Preclinical evaluation of the AR inhibitor enzalutamide in triple-negative breast cancer cells

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Abstract

The androgen receptor (AR) is present in approximately 80% of invasive breast cancer patients and in up to 30% of patients with triple-negative breast cancer (TNBC). Therefore, our aim was to investigate the targeting of AR as a possible hormonal approach to the treatment of TNBC. Analysis of 2091 patients revealed an association between AR expression and poor overall survival, selectively in patients with the basal subtype of breast cancer, the vast majority of which are TNBC. IC₅₀ values for the second-generation anti-androgen enzalutamide across 11 breast cancer cell lines varied from 4 µM to >50 µM. The activity of enzalutamide was similar in TN and non-TN cell lines but was dependent on the presence of AR. Enzalutamide reduced clonogenic potential and cell growth in a 3D matrix in AR-positive cells. In addition, enzalutamide also inhibited cell migration and invasion in an AR-dependent manner. Enzalutamide appeared to mediate these processes through down-regulation of the transcription factors AP-1 and SP-1. The first-generation anti-androgen flutamide similarly blocked cell growth, migration and invasion. AR-positive TNBC cells clustered separately from AR-negative cells based on an androgen-related gene expression signature, independently of TNBC subtype. We conclude that targeting of the AR with drugs such as enzalutamide may provide an alternative treatment strategy for patients with AR-positive TNBC.

Key Words

- ▶ triple-negative
- ▶ breast cancer
- ▶ androgen receptor
- ▶ enzalutamide
- ▶ flutamide

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Introduction

Breast and prostate cancers depend on steroid hormones to drive their growth: oestrogens drive the growth of breast cancers, whereas androgens stimulate the growth of prostate cancers. Consequently, anti-oestrogens are used to treat breast cancer and anti-

androgens to treat prostate cancer (Risbridger *et al.* 2010). However, a proportion of breast cancers contains androgen receptors (AR) (Qu *et al.* 2013, Vera-Badillo *et al.* 2014) and may thus be dependent on androgens.

Although the presence of AR in breast cancer has been known for decades, it has not been widely investigated as a potential therapeutic target in this malignancy. This situation, however, is now changing, especially for the subgroup of breast cancer patients that lack oestrogen receptors (ER), progesterone receptors (PR) and HER2, i.e. those with triple-negative breast cancer (TNBC) (recently reviewed in [Shah et al. 2013](#), [Safarpour & Tavassoli 2014](#), [Proverbs-Singh et al. 2015](#)). Because of the absence of these three predictive biomarkers, TNBC currently lacks a validated targeted therapy ([Duffy et al. 2012](#)). Although lacking ER, PR and HER2, approximately 22–45% of TNBC express AR ([Gucalp & Traina 2010](#), [Park et al. 2010](#), [Collins et al. 2011](#), [Kotsopoulos & Narod 2012](#), [McNamara et al. 2013](#), [Gasparini et al. 2014](#), [Safarpour & Tavassoli 2014](#), [Barton et al. 2015](#)).

Gene expression analysis of patient samples has been used to identify different molecular subtypes of TNBC, including one characterized by androgen receptor signalling and named luminal androgen receptor (LAR) ([Lehmann et al. 2011](#)), which is closely related to a molecular apocrine breast cancer subtype previously identified ([Farmer et al. 2005](#), [Doane et al. 2006](#)). Indeed, at least two clinical trials targeting the AR in patients with TNBC are currently ongoing ([Gucalp et al. 2013](#) and ClinicalTrials.gov Identifier: NCT01889238). Despite these ongoing clinical trials, little detailed preclinical work targeting AR in triple-negative (TN) cell lines has been performed. Therefore, the aim of this study was to investigate the potential of targeting the AR in a large panel of breast cancer cells, focusing particularly on those that possess the TN phenotype. For targeting AR, we used two clinically available anti-androgens: the

first-generation agent, flutamide, and the second-generation agent, enzalutamide.

Material and methods

Cell lines and reagents

HCC1937, HCC1143, Hs578t, BT-20, MDA-MB-231, MDA-MB-453, MDA-MB-468, Sum-159, Cama1, T47D and BT474 cells were obtained from the American Tissue Culture Collection. Hs578t cells were supplied by Dr Susan McDonnell (University College Dublin, Ireland). LnCap cell lysates, used as a positive control for AR, were a gift from Dr Maria Prencipe (University College Dublin). All cell lines were grown in RPMI-1640, supplemented with 10% foetal bovine serum, 1% penicillin/streptomycin and 1% fungizone (all from Invitrogen Life Technologies, Carlsbad, CA, USA) and used for experiments between passages 3 and 15 from original stocks. Cell line identity was confirmed by analysis of short-term repeat loci, and cells were routinely tested for mycoplasma infection. The molecular characteristics of the cell lines investigated are summarised in [Table 1](#). Flutamide was purchased from Sigma Aldrich (Arklow, Ireland) and enzalutamide from SelleckChem (Munich, Germany).

Protein isolation, immunoblotting and ELISA

Cells were lysed in RIPA buffer (150mM NaCl, 50mM Tris-HCl, 1% Triton, 0.5% sodium deoxycholate, and 0.1% SDS) supplemented with a protease and phosphatase inhibitor cocktail (Roche Applied Science, Burgess

Table 1 Pathological and molecular profiles of breast cancer cell lines.

	ER	PR	HER2	PAM50 ^a	Intrinsic subtype ^b	TNBC subtype ^c	Normal-cell subtype ^d
HCC1937	–	–	–	Basal A	Basal	BL1	HR1
HCC1143	–	–	–	Basal A	Basal	BL1	–
BT20	–	–	–	Basal A	Basal	uncl	HR1
MDA-MB-468	–	–	–	Basal A	Basal	BL1	HR1
MDA-MB-231	–	–	–	Basal B	Normal-like	MSL	–
Hs578t	–	–	–	Basal B	Normal-like	MSL	–
SUM159	–	–	–	Basal B	Normal-like	MSL	HR1
MDA-MB-453	–	–	– [+]*	Luminal	HER2+	LAR	HR2
Cama1	+	–	–	Luminal	Luminal	–	–
T47D	+	+	–	Luminal	Luminal	–	HR3
BT474	+	+	+ [+]	Luminal	HER2+	–	HR3

Protein expression of oestrogen receptor (ER), progesterone receptor (PR) and HER2 were derived from [Kao et al. \(2009\)](#) and [Neve et al. \(2006\)](#); HER2 gene amplification is reported in square brackets (*MDA-MB-453 have HER2 amplification without overexpression). Classification of cell lines to breast cancer subtypes by nearest centroid correlation based on different gene expression signatures: ^aPAM50 ([Parker et al. 2009](#)), ^bssp2003 ([Sorlie et al. 2003](#)), ^cTNBC ([Lehmann et al. 2011](#)). ^dClassification according to normal cell type-based ontological method ([Santagata et al. 2014](#)). BL1, basal-like 1; MSL, mesenchymal stem-like; LAR, luminal androgen receptor; HR1-2-3: hormonal differentiation groups.

Table 2 Details of the 2091 patients according to datasets included in the BreastMark database (Madden et al. 2013).

GEO ID	Sample number	Median age	Median size (cm)	Lymph node status	Chemotherapy Info.	Hormone treatment Info.	ER status	HER2 status	PR status	Tumour grade (1/2/3)	DFS (months)	DDFS (months)	OS (months)
GSE3143	158	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	51 ± 31
GSE10510	152	59 ± 12	NA	A	NA	NA	A	NA	A	NA	57 ± 53	NA	87 ± 60
NKI295*	295	44 ± 5	2.25 ± 0.9	A	A	NA	A	NA	NA	NA	84 ± 50	NA	94 ± 47
GSE9893	155	67 ± 10	2.3 ± 0.9	A	NA	A	A	NA	NA	21/94/33	65 ± 32	66 ± 31	72 ± 29
GSE7390	198	46 ± 7	2.2 ± 0.8	NA	NA	NA	A	NA	NA	30/83/83	113 ± 68	114 ± 65	138 ± 61
GSE24450	183	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	72 ± 27
GSE1992	99	55 ± 15	NA	A	NA	NA	A	NA	NA	8/34/57	25 ± 23	NA	29 ± 25
GSE20685	327	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	88 ± 43	94 ± 38
GSE3494	251	62 ± 13	2.3 ± 1.25	A	NA	NA	A	NA	A	67/128/54	NA	NA	98 ± 46
GSE45255	139	55 ± 12	2.9 ± 1.3	A	A	A	A	A	A	17/52/67	48 ± 22	51 ± 25	54 ± 21
GSE1456	159	NA	NA	NA	NA	NA	NA	NA	NA	28/58/61	72 ± 29	NA	77 ± 23

*, Chang et al. 2005; A, available; NA, not available; ER, oestrogen receptor; HER2, human epidermal growth factor receptor 2; PR, progesterone receptor; DFS, disease-free survival; DDFS, distant disease-free survival; OS, overall survival, tumour grade (1/2/3); 1 refers to the number of grade 1 tumours, 2 refers to the number of grade 2 tumours and 3 refers to the number of grade 3 tumours.

Hill, UK) and 1 mM PMSF (Sigma Aldrich). Western blot was performed as described previously (Caiazza et al. 2015) using a mouse monoclonal antibody for AR (clone 441; Santa Cruz Biotechnology, Heidelberg, Germany), with normalization against β -actin (Sigma Aldrich). AR levels were quantified with an ELISA assay (Abcam, Cambridge, UK) following manufacturer's instructions and using a LnCap cell lysate as positive control.

Clonogenic assays

Colony formation assays were performed as described previously (Caiazza et al. 2015). Image analysis was performed using ImageJ software (NIH, Bethesda, MA, USA; <http://imagej.nih.gov/ij/>) to determine colony size and area (Cai et al. 2011). Plating efficiency was calculated by dividing the number of colonies by the original seeding density. The surviving fraction was determined by comparing the plating efficiency of treated vs control wells. The total colony area was calculated for each biological replicate by averaging the area of all colonies in replicate wells.

Cell invasion assays

Cell motility was assessed as described previously (Caiazza et al. 2015) using Matrigel-coated inserts (8- μ m pore size; BioCoat, BD Biosciences, Erembodegem-Dorp, Belgium) or non-Matrigel-coated control inserts and fibroblast-conditioned medium as chemoattractant.

Three-dimensional cell culture

For 3D cultures, 5×10^5 cells were seeded in Alvetex scaffolds (Reinervate, Sedgefield, Co. Durham, UK) in low serum (2%) medium and cultured for 7 days. At the end of the incubation, scaffolds were washed in PBS and stained with a 0.25% solution of neutral red (Sigma Aldrich) in PBS for 5 min at room temperature. Scaffolds were imaged on a bright field microscope at 4 $\times$ magnification, averaging four random fields for each scaffold. A 1 $\times$ magnification image of the entire scaffold was acquired with a standard digital camera. Neutral red staining was quantified with ImageJ software.

RT2 profiler PCR array analysis

Total RNA isolated using the RNeasy Mini kit (Qiagen, Manchester, UK) was transcribed into cDNA using the RT² First-strand Kit (Qiagen). The cDNA (0.5 μ g of template) was used on the real-time Human Androgen Receptor

Figure 1

Effect of enzalutamide in breast cancer cells is related to AR protein expression. (A) Colony formation was measured for 11 cell lines in the presence of different concentrations of enzalutamide (0.05–100 μ M). Bar charts indicate the percentage inhibition of cell growth after treatment with 10 μ M enzalutamide and the corresponding IC₅₀ values for all cell lines. Black bars=TNBC, grey bars=HER 2+, white bars=luminal breast cancer cell lines. (B) Bivariate scattergram illustrating the direct negative correlation between IC₅₀ values and percentage growth inhibition following treatment with 10 μ M enzalutamide. Data was analysed using the Pearson correlation test. (C) IC₅₀ values for enzalutamide were compared in TN cells ($n=8$) vs non-TN cells ($n=3$). (D and E) Western blot and ELISA analysis for the AR was performed on total cell lysates from the indicated cell lines. The prostate cancer cell line LnCap was used as a positive control. (F) Bivariate scattergram illustrating the direct negative correlation between IC₅₀ values for enzalutamide and AR protein expression in TN cells. A full colour version of this figure is available at <http://dx.doi.org/10.1530/ERC-16-0068>.

Signaling Targets RT² Profiler PCR Array (Qiagen) in combination with RT² SYBR Green qPCR Mastermix. Cycle threshold (C_t) values were normalized using three housekeeping genes and analysed using the data analysis web portal (<http://www.qiagen.com/geneglobe>) following manufacturer's instructions. The fold change gene expression was calculated with the delta C_t method. Confirmatory RT-PCR analysis of *FOS* and *JUN* mRNA was performed in triplicate using the RT² qPCR Primer Assay (Qiagen) and GAPDH as housekeeping gene.

Hierarchical clustering of breast cancer cells

A gene signature for the LAR subtype of TNBC was derived from two publicly available datasets describing ER-/AR+ breast cancer, based on the analysis of 41 (Doane *et al.* 2006) and 49 (Farmer *et al.* 2005) patients. The list of the 100 genes most differentially expressed in the ER- class A tumours from the Doane cohort was compared with the list of genes that discriminate for group 3 (molecular apocrine) in the Farmer cohort. The resulting list of 76 genes commonly discriminating both groups of LAR patients is reported in [Supplementary Table 1](#) (see section

on [supplementary data](#) given at the end of this article). This 76 gene list was used to perform unsupervised hierarchical clustering of a publicly available breast cancer cell line dataset (Neve *et al.* 2006) containing all 11 cell lines of our original panel. To cluster data, we used CIMiner software (<http://discover.nci.nih.gov/ciminer/home.do>) (Weinstein *et al.* 1997) with the Euclidean distance method; the average linkage was used as the measure of proximity between clusters to construct dendrograms.

Mining of publicly available breast cancer microarray data

Analysis of breast cancer datasets was carried out using BreastMark (Madden *et al.* 2013). Kaplan–Meier survival curves were generated at <http://glados.ucd.ie/BreastMark/index.html>. The key characteristics of the different cohorts of patients in this database are summarised in [Table 2](#).

Statistical analysis

Statistical analysis of the data was performed with paired Student's *t*-test or one-way ANOVA for differences

Figure 2

Molecular clustering of breast cancer cell lines. Hierarchical clustering was performed on 11 breast cancer cells based on the 76 genes signature of LAR subtype. The dendrogram represents the relationship among samples, with the length of branches representing the correlation coefficient between samples. Cell lines are arranged in columns and genes are arranged in rows. Gene expression levels are pseudocolored according to the legend reported at the bottom of the figure. A full colour version of this figure is available at <http://dx.doi.org/10.1530/ERC-16-0068>.

between experimental group mean values. Pearson's coefficient was used to measure the correlation between linear variables. Survival curves were calculated according to the Kaplan–Meier method and compared using the Cox–Mantel log-rank test. The AR expression was categorised as high or low using the 'low' cut-off point from BreastMark (Madden *et al.* 2013), and overall survival (OS) was chosen as the survival endpoint. Two-tailed *P*-values <0.05 were considered statistically significant. All analyses were performed using Prism 5 (Graphpad Software, La Jolla, CA, USA).

Results

Effect of enzalutamide on growth of breast cancer cells

The effect of enzalutamide on clonogenic growth of 11 breast cancer cell lines (eight TNBC and three non-TN) is summarised in Fig. 1A. Sensitivity to enzalutamide treatment was widely variable, with IC₅₀ values ranging from 4 μM to >50 μM. The percentage of cell growth inhibition at 10 μM varied from 0% to 49%, with the highest inhibition seen in MDA-MB-453 and HCC1937 cells. Overall, a strong and significant correlation was found between the IC₅₀ values for enzalutamide and percentage growth inhibition ($r = -0.8749$, $P = 4.00 \times 10^{-4}$; Fig. 1B). The cell lines found to be most sensitive to

enzalutamide were MDA-MB-453 and HCC1937, both of which are TN. Overall, however, the response to enzalutamide was cell line dependent and unrelated to whether cell lines were TN or non-TN ($P = 0.118$; Fig. 1C). Western blot and ELISA analysis of the AR expression by 11 BC cell lines (Fig. 1D and E respectively) revealed a significant correlation between the level of AR protein expression and sensitivity to enzalutamide selectively in TN cells ($r = -0.804$, $P = 0.016$; Fig. 1F).

Characterization of AR-positive TNBC cells

In order to verify that the cell line models we used were representative of AR-positive TNBC, we selected a 76-gene signature from genes highly deregulated in LAR TNBC patients (Farmer *et al.* 2005, Doane *et al.* 2006) and used it to perform hierarchical clustering of the 11 BC cell lines (Fig. 2). MDA-MB-453 was the cell line most representative of the LAR subtype, as previously reported, and it closely clustered with luminal cell lines. The AR-positive cells HCC1937, HCC1143 and MDA-MB-468 clustered together, whereas the AR-negative cells BT20, Hs578t and MDA-MB-231 were the least representative of the LAR subtype. The only exception to this was SUM159, which clustered with the AR-negative cells despite showing AR expression.

Figure 3

Growth inhibitory effect of AR inhibition in TNBC cells. (A and B) Colony formation was measured for the indicated four cell lines in the presence of (A) enzalutamide (10 μ M) or (B) flutamide (1 μ M). Bar charts indicate the surviving fraction and mean colony area for each compound. (C) HCC1937 or (D) MDA-MB-453 cells were cultured for 7 days on 3D scaffolds in the presence of flutamide (1 μ M) or enzalutamide (10 μ M). Neutral red staining was quantified and reported with representative pictures. Data are mean values $\pm$ S.E.M. of three independent experiments. * P < 0.05, ** P < 0.001, *** P < 0.0001 compared with vehicle control (ANOVA). A full colour version of this figure is available at <http://dx.doi.org/10.1530/ERC-16-0068>.

Effect of enzalutamide on growth of TNBC cells

As our primary interest was to develop an alternative treatment strategy for TNBC, our subsequent *in vitro* investigations focused exclusively on AR-positive cells of this molecular subtype (MDA-MB-453, HCC1937 and HCC1143) as well as the AR-negative cell line, Hs578t, as control. As shown in Fig. 3A, enzalutamide reduced clonogenic potential as measured by both decreased number of colonies and mean colony area in the two cell lines expressing high levels of AR, i.e. in MDA-MB-453 and HCC1937 cells. In contrast, there was little (non-significant) effect on growth in the cell line with low AR expression (HCC1143 cells) and no detectable changes in the AR-negative cell line Hs578t. Similar to our findings with enzalutamide, the first-generation anti-androgen flutamide also decreased clonogenic growth in an AR-dependent manner (Fig. 3B). Although both AR antagonists were investigated in only four cell lines, there was a high correlation between the IC₅₀ values for the two inhibitors ($r=0.9722$, $P=0.028$). Enzalutamide also reduced clonogenic potential in two other TN cell lines showing detectable but low AR expression, MDA-MB-468 and SUM159 (Fig. 4A and B).

In order to confirm our findings in a more biologically relevant system, we investigated the anti-growth effects of enzalutamide in AR-positive cells cultured in 3D scaffolds (Fig. 3C and D). Using this system, the effect of the two

inhibitors was cell line-specific, with both flutamide and enzalutamide reducing the growth of HCC1937 cells (Fig. 3C), whereas only flutamide significantly reduced growth of MDA-MB-453 cells (Fig. 3D). There was, however, a non-significant trend for enzalutamide to reduce growth in MDA-MB-453 cells ($P=0.169$). In contrast, enzalutamide showed no effect on the three-dimensional growth of the AR-negative cell line Hs578t or the low AR-expressing cell line MDA-MB-468 (Fig. 4C and D).

Transcriptional effects of enzalutamide in TNBC cells

In order to clarify the mechanism by which enzalutamide reduced cell proliferation in AR-positive cells, we investigated the effects of the anti-androgen on gene expression in the MDA-MB-453 cell line. Using a threshold of fold change in gene expression of two, five genes were found to be significantly down-regulated following treatment with enzalutamide (Table 3). Among these genes were *FOS* and *JUN*, which are known to heterodimerize and form the transcription factor AP-1 (Vesely et al. 2009).

AP-1 has previously been implicated in a variety of cellular processes, including proliferation and migration (Vesely et al. 2009), as well as being linked to enhanced AR transactivation in prostate cancer cells (Chen et al. 2006). Therefore, we validated the *FOS* and *JUN* array

Figure 4

Growth inhibitory effect of AR inhibition in additional TNBC cells. Colony formation was measured for (A) MDA-MB-468 and (B) SUM159 in the presence of enzalutamide (10 µM). Bar charts indicate the surviving fraction and mean colony area for each compound. (C) Hs578t or (D) MDA-MB-468 cells were cultured for 7 days on 3D scaffolds in the presence of enzalutamide (10 µM). Neutral red staining was quantified and reported with representative pictures. * $P < 0.05$, ** $P < 0.001$. A full colour version of this figure is available at <http://dx.doi.org/10.1530/ERC-16-0068>.

results by RT-PCR. Treatment of the AR-positive cell line, MDA-MB-453, with enzalutamide was found to reduce expression of *FOS* and *JUN* by 2.6- and 1.7-fold respectively (Fig. 5A). In contrast, enzalutamide had no effect on the expression of these two genes in the AR-negative cell line, BT-20 (Fig. 5B).

Effect of AR inhibition on TNBC cell motility

As most TNBC have a basal phenotype and are highly invasive, we next examined whether AR inhibition impacted on their invasive potential. As shown in Fig. 6, enzalutamide reduced cell migration and invasion in the three AR-positive cell lines MDA-MB-453 (Fig. 6A), HCC1937 (Fig. 6B) and HCC1143 (Fig. 6C). This effect, however, was again cell line dependent and compound specific. For example, enzalutamide significantly reduced migration in MDA-MB-453 cells but not in HCC1143 or HCC1937 cells, while reducing cell invasion in all three cell lines. In contrast to our findings in the AR-positive cell lines, enzalutamide did not affect migration or invasion in the AR-negative cell line Hs578t (Fig. 6D). Similarly, flutamide also reduced cell migration and invasion in

AR-positive cell lines MDA-MB-453 (Fig. 7A), HCC1937 (Fig. 7B) and HCC1143 (Fig. 7C) but not in the control AR-negative cells Hs578t (Fig. 7D).

Prognostic significance of AR in breast cancer samples

To evaluate the potential prognostic significance of AR expression in TNBC, we analysed a pooled gene expression database of 2091 patients. Clinical details of the patient cohorts included in the database are outlined in Table 2. In the total population of patients, high AR expression was found to be associated with good prognosis (Fig. 8A, HR=0.706, 95% CI: 0.587–0.849, $P = 1.928 \times 10^{-4}$). However, following separation of the total population of patients into their molecular subtype using the ssp2003 classifier (Sorlie et al. 2003), AR retained its positive prognostic significance in the luminal A group (Fig. 8B, HR=0.490, 95% CI: 0.314–0.766, $P = 0.001$, $n = 812$). Conversely, in patients with basal tumours, AR was a marker of bad prognosis (Fig. 8C, HR=1.709, 95% CI: 1.021–2.862, $P = 0.039$, $n = 273$). In contrast, AR lacked a prognostic impact in the luminal B and HER2-positive subtypes (data not shown).

Table 3 Gene expression changes induced by enzalutamide in MDA-MB-453 cells.

Gene symbol	Gene name	Fold change
<i>FOS</i>	FBJ murine osteosarcoma viral oncogene homolog	-2.85
<i>JUN</i>	Jun proto-oncogene	-3.83
<i>ORM2</i>	Orosomucoid 2	-2.66
<i>SP1</i>	Sp1 transcription factor	-2.17
<i>TMPRSS2</i>	Transmembrane protease, serine 2	-2.42

Discussion

To our knowledge, this is one of the largest preclinical studies to investigate the effects of the second-generation AR antagonist enzalutamide in TNBC cell lines. Unlike the first generation of AR antagonists, enzalutamide lacks AR agonistic activity (for review, see [Ha & Kim 2014](#)) and, furthermore, has been reported to bind to AR with approximately eight-fold higher affinity than bicalutamide ([Tran et al. 2009](#)).

Although previous reports have investigated the anti-proliferative effects of anti-androgens on breast cancer cell line growth *in vitro*, until recently, most studies used only one cell line ([Doane et al. 2006](#), [Ni et al. 2011](#), [Cochrane et al. 2014](#)). While this manuscript was in preparation, Barton and coworkers reported the anti-proliferative effects of enzalutamide in four TNBC cell lines ([Barton et al. 2015](#)). In contrast, our study investigated the potential growth inhibitory effects of enzalutamide in 11 cell lines (eight of which were TN) and flutamide in four cell lines (all TN). Our results show that growth inhibition with both anti-androgens was dependent on the expression of the AR, i.e. high AR levels were associated with low IC₅₀ values. Although such a finding might be expected, our study appears to be the first to report this observation.

Although frequently regarded as a homogeneous subgroup, it is important to stress that both TNBC and TNBC cell lines are highly heterogeneous ([Lehmann et al. 2011](#), [Santagata et al. 2014](#), and [Table 1](#)). Previously, most of the preclinical work investigating the anti-growth potential of enzalutamide on breast cancer cell lines focused on luminal AR (LAR) subtype, especially the MDA-MB-453 cell line. However, we here show that some cell lines not originally classified as LAR, expressed AR at the protein level and were responsive to AR-targeted therapy. Specifically, three basal-like (BL1) cell lines (HCC1143, HCC1937 and MDA-MB-468) and one mesenchymal stem-like (MSL) cell line (SUM159) were found to be

Figure 5

Effect of enzalutamide on gene expression. MDA-MB-453 (A) or BT-20 (B) cells were treated with enzalutamide (10 μ M) or vehicle control for 24 h. mRNA was extracted and was reverse transcribed and analysed by real-time qRT-PCR using *FOS*- and *JUN*-specific primers. Data represents mean $\pm$ S.E.M. values of three experiments.

sensitive to enzalutamide. We also showed that these AR-positive, non-LAR cells clustered separately from the AR-negative TN cells, based on the expression of 76 genes highly related to the LAR subtype of TNBC. Such results support the use of these cell lines as preclinical models of AR-positive TNBC. Our findings are thus in accordance with a recent report showing enzalutamide-mediated growth inhibition in four different non-LAR TNBC cell lines of the mesenchymal-like MSL and BL2 subtypes ([Barton et al. 2015](#)).

Some differences exist between our results and those previously reported with enzalutamide in breast cancer cell lines. Thus, although MDA-MB-231 cells were not sensitive to enzalutamide in our study, a previous report found that growth of these cells was partially inhibited by enzalutamide ([Barton et al. 2015](#)). These different findings may relate to the different biological assay used to assess cell viability. It should also be stated that the Hs578t cell line, which is AR-negative and unresponsive to either flutamide or enzalutamide in our study, was previously reported to have mRNA expression of AR and to be partially responsive to bicalutamide ([Lehmann et al. 2011](#)). These apparently discordant results could be explained by the lack of concordance between transcript and corresponding protein expression, which was previously reported for steroid receptors

Figure 6

Effect of enzalutamide on TNBC cell migration and invasion. MDA-MB-453 (A), HCC1937 (B), HCC1143 (C) and Hs578t (D) cells were cultured in Matrigel-coated Boyden chambers or in control Boyden chambers without Matrigel coating, using fibroblast-conditioned media as a chemoattractant. Cells were treated with enzalutamide (10 μ M) or vehicle control for 72h and then invaded/migrated cells were fixed, stained and counted. Data represent mean $\pm$ S.E.M. values of three experiments. * P < 0.05, ** P < 0.001, *** P < 0.0001 compared with vehicle control (Student's t -test). A full colour version of this figure is available at <http://dx.doi.org/10.1530/ERC-16-0068>.

(Caiazza *et al.* 2007, Vogel & Marcotte 2012). A further potential reason for these discordances could be the different mechanisms of action between first- and second-generation anti-androgens.

In addition to the effects on cell proliferation, we also showed that both flutamide and enzalutamide inhibited cell invasion and migration of TNBC cells. The effect on these endpoints was again cell line and compound dependent. Thus, although both flutamide and enzalutamide significantly decreased migration in MDA-MB-453 cells, only flutamide impacted on this endpoint in HCC1937 and HCC1143 cells, whereas both compounds reduced cell invasion in all three AR-positive cell lines. Neither flutamide nor enzalutamide affected invasion or migration in the AR-negative cell line, Hs578t.

The involvement of AR signalling in metastasis and tumour invasiveness of non-prostate malignancies is still relatively unexplored. Previously, Castoria *et al.* (2013) reported that the anti-androgen bicalutamide blocked migration in several different cancer cell lines. However, in that study, TNBC cell lines were not investigated. In agreement with our findings,

Barton *et al.* recently reported that enzalutamide reduced migration and invasion of the TNBC cell line (BT549) (Barton *et al.* 2015).

In order to identify a putative mechanism to explain the effects of enzalutamide on the growth of AR-positive TNBC cells, we used a gene expression profiling tool to analyse a focused panel of genes in the AR signalling pathway. Five genes were down-regulated by enzalutamide treatment, including *FOS* and *JUN* (components of the early-response transcription factor AP-1) and the transcription factor SP-1. Both AP-1 and SP-1 are critical for the regulation of genes involved in proliferative signalling, replicative immortality, cell metabolism, as well as cell invasion and metastasis (Vesely *et al.* 2009, Beishline & Azizkhan Clifford 2015). Both *FOS* and *JUN* were further validated by RT-PCR as being significantly down-regulated by enzalutamide, specifically in AR-positive cells. These results suggest that inhibition of transcriptional regulation through AP-1 (and possibly SP-1) could be involved in the anti-proliferative actions of enzalutamide in TNBC.

In order to establish a possible clinical role for AR in breast cancer, especially in the TN subtype, we evaluated

Figure 7

Effect of flutamide on TNBC cell migration and invasion. MDA-MB-453 (A), HCC1937 (B), HCC1143 (C) and Hs578t (D) cells were cultured in Matrigel-coated Boyden chambers or in control Boyden chambers without Matrigel coating, using fibroblast-conditioned media as a chemoattractant. Cells were treated with flutamide (1 μ M) or vehicle control for 72 h and then invaded/migrated cells were fixed, stained and counted. Data represent mean $\pm$ s.e.m. values of three experiments. * $P < 0.05$, ** $P < 0.001$, *** $P < 0.0001$ compared with vehicle control (Student's *t*-test). A full colour version of this figure is available at <http://dx.doi.org/10.1530/ERC-16-0068>.

the prognostic potential of AR in a pooled database of 2091 patients (Madden *et al.* 2013). In agreement with a previous meta-analysis (Vera-Badillo *et al.* 2014), we found that high AR expression predicted a favourable outcome in the total population of patients. In contrast, in the subgroup of patients with basal disease (approximately 70–80% of which are TN (Duffy *et al.* 2012)), the presence of AR was associated with poor outcome. A possible explanation for this difference between the total population and those with basal/TN disease is that AR inhibits growth of ER-positive breast

cancer cells by antagonizing oestrogen receptor-induced signalling (Peters *et al.* 2009) but stimulates growth via an androgen-dependent mechanism in ER-negative and AR-positive cells (Doane *et al.* 2006, Hickey *et al.* 2012). A further possible explanation is that AR-positive TNBC may be less likely to benefit from chemotherapy than other forms of breast cancers (Masuda *et al.* 2013, Mayer *et al.* 2014).

It should be stated that conflicting results exist in the literature regarding the prognostic impact of AR in basal/TN breast cancer. In agreement with our finding,

Figure 8

Prognostic effect of AR. Prognostic value of AR expression in breast cancer patients from the BreastMark database (Madden *et al.* 2013), using OS as the survival endpoint and 'low' as the expression cut-off. Kaplan-Meier estimates for (A) unselected patients ($n = 2091$, $HR = 0.706$, $P = 1.928 \times 10^{-4}$), (B) patients with luminal A subtype ($n = 812$, $HR = 0.49$, $P = 1.37 \times 10^{-3}$) or (C) patients with basal subtype ($n = 273$, $HR = 1.709$, $P = 0.039$). Survival curves were compared with the Cox-Mantel log-rank test and the corresponding *P*-value is indicated. A full colour version of this figure is available at <http://dx.doi.org/10.1530/ERC-16-0068>.

Hu *et al.* (2011) reported that high AR levels predicted poor patient outcome. In contrast, a recent meta-analysis found that increased AR expression predicted good outcome in this subset of breast cancer patients (Qu *et al.* 2013). Another confounding factor is the fact that basal and TN breast cancer are not synonymous, and standardised criteria for defining both subgroups have not been defined.

In conclusion, we have shown that two structurally different anti-androgens can inhibit cell growth, invasion and migration in AR-positive TNBC cell lines. Our finding should inform ongoing clinical trials investigating the targeting of AR in patients with TNBC. Indeed, promising preliminary results from these trials have been reported in patients with advanced AR-positive TNBC (Cortes *et al.* 2015, Traina *et al.* 2015). Thus, in a phase II study, administration of enzalutamide to AR-positive patients who were also positive for a specific androgen-driven gene signature (PREDICT AR) was reported to result in a superior outcome, compared with patients negative for this signature (Cortes *et al.* 2015). As anti-androgens are already widely used to treat metastatic prostate cancer, repurposing them for use in TNBC could proceed rapidly. Finally, as the AR is expressed in several different types of cancer (Munoz *et al.* 2014), targeting with androgen antagonists may have wide application in cancer treatment.

Supplementary data

This is linked to the online version of the paper at <http://dx.doi.org/10.1530/ERC-16-0068>

Declaration of interest

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Author contribution

FC, AM and MJD designed the study and developed the methodology. FC, AM, SFM, NCS and EJR performed the experimental procedures and analysed the data. FC, AM and MJD prepared the manuscript. NOD, JC, SFM and MJD contributed reagents, materials and analysis tools.

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