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Enzalutamide and EPI-001 Modulate Cell Proliferation and Metastasis Markers in T47D By Targeting AR/ARV7

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ABSTRACT

Androgen receptor (AR) and its splicing variant 7 (ARv7) play vital roles in the pathobiology of breast cancer (BC) but their roles in the estrogen receptor-positive (ER+) type is controversial. Hence, we studied the influence of the blockers of AR (Enzalutamide) and ARv7 (EPI-001) on tumorigenesis processes using T47D, an ER+ BC cell line. Several techniques were employed: Sulphorhodamine assay (SRB), Flow cytometry, Immunostaining, Scratch wound healing assay, Enzyme Linked Immunosorbent assay (ELISA), and Western blot. Mechanistically, the drugs caused cell cycle arrest at S-phase and downregulated the protein expression of cyclins A, E, and C. Additionally, they inhibited the cell proliferation stimulator nuclear factor kappa B (NF-κB), whereas only EPI-001 reduced the cell regulatory marker c-Myc. They also opposed the endothelial-to-mesenchymal transition (EMT) process, by boosting the epithelial marker E-Cadherin and reducing the protein expression of the mesenchymal marker fibronectin. Their anti-metastatic potential was evidenced by the hindrance of cell migration using the wound healing assay and further confirmed by the downregulation of metalloproteinase (MMP) 2 and 9 protein expression, and protein content of Rho kinase (ROCK) 1 and 2. Besides, by downregulating the protein expression of vascular endothelial growth factor (VEGF) the drugs point to their anti-angiogenic aptitude. In conclusion, the current investigation highlights the importance of targeting AR/ARv7 via Enzalutamide and EPI-001 in reducing proliferating cell markers, EMT and metastasis in ER+ BC cells, findings that may have great impact in the treatment of ER+ BC.

Keywords: Androgen receptor, Androgen receptor variant 7, metastasis, Epithelial to mesenchymal transition, breast cancer

INTRODUCTION

Androgen receptor (AR) is a vital element in breast cancer (BC) pathogenesis, the most common type of women cancer (You *et al*., 2022). AR expression is evident in more than 60% of BCs and around 90% of estrogen receptor (ER)+ tumors, indicating possible responsiveness to androgen (Li *et al*., 2016), however this role is controversial and still far from clarity which restrains the application of AR-directed therapies.

AR undertakes a tumor suppressor part in ER+ BC (Hickey *et al*., 2021) by binding to a group of estrogen response elements, thereby preventing the stimulation of target genes that facilitate the effects of 17-β-estradiol on BC cells (Peters *et al*., 2009). However, blocking AR hindered the estradiol-induced proliferation of an ER+/AR+ cell line model (Cochrane *et al*., 2014) and suppressed the proliferation of MCF7 cells (Yeh *et al*., 2003). In the same context, constitutively active AR

sustained the metastasis of resistant hormone receptor positive BC (Bahnassy *et al*., 2020). Moreover, some researchers suggest the oncogenic impact of AR and its potential role in the acquired resistance against ER+ treatments for BC (Hu *et al*., 2011). Thus, sustaining the balance between AR and estrogen receptor pathways to acquire an optimum therapy necessitates significant considerations for targeting AR in the management of BC (Chan-Ping *et al*, 2022).

The controversial AR role has also been described for other BC subtypes. Indeed, while some reports showed that AR expression in HER2+ BC was linked with a worse prognosis (Micello *et al*., 2010); others found that overall survival improved in case of the expression of AR mRNA (Bozovic-Spasojevic *et al*., 2017; Doane *et al*., 2006). The contradiction for AR role was also extended toward the Triple negative breast cancer (TNBC) subtype where AR was associated with lower histological grade and clinical stage (Mrklic *et al*., 2013; Ogawa *et al*., 2008). Other studies however, demonstrated that deficiency of AR increased the risk of TNBC metastasis and recurrence (Rakha *et al*., 2007; Sutton *et al*., 2012). Interestingly, AR targeting therapies combined with other treatments, such as tamoxifen in ER+ BC or radiation therapy in TNBC, have presented encouraging results in several clinical trials. Harnessing the AR targeting full potential requires a better knowledge of the role of AR in each BC subtype (Emily *et al*, 2022).

The AR variants arise owing to rearrangements or splicing of the AR gene. They have several structures, but each is deficient in all or a part of the ligand-binding domain. This produces constitutively active, ligand independent variants resistant to drugs that decrease androgen biosynthesis or production (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 (Wang *et al*., 2020). Moreover, their role in BC has been recognized and is considered an area of active research (Ferguson *et al*., 2022).

The role of aberrant expression of AR/ARv7 in the progression of different cancers, including breast (Ferguson *et al*., 2022) and prostate (Sharp *et al*., 2019) cancers, has been evaluated by endorsing tumor cell growth, however, the impact of AR/ARv7 on cell proliferation, epithelial to mesenchymal transition (EMT) process and metastasis was scarcely appraised. It's noteworthy that EMT is involved in carcinogenesis and provides metastatic properties to cancer cells (Sistigu *et al*., 2017) and the EMT-derived tumor cells attain stem cell characteristics and display a noticeable therapeutic resistance (Yin *et al*., 2017).

Accordingly, we evaluated the effects of AR and ARv7 blockers using Enzalutamide and EPI-001, respectively, on cell cycle, EMT, and metastasis markers using T47D (ER+ BC) cell line. At a mechanistic level, we proposed that blocking AR/ARV7 might modulate proliferation and EMT related pathways via inhibiting [nuclear factor kappa B (NF-κb)/ c-Myc/ Rho kinase (ROCK) hub] (Holmes *et al*., 2022; Yin, *et al*., 2017) which consequently influence [c-Myc/ vascular endothelial growth factor (VEGF) / matrix metalloproteinases (MMPs) trajectory] (Najafi *et al*., 2019), ROCK/cadherin and fibronectin axis (de Toledo *et al*., 2012; Zhu *et al*., 2013) or ROCK/cyclin pathways (Kumper *et al*., 2016). Thereby, we paid special attention to these markers aiming to uncover some of the mechanisms by which AR and ARv7 blockers may potentially influence ER+ BC.

MATERIALS AND METHODS

Cell culture

T47D cells were attained from the American Type Culture Collection (ATCC, MN, USA) and were routinely checked and maintained at the tissue culture facility of National Cancer Institute, Cairo University. The cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum (FCS), L-glutamine (2 mM), sodium bicarbonate (1.5 g/L), and penicillin/streptomycin (1%) and kept in a humid atmosphere with 5% CO2 at 37°C.

Ethical statement

The present research was conducted on T47D cell line. The methods comply with pertinent guidelines and regulations and the protocol was approved by the Committee for the Safe Handling and Disposal of Chemical and Biological Substances affiliated with the Research Ethics Committee of the Faculty of Pharmacy at Cairo University (Cairo, Egypt; PT 2126).

Sulphorhodamine-B (SRB) assay

The antitumor activity of Enzalutamide and EPI-00 (ApexBio, TX, USA) on T47D cells was evaluated by SRB assay. Cells were seeded for 24h in a 96-well plate at a density of 3×10^3 cells per well and were treated with different concentrations

(6.25, 12.5, 25, 50, 100, and 200 μM) of Enzalutamide or EpI-001. Drugs were dissolved in dimethylsulfoxide to prepare a stock solution of 1 mM. For each concentration, two wells were used to be incubated for 48 hr, 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 an ELISA microplate reader (TECAN sunrise, Germany). The cell survival fraction was calculated by dividing the O.D. of treated cells over that of control cells.

For 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. These doses were chosen based on previous reports (Brand *et al*., 2015; Cochrane *et al*., 2014).

Immunocytostaining of ARv7

Cells were collected and washed with PBS, resuspended in DMEM, and incubated in H_2O_2 for 15 min over a glass slide, then washed again using PBS to be incubated with a monoclonal antibody against ARv7 for an hr. Afterwards, biotinylated secondary antibody was added, kept at room temperature for 10 min, then washed with PBS, and 3,3′-Diaminobenzidine (DAB) was dropped and incubated for 5 min. followed by counterstaining the slides with hematoxylin. The ARv7 expression was demonstrated by brown nuclear color.

Determination of cell cycle phase using flowcytometry

Treated and control cells were collected after 24h and fixed with 70% cold ethanol, washed and the pellets were resuspended in trypsin buffer and left for 10 min. Afterwards, trypsin inhibitor and 1% RNase buffer were added and incubated for 10 min. Propidium iodide (100 g/ml) was then added and the samples were kept in dark for 30 min. at 4°C and the distribution of each cell cycle phase was then determined using an EPICS® C Flow cytometer (FLA, USA).

Scratch wound healing assay

The cells were seeded in six well plates and allowed to adhere. The formed monolayer was then slowly scratched with a 1 ml pipette tip through each well center in one direction. The wells were washed and treated with Enzalutamide or EPI-001 for 24h to be fixed with 3.7 % paraformaldehyde for 30 min. Finally, the cells were stained with 1 % crystal violet for 30 min. The stained monolayer was photographed and the reduction in gap area was measured (ImageJ software, MD, USA).

Assessment of protein content of AR, ARv7, NF-κB, c-Myc, E-Cadherin, N-Cadherin, ROCK1, and ROCK2 using the ELISA technique

The ELISA kits were obtained from Innova Biotectech (BJ, China) for AR (cat #: In-Hu4116), ARv7 (cat #: In-Hu4117), c-Myc (cat #: In-Hu1853), NF-κB (cat #: In-Hu2637), E-Cadherin (cat #: In-Hu1892), and N-Cadherin (cat #: In-Hu4118), whereas for ROCK1 (Catalog #: OKEH06554) and ROCK2 (catalog #: LS-F22011) the ELISA kits were purchased from Aviva Systems Biology, (LA, USA) and LifeSpan BioSciences, (WA, USA), respectively. The cells were harvested, and their protein was quantified using Bradford assay kit (Pierce, Rockford, IL, USA). ELISA quantification was carried out in accordance with the manufacturer's instructions and the protein contents were calculated from the constructed calibration curves using second-order polynomial curve-fitting models.

Determination of AR, ARv7, cyclins A, C, and E, fibronectin, MMP2 and 9, and VEGF protein expression using Western blot analysis

The primary antibodies used for AR (cat #: sc-7305), cyclins A (cat #: sc-271682), cyclin E (cat #: sc-377100), fibronectin (cat #: sc-8422), matrix metalloproteinase (MMP)2 (cat #: sc-13595), MMP9 (cat #: sc-393859), and VEGF (cat #: sc-7269) were purchased from Santa Cruz Biotechnology, (TX, USA), whereas those for cyclin C (catalog #: 26464-1-AP) and ARv7 (catalog #: AG10008) were procured from Proteintech (IL, USA) and A and G Precision Antibody (MD, US), respectively. The Ready PrepTM protein extraction kit (Bio-Rad Inc., CA, USA) was used to extract protein from the pellets obtained from the collected cells and protein analysis was conducted using the Bradford assay kit. The extracted proteins were separated by SDS-PAGE using TGX Stain-Free™ FastCast™ (Bio-Rad Inc., CA, USA) and blotted onto PVDF membranes that were blocked with 5% non-fat dry milk and incubated at 4°C with primary antibodies overnight followed by incubation with peroxidase-conjugated secondary antibodies. The β-actin antibody was probed in the membrane for normalization**.** The intensities of the bands were normalized against β-actin and were analyzed on the ChemiDoc MP imager.

Statistical analysis

The data were presented as mean ± S.E.M and statistically analyzed using one-way analysis of variance (ANOVA) followed by Tukey's post hoc test ($p \leq 0.05$) and F values were expressed to indicate the ratio between and within group variances using IBM SPSS Statistics for Windows, Version 26.0 (NY, USA). Sketching of graphs was done using GraphPad InStat, version 5.0 (LA, USA).

RESULTS AND DISCUSSION

Although the expression of AR in BC has appointed it an attractive therapeutic target, its role in ER+ type is still controversial since it can either inhibit or promote breast tumor growth and metastasis (Hickey *et al*., 2021; Li *et al*., 2013). Among the AR variants, ARv7 is drawing attention as a prospective marker for one of the resistance mechanisms to anti-AR therapy in castration-resistant prostate cancer patients (CRPC) (Sharp *et al*., 2019) and has also been recently detected in BC subtypes (Ferguson *et al*., 2022). However, the role of both AR and ARv7 in ER+ BC needs further clarification.

It is well documented that the tumorigenesis events of cancer instigation and advancement involve several correlated steps including tumor growth, angiogenesis, neovascularization, invasion, and metastasis. Here, we have evaluated the impact of inhibiting AR and ARv7 by Enzalutamide or EPI-001 on the tumorigenesis multi-steps using T47D, an ER+ BC cell line. The choice of T47D was based on preliminary studies in our lab screening the expression of AR/ARv7 in different ER+ BC cell lines using immunocytochemistry and/or Western blot analysis. T47D displayed more detectable expression and therefore was chosen as our study model. This was endorsed by previously published data which showed ARV7 transcript expression was higher in T47D than MCF7 cell line (Hu *et al*., 2014), The two blockers didn't significantly alter their corresponding receptor protein contents, only Enzalutamide reduced ARV7 (Figure 3 a,b).

Examining the cytotoxicity of the tested drugs, we showed that the two inhibitors didn't significantly affect the cell survival of the ER+ cell line T47D (Figure 1 c,d). Although this was not expected, another study supported our findings, where Caiazza and co-workers (2016) reported that the sensitivity of BC proliferation to treatment with Enzalutamide was widely variable with a marginal effect of Enzalutamide on the T47D cell line (IC50 >50 μM).

Population 56.43% and 24.72% (Figure 2 a and b) of the cell were arrested in the S-phase after treatment with EPI-001 and Enzalutamide, respectively as compared to 4.36% in the untreated control group. Furthermore, they caused (c and d) a sharp decline in cyclins A [$F(2,8)= 163.35$, $p<0.001$], E [$F(2,8)= 352.8$, $p<0.00$)], and C [$F(2,8)= 123.11$, $p<0.001$], compared to the untreated control cells. These results are in harmony with a past study which indicated that the blockade of AR signaling blunted the G1-S cyclins in a resistant BC cell line (Wenfei *et al*., 2019). Although it is not clear how the effect on the cell cycle was not translated into a decrease in cell survival, this can be clarified by the report of Sherr and Roberts (2004), who in their review argued about the necessity of cyclins D and E and their cyclin-dependent kinases (CDKs) in cell survival and stated that despite the disruption of the genes encoding them in the mouse germ line, much of fetal development occurs normally in their absence (Sherr and Roberts, 2004). Other researchers also underlined the existence of an 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 skewed 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 cell cycle (Gerard and Goldbeter, 2016).

On the molecular level, both drugs were able to affect growth-related biomarkers; the first is the transcription factor NF-κB, which is activated in some forms of cancer cells and possesses pleiotropic roles in different aspects of tumorigenesis, such as enhancing cancer-cell proliferation, increasing angiogenesis, and enhancing metastatic potential (Okamoto *et al*.; 2007, Park and Hong, 2016). As depicted in Figure 3c, both EPI-001 and Enzalutamide significantly reduced the transcription factor NF-κB protein expression in the T47D-treated cells. The link between NF-κB and AR was recounted previously, where the promoter region of the AR gene possesses a NF-κB response element and it was established that the activation of the latter raises AR in prostate cancer cells (Zhang *et al*., 2009).

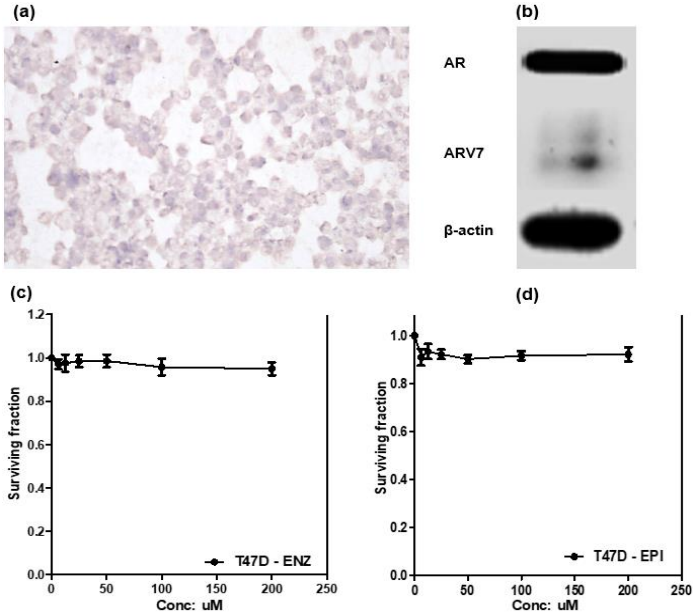

Figure 1. Screening of AR and ARv7 protein expression and determination of IC50 of EPI-001 and Enzalutamide in the T47D cell line (a) Immunocytochemistry showing ARv7 positive nuclear expression (b) Western blot analysis showing the expression of AR and ARv7 protein levels in the T47D cell line (c and d) Effect of EPI-001 and Enzalutamide on the survival of the T47D cells measured by SRB assay after treatment for 48 hr, respectively. Values are indicated as the mean of three independent experiments ± S.E.M and data were plotted using GraphPad InStat, version 5.0. SRB: Sulphorhodamine assay.

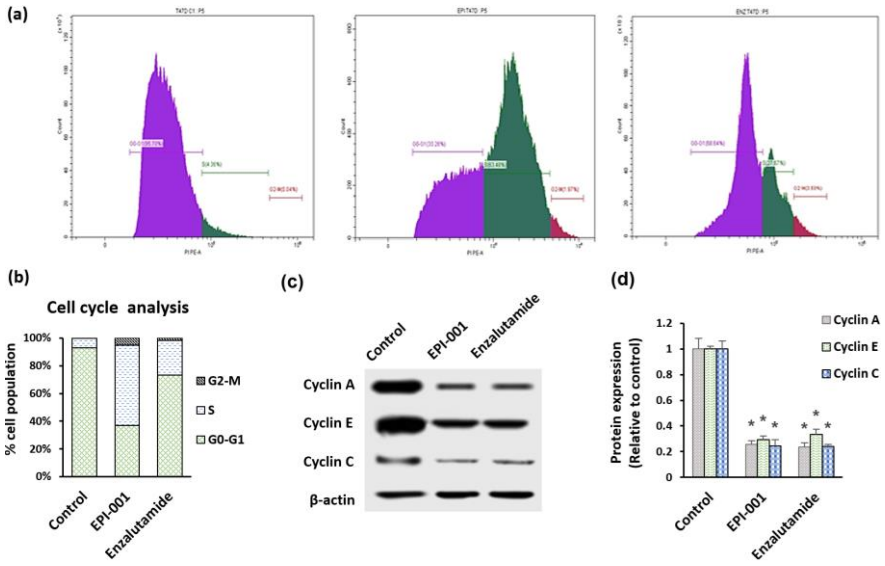

Figure 2. Effect of EPI-001 and Enzalutamide on cell cycle phases and protein expression of cyclins A, E, and C in the T47D cell line (a) Flow cytometry data analysis (b) Graphical representation of cell population percentage at each cell cycle stage (c and d) Effect of EPI-001 and Enzalutamide on protein expression of cyclin A, E, and C. Values are indicated as mean of three independent experiments ± S.E.M. Data were analyzed using one-way ANOVA followed by Tukey's post hoc test. As compared to (*) the corresponding untreated control group ($p \leq 0.05$). EPI-001 was used at a concentration of 50µM while Enzalutamide was used at a concentration of 10µM.

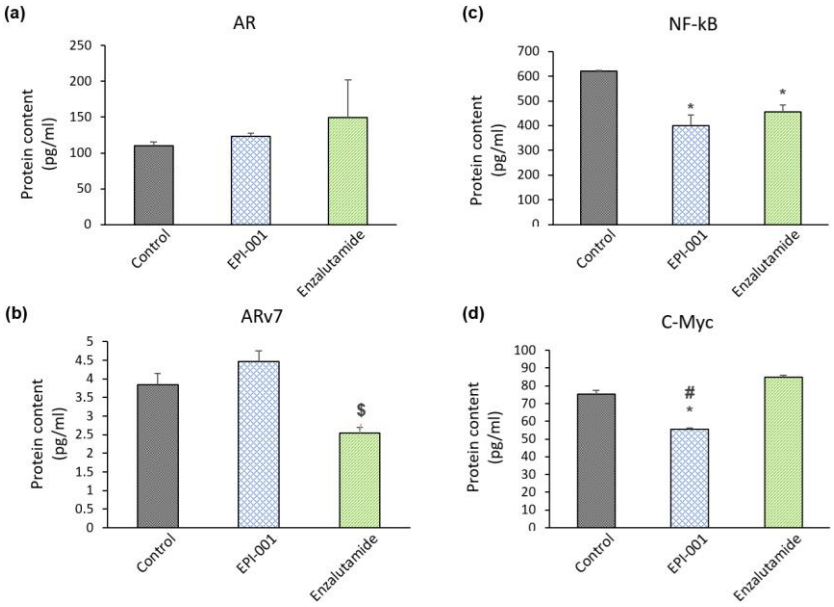

Figure 3. Effect of EPI-001 and Enzalutamide on the protein contents of (a) AR, (b) ARv7, (c) NF-κB, and (d) c-Myc in the T47D cell line. Values are indicated as the mean of four independent experiments ± S.E.M. Data were analyzed using one-way ANOVA followed by Tukey's post hoc test. As compared to (*) the untreated control, and (#) Enzalutamide and ($) EPI-001 treated groups ($p \leq 0.05$). EPI-001 was used at a concentration of 50μM while Enzalutamide was used at a concentration of 10μM. AR: Androgen receptor; ARv7: Androgen receptor variant 7; c-Myc: Cellular myelocytomatosis oncogene; NF-κB: nuclear factor kappa B.

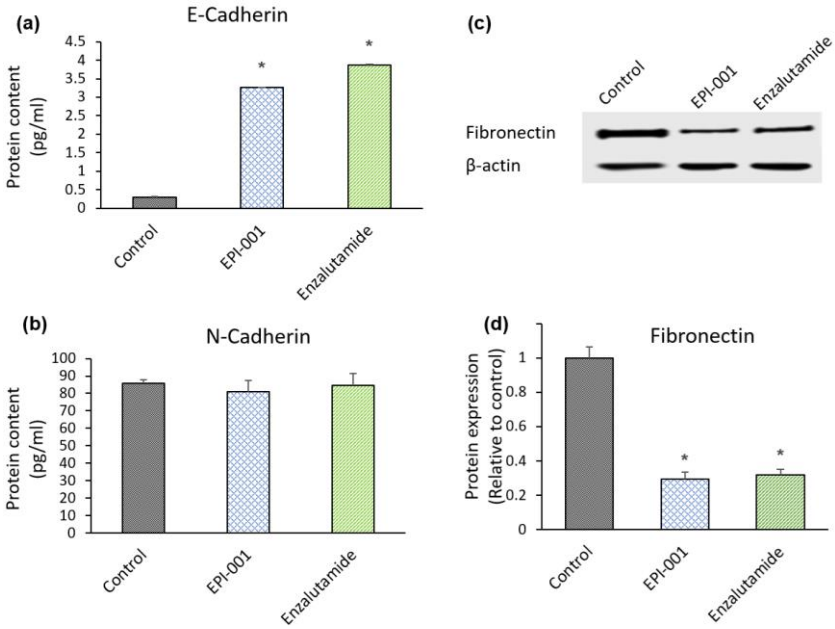

Figure 4. Effect of EPI-001 and Enzalutamide on the protein contents of (a) E-Cadherin and (b) N-Cadherin and protein expression of (C and D) fibronectin in the T47D cell line. Values are presented as the mean of at least three independent experiments ± S.E.M. Data were analyzed using one-way ANOVA followed by Tukey's post hoc test. As compared to (*) the untreated control group ($p \leq 0.05$). EPI-001 was used at a concentration of 50μM while Enzalutamide was used at a concentration of 10μM.

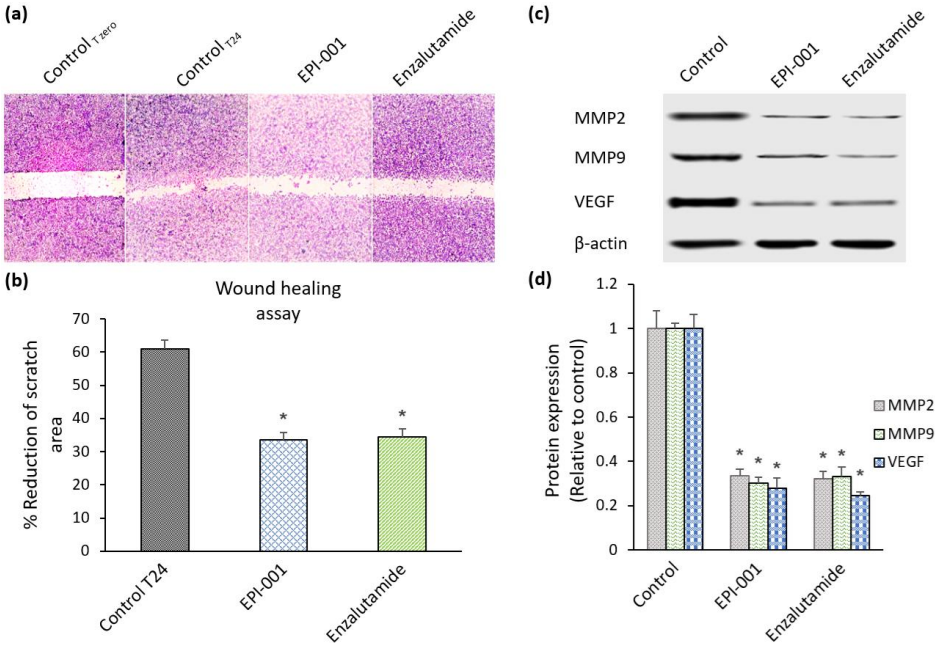

Figure 5. Effect of Enzalutamide and EPI-001 on cell migration and angiogenesis in T47D cell line Panel (a) shows the wound healing of cells in the untreated control group at zero and 24h and after treatment with Enzalutamide and EPI-001 Panel (b) represents the percent reduction in the scratch area and panels (c and d) show the effect of EPI-001 and Enzalutamide on the protein contents of MMP2, 9 and VEGF in T47D cells. Six representative images of the scratch area for each treatment well were analyzed using ImageJ software and the values of gap area reduction were expressed as mean ± S.E.M. Values of protein contents are indicated as mean of at least three independent experiments ± S.E.M. Data were analyzed using one-way ANOVA followed by Tukey's post hoc test. As compared to (*) the untreated control after 24h ($p \leq 0.05$). EPI-001 was used at a concentration of 50µM while Enzalutamide was used at a concentration of 10µM. MMP: metalloproteinase; VEGF: vascular endothelial growth factor.

These results implicate that NF-Kb may be a target for BC therapy through the regulation of AR and can be partly responsible for the decrease of the investigated cell cycle and EMT regulators. However, extra studies are necessary to endorse these direct implications.

A down target of NF-κB is c-Myc which is also a transcription factor for a miscellaneous array of genes regulating the cell cycle, cell growth, and apoptosis. It has a proliferative task and imparts chemotherapy resistance in ER+ BC cell lines (Holmes *et al*., 2022). The link between c-Myc and AR has been stated earlier, where c-Myc has a critical role in regulating AR and AR variants in CRPC and through the upregulation of c-Myc, AR promotes ligand independent prostate cancer progression (Bai *et al*., 2019). Moreover, it was reported that AR collaborates with c-Myc in tumorigenesis induction (Ni *et al*., 2013) and that the levels of c-Myc positively correlated with ARv7 in prostate cancer (Bai *et al*., 2019). Despite these verities, the current investigation presented that c-Myc was only inhibited by the variant inhibitor EPI-001 [$F(2,11)= 211.097$, $p<0.001$] but not by Enzalutamide. Indeed, a recent study reported that the use of a Myc inhibitor has sensitized the prostate cancer cells to Enzalutamide to inhibit cell viability (Holmes *et al*., 2022), a finding that may highlight the necessity of another factor to help Enzalutamide inhibit c-Myc.

Aside from cell growth regulatory role, c-Myc overexpression was reported to activate the EMT process in the triple-negative MDA-MB-231 cells (Yin *et al*., 2017). BC cells undergo EMT modifications, a process that aids in enhancing tumor progression, dissemination, metastasis, and therapy resistance (Yin *et al*., 2017).

This process is also under the control of the upstream transcription factor NF-κB, which was found to modulate EMT in BC via controlling several markers involved in EMT as cadherins and fibronectin (Chua *et al*., 2007) and is associated with high ER expression, invasion, and metastasis (Zhou *et al*., 2014). Although the contribution of AR and ARv7 in the EMT process was previously evaluated in prostate cancer, however as far as we know, their impact on ER+ (T47D) BC cells has first been assessed herein by the assessment of E-Cadherin, N-Cadherin and fibronectin. Our data showed that the receptor blocker and the variant inhibitor have boosted the content of the epithelial marker E-Cadherin to strengthen cell-cell adhesion and hinder the invasion and metastasis of the tumor, an effect that can be related to the inhibited NF-κB which is important in the regulation of E-Cadherin (Chua *et al*., 2007). Despite their ability to suppress the protein expression of the mesenchymal marker fibronectin, the tested inhibitors didn't alter the protein content of the second mesenchymal marker N-Cadherin. Indeed, the decrease in E-Cadherin and the increase in N-Cadherin correlate with increased invasiveness and metastasis of tumors, where N-Cadherin is regarded as an oncoprotein that stimulates invasion, proliferation, angiogenesis, and metastasis (Paolillo and Schinelli, 2019). Similar to N-Cadherin, fibronectin glycoprotein is associated with metastatic tumors and poor prognosis in many cancers including BC (Efthymiou *et al*., 2020). In 2008, Liu *et al*. suggested that activated AR can downregulate the expression of E-Cadherin and can promote activation of EMT and metastasis in BC patients through binding to E-Cadherin regulatory sequences (Liu *et al*., 2016), results that can support our data. In prostate cancer cells, it was also reported that the constitutively active AR variants upregulate the expression of several mesenchymal markers including N-Cadherin (Liu *et al*., 2008). Later, Das *et al*. (2016) identified a pathway in prostate cancer whereby miR-1207-3p regulates the AR via fibronectin (Das *et al*., 2016).

Our study also evaluated the anti-angiogenic potential of the two inhibitors in T47D and our data documented the aptitude of both drugs to sharply downregulate the protein expression of the angiogenic marker VEGF band densities by 72.21% and 75.49%, respectively [$F(2,8)= 82.74$, $p<0.001$] relative to the control group. The decreased content of NF-κB by the two treatments can be one reason behind the severely down regulated VEGF being one of its downstream targets (Wang *et al*., 2006). In line with the present data, a close tether between the angiogenic cytokine VEGF and AR in prostatic tumors has been identified; where the androgen stimulating effect on VEGF was found to be significant and part of the antitumor influence of antiandrogens was mediated by its down regulatory effect on VEGF (Woodward *et al*., 2005). Furthermore, it has been suggested that alterations in cellular pathways related to both endocrine and VEGF may contribute to the progression of BC progression through EMT. Therefore, in the current investigation, it is probable that the tested blockers ameliorated EMT by downregulating the expression of VEGF protein.

Besides its known angiogenic role, VEGF partakes in tumor metastasis, where it shares in the breakage of ECM by enhancing the release of the proteolytic enzymes MMP2 and MMP9 from tumor cells (Najafi *et al*., 2019). These enzymes are implicated also in angiogenesis, invasion and metastasis in various tumors including BC (Quintero-Fabian *et al*., 2019). Moreover, elevated levels of MMPs in ECM significantly increased EMT (Scheau *et al*., 2019), and their expression was associated with AR in epithelial ovarian tumors and hepatocellular carcinoma to be one risk factor for overall survival or predictive of invasive and metastatic possibilities (Morales-Vasquez *et al*., 2020).

In the same context, the androgen inhibitor Bicalutamide was demonstrated to abolish MMPs in prostate cancer (Pang *et al*., 2004). Despite BC exhibiting a highly metastatic potential, few studies have evaluated the AR expression in primary tumors and metastatic samples. A previous study showed that in BC patients, the level of some MMPs was higher in AR-positive than in AR-negative tumors (Gonzalez *et al*., 2008). Moreover, androgens were stated to regulate the motility and invasion of the T47D cells (Montt-Guevara *et al*., 2016). On the other side, the ARv7 expression was reported to be associated with metastasis in CRPC (Sharp *et al*., 2019). These studies concur with our results in which the anti-metastatic effect of the two blockers Enzalutamide and EPI-001 was evidenced by the curb in the protein level of MMP2 and MMP9 in T47D cells (Figure 5 c, d) and the widened gap in the Scratch wound healing assay (Figure 5 a) to harmonize with a previous study which found that Enzalutamide inhibited cell migration and invasion in TNBC cell line in an AR-dependent manner (Caiazza *et al*., 2016).

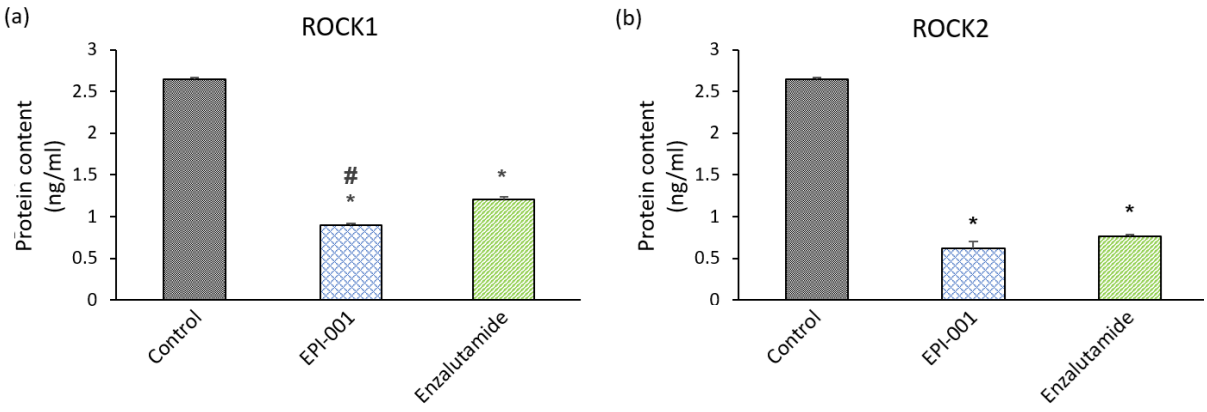

Figure 6. Effect of EPI-001 and Enzalutamide on the protein contents of (a) ROCK1 and (b) ROCK2 in the T47D cell line. Values are indicated as the mean of four independent experiments ± S.E.M. Data were analyzed using one-way ANOVA followed by Tukey's post hoc test. As compared to the (*) untreated control and (#) Enzalutamide treated groups ($p \leq 0.05$). EPI-001 was used at a concentration of 50 µM while Enzalutamide was used at a concentration of 10 µM. ROCK: Rho-kinase.

The last assessed markers herein are ROCK1 and 2 which intermingle with the investigated markers and play a crucial role in controlling many tumorigenic processes including tumor metastasis and invasion. For example, the ROCK has been reported to regulate EMT in nasopharyngeal and renal carcinoma cells (Yuan *et al*., 2019). Also, through causing cytoskeletal rearrangement, it performs a key role in BC cells and their disrupted tissue architecture. Thereby, therapy targeting the ROCK signaling cascade may open a therapeutic window for BC (Matsubara and Bissell, 2016). Connections have been described for androgens, AR, and ROCK pathways (Kroiss *et al*., 2015), where androgens induce the remodeling of actin cytoskeleton by regulating the expression and activation of moesin, causing augmented endothelial cell migration in hUVECs cells (Liao *et al*., 2013). In line with that, the AR inhibitors Enzalutamide and EPI-001 in the current investigation markedly reduced the protein levels of ROCK1 and ROCK2 in T47D cells to further support their anti-metastatic effect (Figure. 6a, b). Moreover, the inhibition of ROCKs can participate in reducing cell proliferation by reducing c-Myc which was affected herein by the variant inhibitor only. A previous study has highlighted the potential antiproliferative role of ROCK inhibitors by preventing the transcriptional activity of the oncogenic marker c-Myc in BC (Yin *et al*., 2017).

This study was limited by the availability of some resources. More migration and invasion assays could have been performed, eg those based on chemotaxis (the Dunn chamber), Boyden Chamber principle (Transwell migration/invasive test), or microfluidic devices with three-dimensional (3D) microscopy visualization (Pavla and Pavel, 2022). Moreover, no invivo study was performed which can better reflect interactions between cells and biochemical processes that occur after administration of Enzalutamide and EPI-001.

CONCLUSION

Based on these findings, our study indicated that blocking AR/ARv7 by Enzalutamide or EPI-001 decreased markers of proliferation (NF-κB, c-Myc), cell cycle (Cyclins A, C, E), EMT (E-Cadherin, fibronectin), angiogenesis (VEGF), and hence metastasis (MMP2, 9, ROCK1 and 2) in ER+ BC cells. This highlights the significance of targeting AR/ARv7 in ER+ BC where there has been much controversy. Extra preclinical and clinical studies are recommended in this area which may have strong clinical benefits in the future.

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