-phase cell cycle arrest. Most importantly, there was decrease in metastasis by modulating key biomarkers and proteins involved in regulating the ECM and remodeling of EMT. Thus, we suggest that blocking AR/ARV7 may have beneficial antimetastatic effect in ER + breast cancer subtype.

Declarations

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Competing interests

The authors declare no competing interests.

Author contribution

All authors agreed to the study conception and design and read and approved the final manuscript. The authors declare that all data were generated in-house and that no paper mill was used.

BAM: methodology, analysis, investigation, drafting

HSE: design, supervision, review

GM: methodology

MS: methodolgy

SS: design, data analysis, supervision, review

MK; design, supervision, methodology, data analysis, drafting and revision

Ethics approval

Not applicable

Consent to participate

Not applicable.

Competing interests

The authors declare no competing interests

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Figures

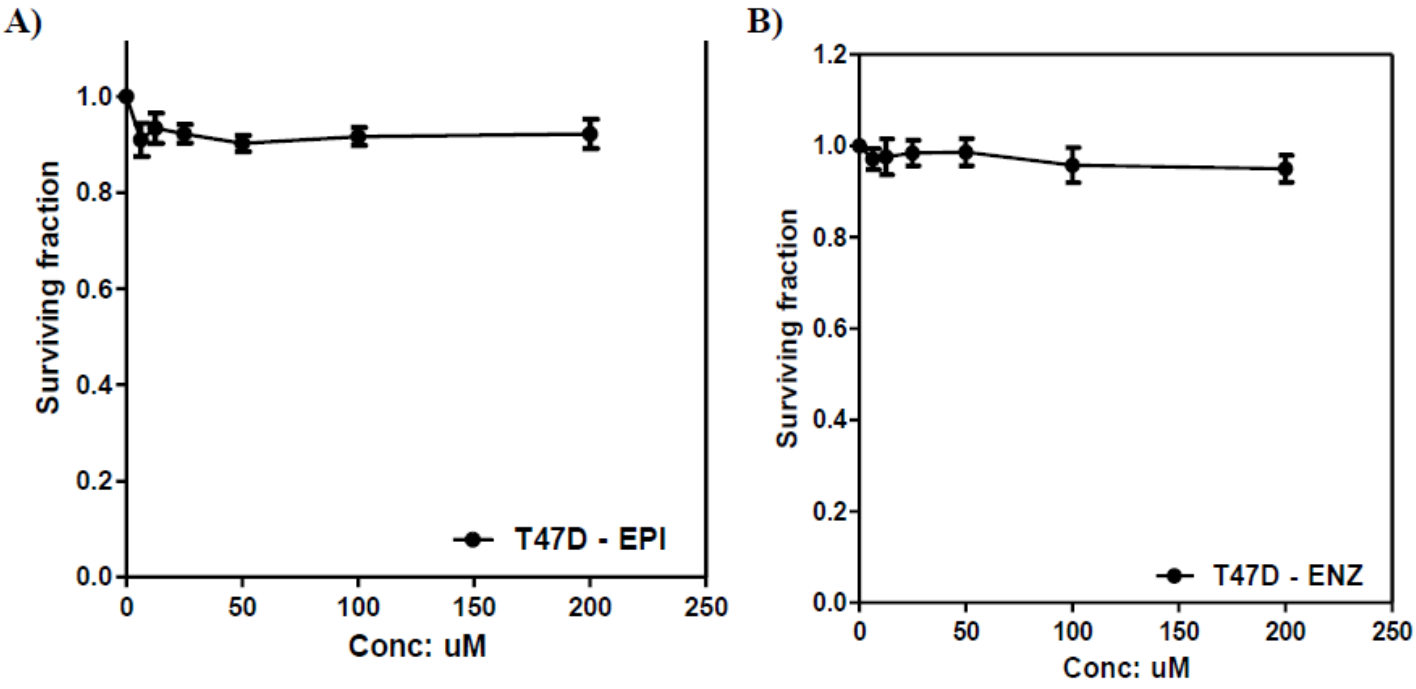

Figure 1

Effect of A) Enzalutamide and B) EPI-001 on the survival of T47D cells measured by SRB assay: Data analysis was performed using GraphPad InStat, version 5.0. The represented values indicated the mean of three independent experiments ± S.E.M.

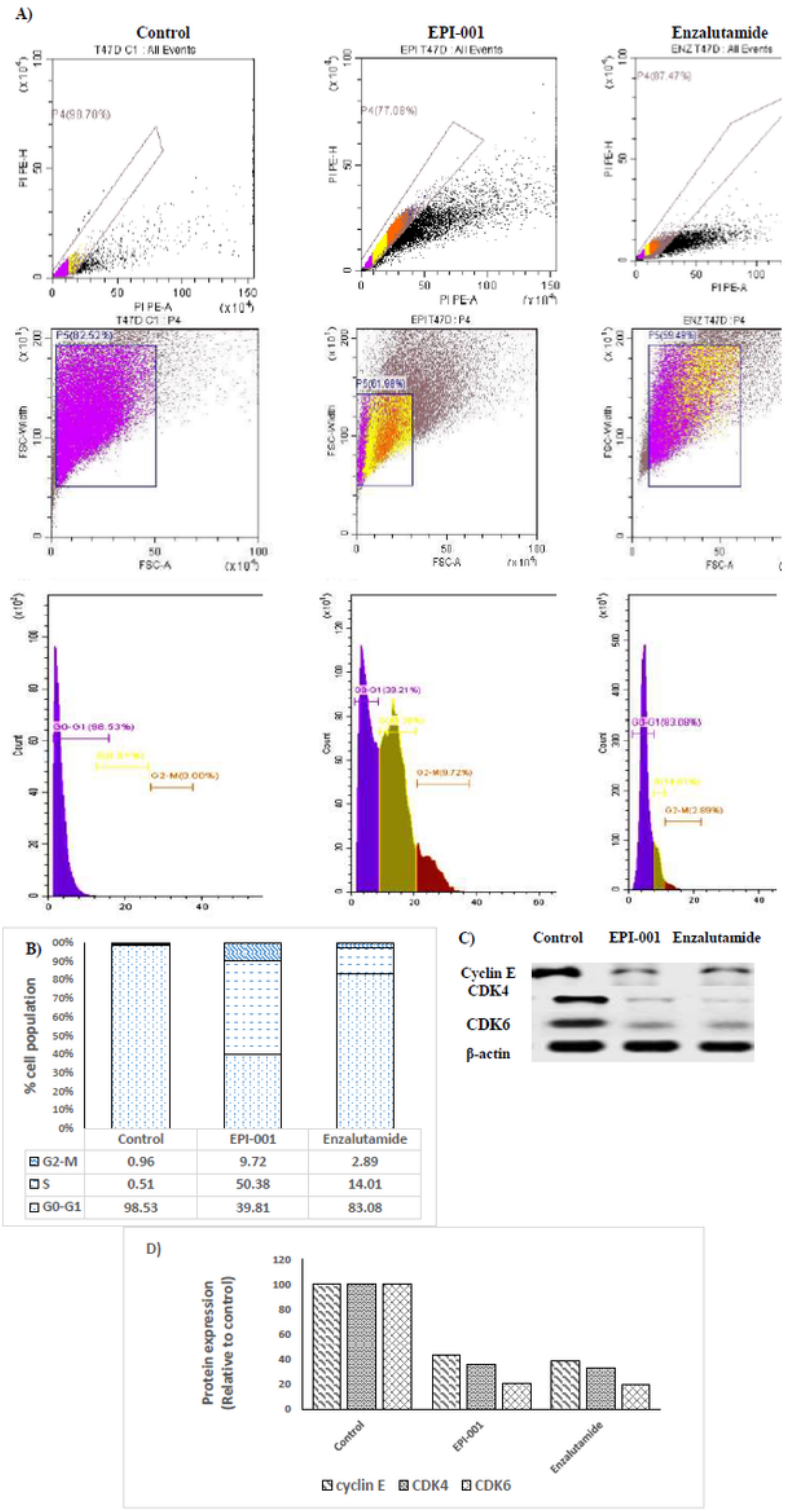

Figure 2

Effect of EPI-001 and Enzalutamide on cell cycle in T47D cells A) Flow cytometry data analysis B) Graphical representation of cell population percentage at each cell cycle stage. C) Western blot analysis of CDK4, CDK6 and Cyclin E in different treatment groups. D) Histogram demonstrating CDK4, CDK6 and Cyclin E expressions relative to control in different treatment groups. The bands intensities were

normalized against β-actin and analyzed by ChemiDoc MP imager. Enzalutamide was used at concentration of 10 uM while EPI-001 was used at concentration of 50 uM.

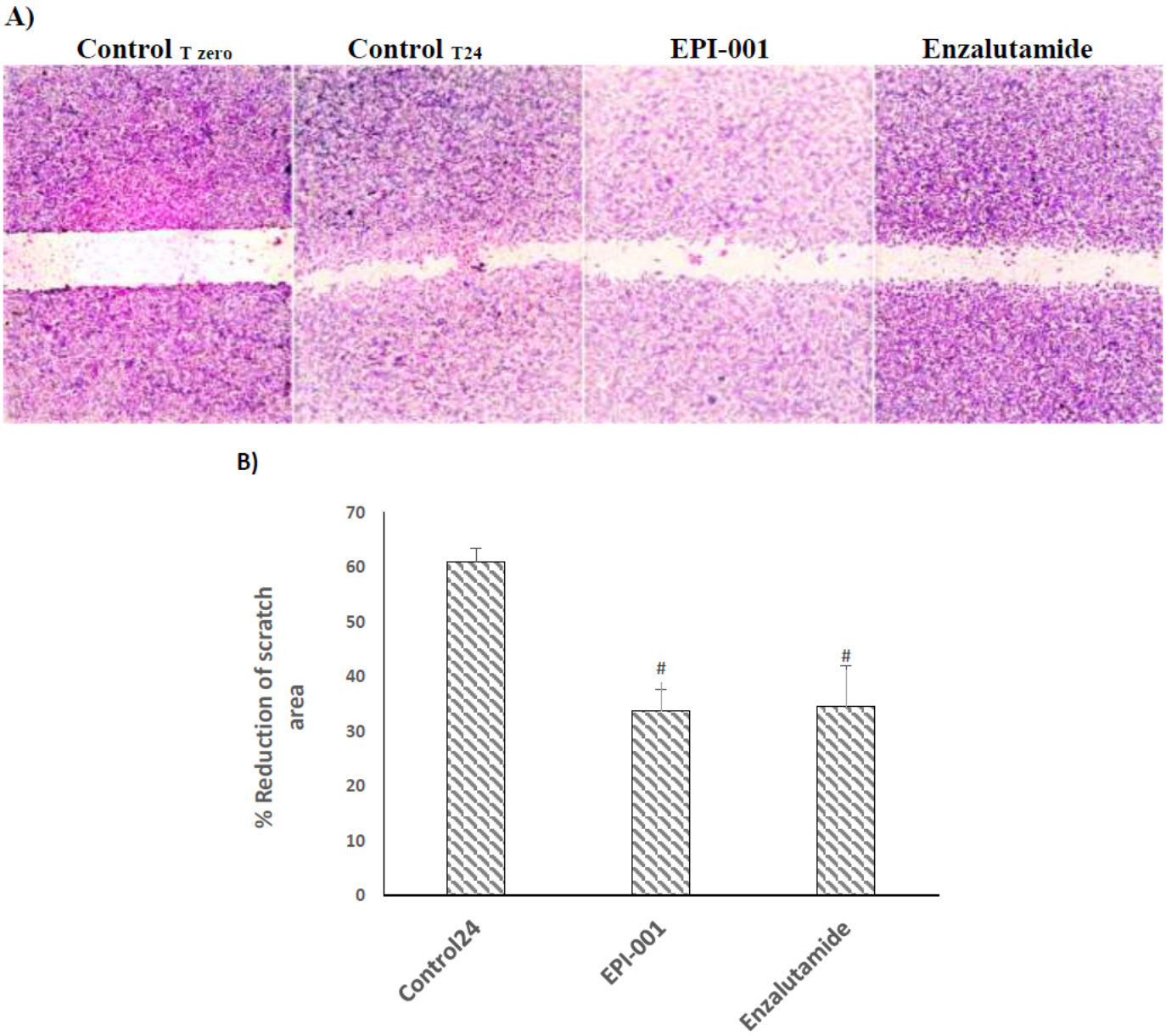

Figure 3

Effect of Enzalutamide and EPI-001 on metastasis in T47D as indicated by Scratch wound healing assay). A) Images of different treatment wheels and control after 24 hr. The white area represents the scratch area. B) Histogram demonstrating the percentage reduction of scratch area induced by Enzalutamide and EPI-001. Six representative images of scratch area for each treatment well was analyzed using ImageJ software and the values of gap area reduction were expressed as mean ± S.E.M. statistical significance of the results was analyzed using ANOVA followed by Tukey as a post hoc test. #

Significantly different from control 24 group ($p \leq 0.05$). EPI-001 was used at concentration of 50 uM while Enzalutamide was used at concentration of 10 uM.

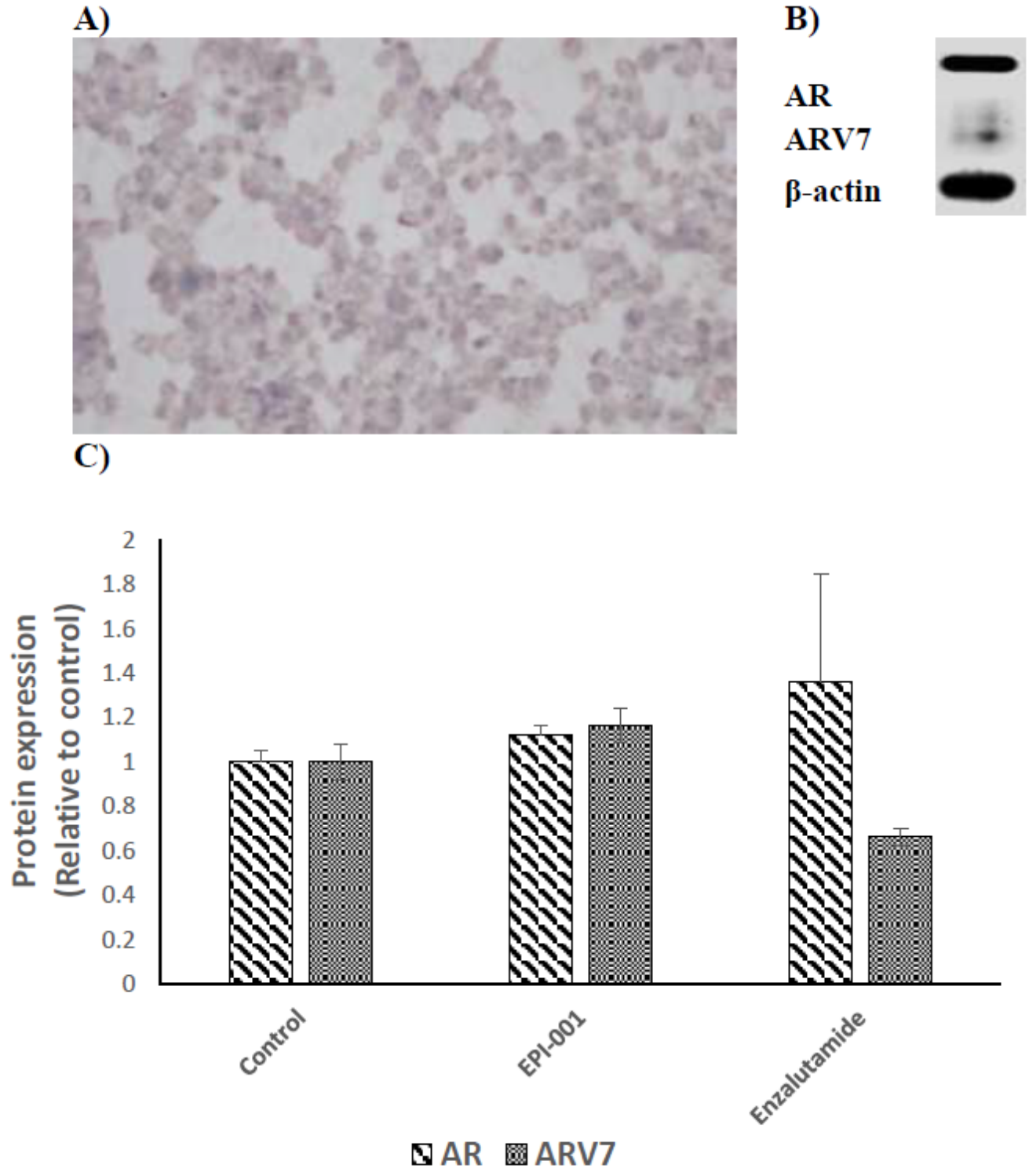

Figure 4

Evaluation of AR and ARV7 expression and blockers effect on its protein level A) Immunocytochemistry showing ARV7 positive nuclear expression. B) Western blot analysis showing the expression of AR and ARV7 protein levels in T47D cell line. C) Histogram demonstrating the effect of Enzalutamide and EPI-001 on AR and ARV7 level as measured by ELISA. The data of ELISA represent the mean value of two independent experiments ± S.E.M. Statistical significance of the results was analyzed using ANOVA followed by Tukey as a post hoc test. EPI-001 was used at concentration of 50 uM while Enzalutamide was used at concentration of 10 uM.

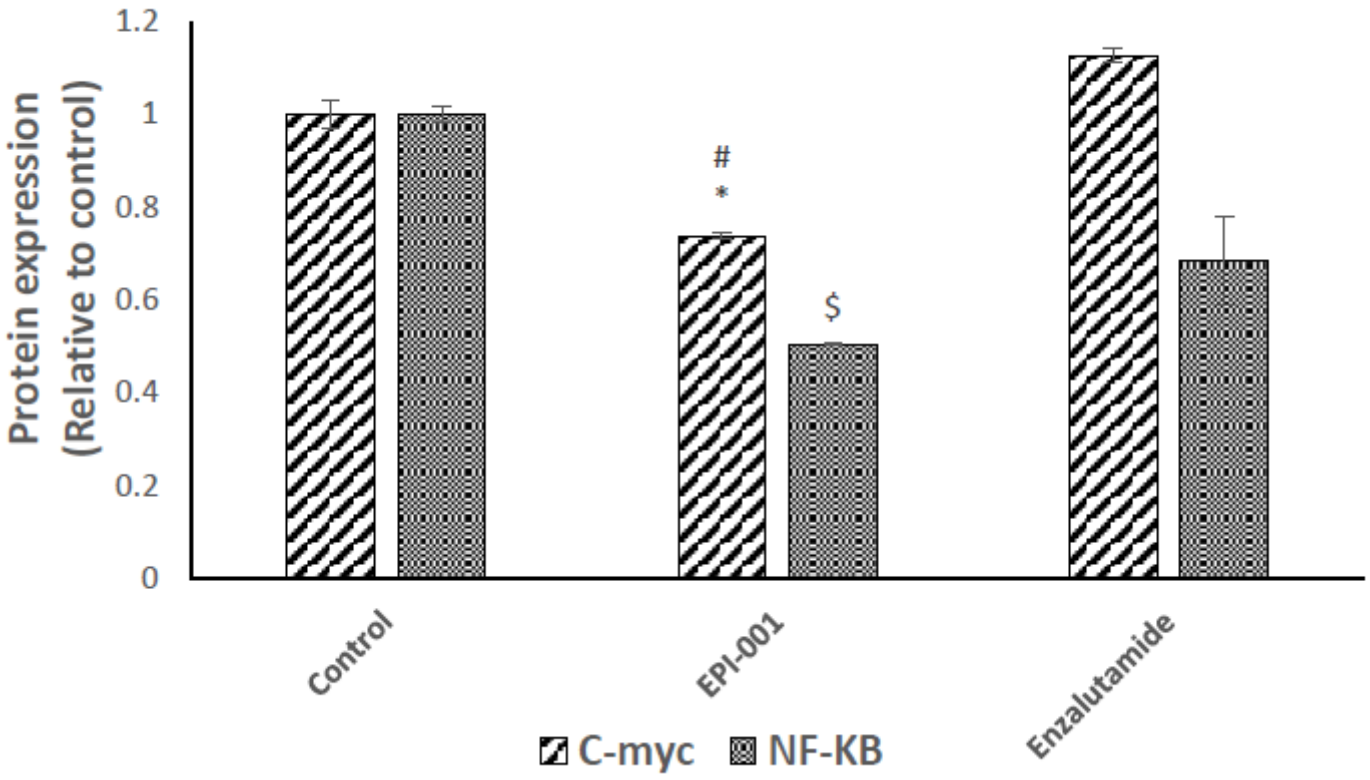

Figure 5

Effect of EPI-001 and Enzalutamide on C-myc and NF-κB expression levels in T47D cell line as indicated by ELISA assay: The data represent the mean value of at least two independent experiments ± S.E.M. Statistical significance of the results was analyzed using ANOVA followed by Tukey as a post hoc test. $^{\#}$ Significantly different from C-myc control group ($p \leq 0.05$). * Significantly different from C-myc Enzalutamide group ($p \leq 0.05$). $^{\$}$ Significantly different from NF-KB control group ($p \leq 0.05$). EPI-001 was used at concentration of 50 uM while Enzalutamide was used at concentration of 10 uM.

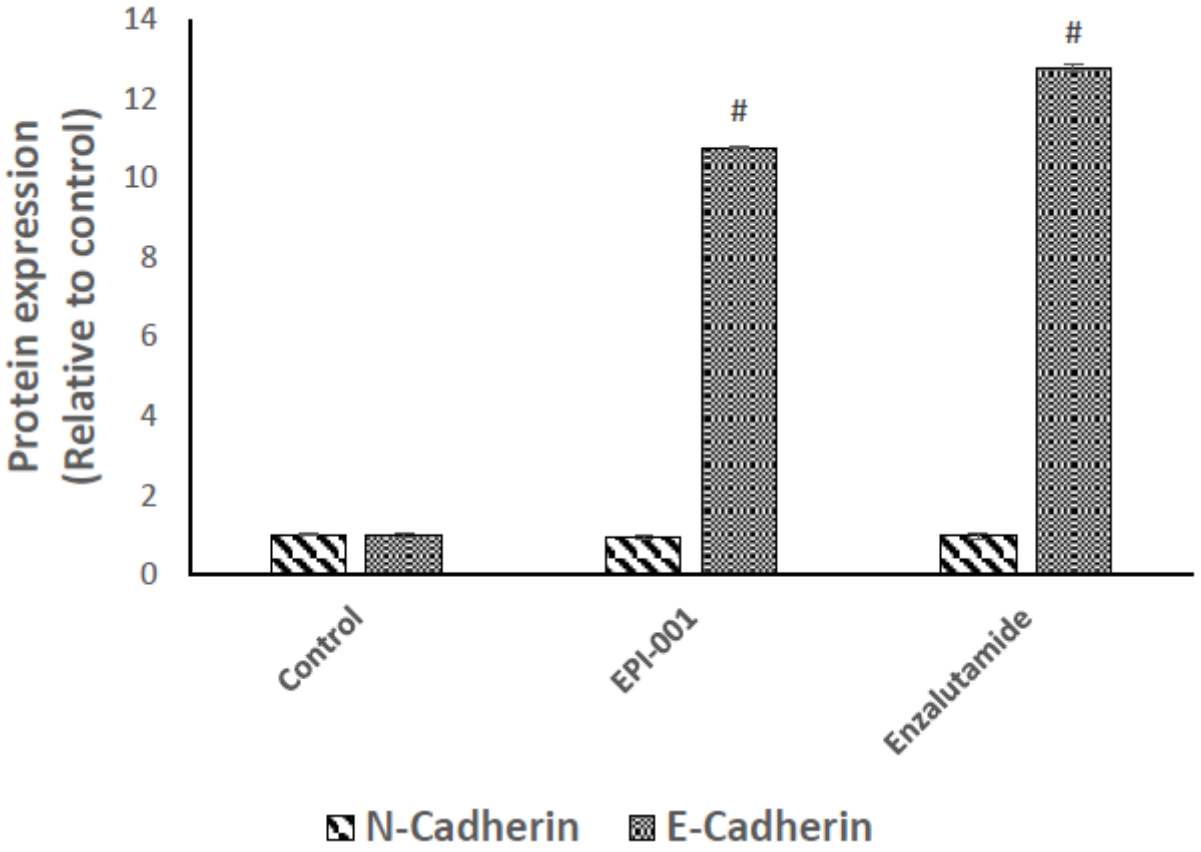

Figure 6

Effect of EPI-001 and Enzalutamide on N-Cadherin and E-Cadherin in different treatment groups as indicated by ELISA assay: The data represent the mean value of at least two independent experiments ± S.E.M. Statistical significance of the results was analyzed using ANOVA followed by Tukey as a post hoc test. # Significantly different from E-Cadherin control group ($p \leq 0.05$). EPI-001 was used at concentration of 50 uM while Enzalutamide was used at concentration of 10 uM.

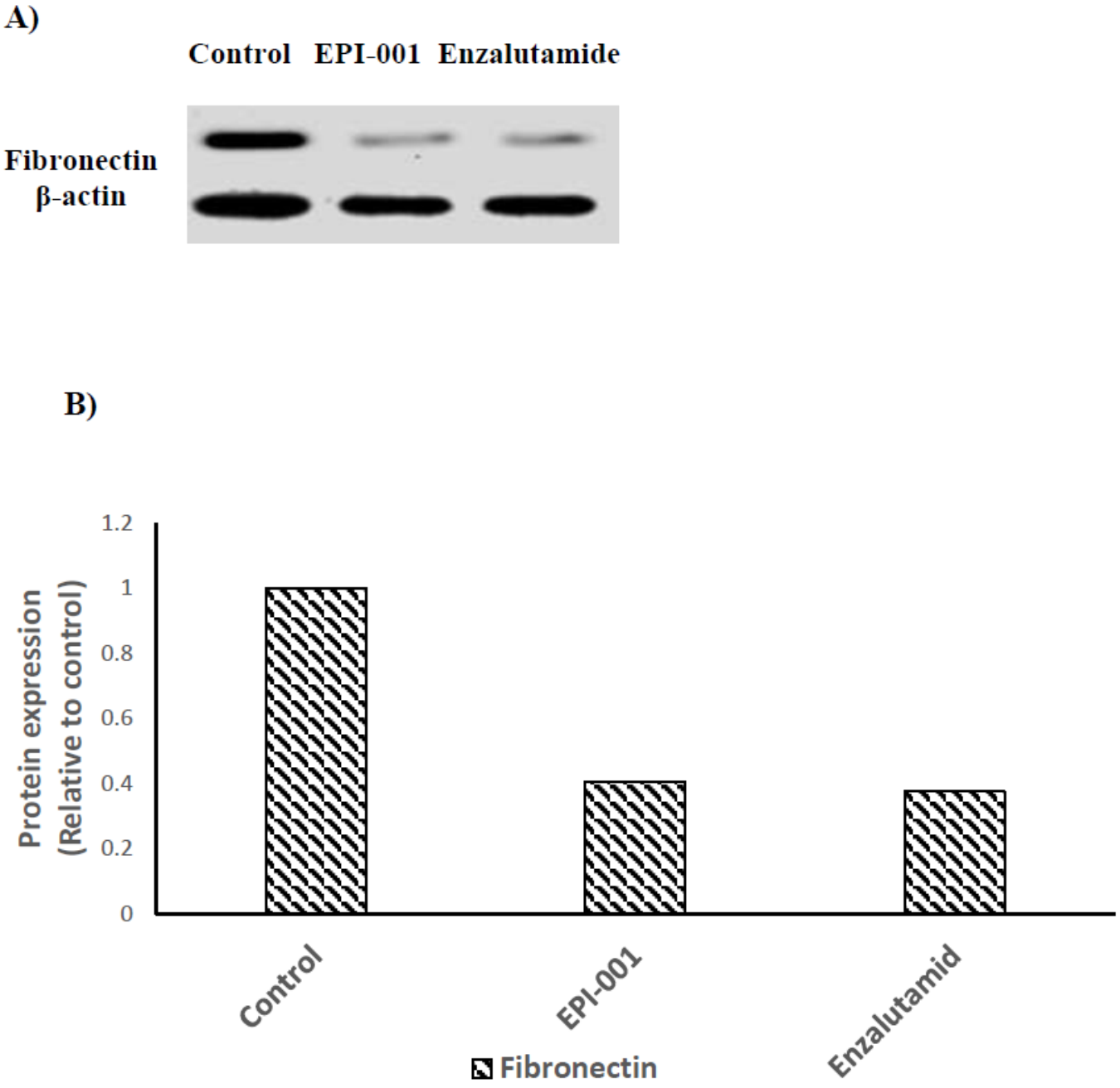

Figure 7

Effect of EPI-001 and Enzalutamide on Fibronectin in different treatment groups: A) Western blot bands of Fibronectin in different treatment groups. B) Histogram demonstrating Fibronectin expression relative to control in different treatment groups. The bands intensities were normalized against β-actin and analyzed by ChemiDoc MP imager. EPI-001 was used at concentration of 50 uM while Enzalutamide was used at concentration of 10 uM.

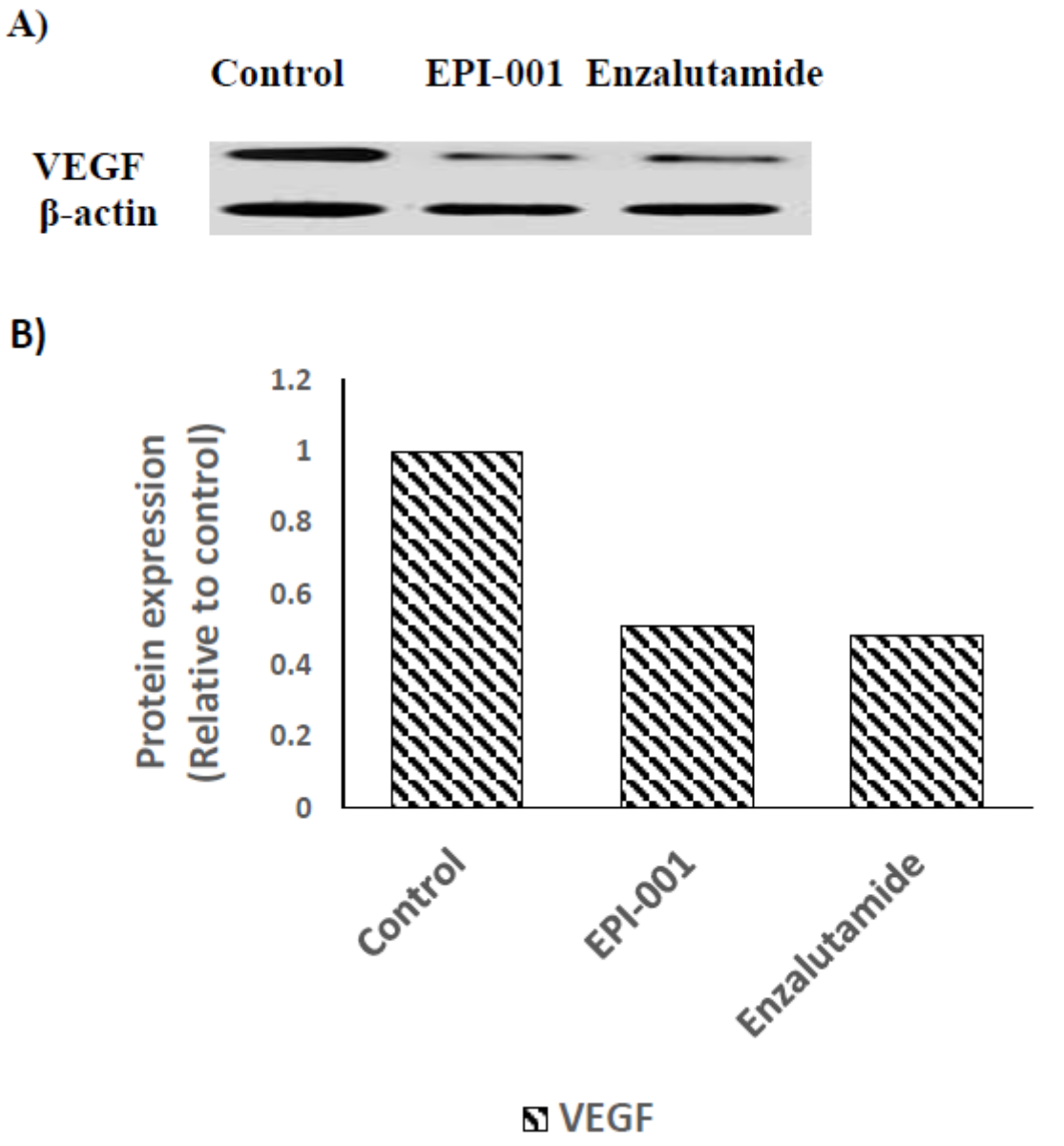

Figure 8

Effect of EPI-001 and Enzalutamide on VEGF level in T47D cell line: A) Western blot bands of VEGF in different treatment groups. B) Histogram demonstrating the VEGF expression relative to control in different treatment groups. The bands intensities were normalized against β-actin and analyzed by ChemiDoc MP imager. EPI-001 was used at concentration of 50 uM while Enzalutamide was used at concentration of 10 uM.

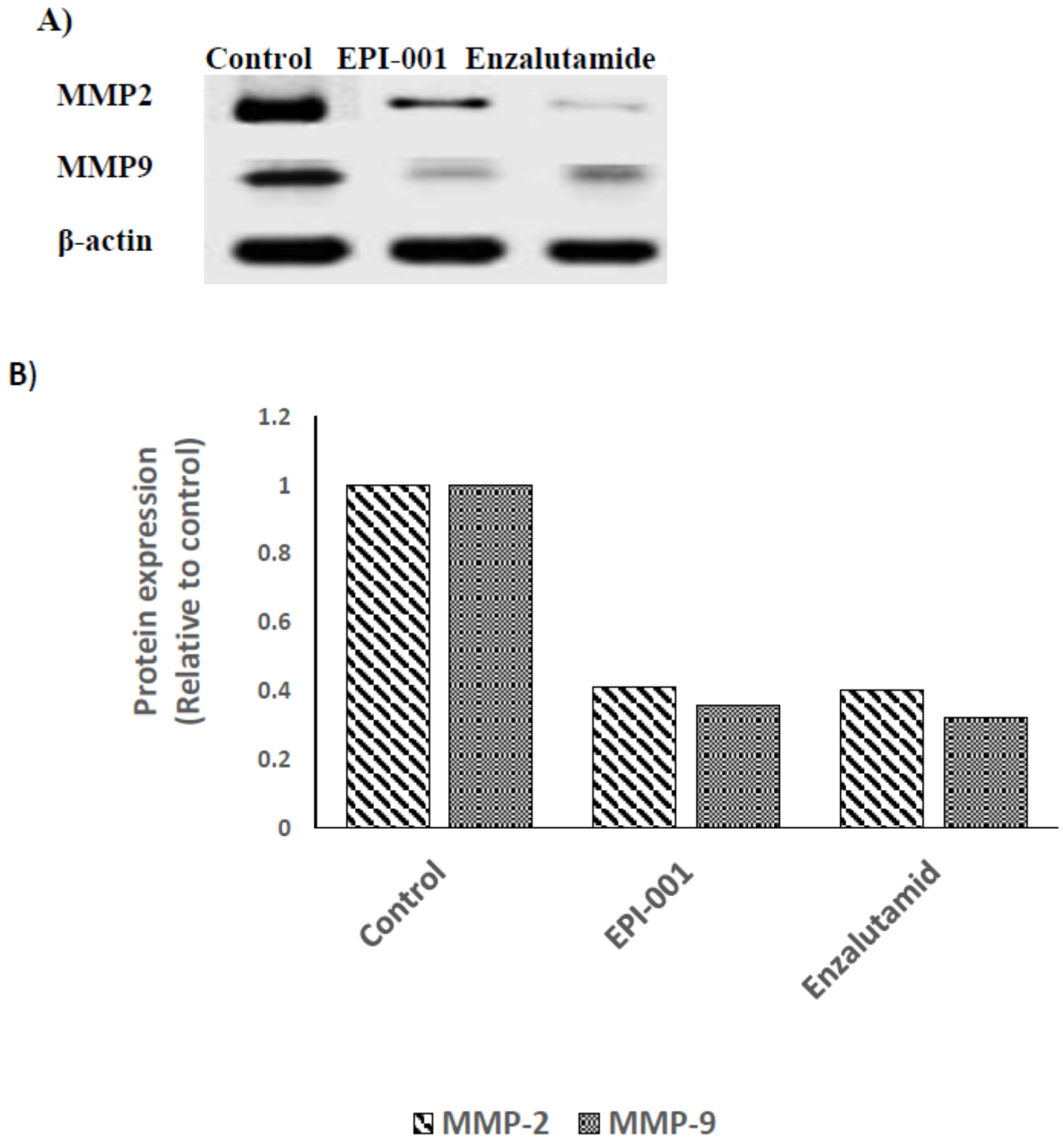

Figure 9

Effect of EPI-001 and Enzalutamide on MMPs 2 and 9 in different treatment groups: A) Western blot bands of MMP2 and 9 in different treatment groups. B) Histogram demonstrates MMP2 and 9 expressions relative to control in different treatment groups. The bands intensities were normalized against β-actin and analyzed by ChemiDoc MP imager. EPI-001 was used at concentration of 50 UM while Enzalutamide was used at concentration of 10 uM.

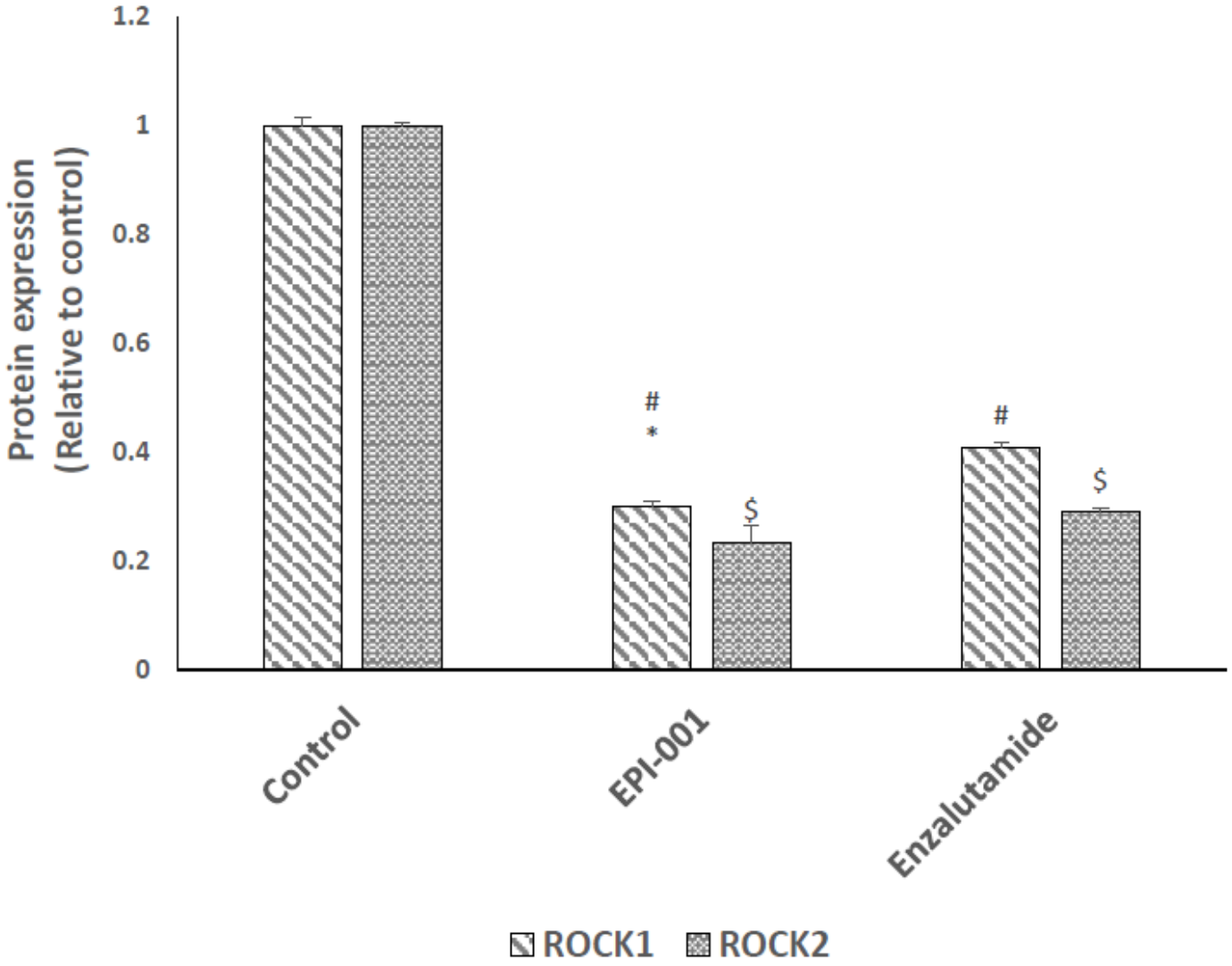

Figure 10

Effect of EPI-001 and Enzalutamide on ROCK 1 and 2 levels in T47D cell line as indicated by ELISA assay. The data represent the mean value of two independent experiments ± S.E.M. statistical significance of the results was analyzed using one way ANOVA followed by Tukey as a post hoc test. # Significantly different from ROCK 1 control group ($p \leq 0.05$). * Significantly different from ROCK 1 Enzalutamide group ($p \leq 0.05$). $ Significantly different from ROCK 2 control group ($p \leq 0.05$). EPI-001 was used at concentration of 50 UM while Enzalutamide was used at concentration of 10 uM.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- 1Sulphorhodamineassay.pdf

- 2Woundhealingassay.xlsx
- 3ELISAcaliberationcurves.pdf
- 4ELISArawdata.xlsx
- 5ROCK1data.xlsx
- 6ROCK2data.xlsx
- 7Westernblot.pdf
- 8opticaldensitiesofWesternblot.xlsx