The role of the glucocorticoid receptor in anti-hormone resistance in breast cancer

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ABSTRACT

Background: Approximately 75% of all breast cancer diagnoses are oestrogen receptor (ER) positive. In such ER positive subtypes, anti-hormones such as fulvestrant and tamoxifen are a mainstay therapy. However, the efficacy of these agents is severely limited by subsequent development of resistance. The glucocorticoid receptor (GR) has been implicated as a possible resistance mechanism owing to transcription of pro-proliferative and anti-apoptotic genes in breast cancer cells. A similar contributory role to resistance has also been observed in anti-hormone resistant prostate cancer suggested by increased GR expression and tumour progression. These associations are of particular concern given the use of glucocorticoids as an adjuvant treatment in breast cancer. This research aims to assess the impact of fulvestrant and tamoxifen on GR expression in the anti-hormone treated and resistant MCF-7 ER positive breast cancer cell line.

Methods: mRNA and protein expression of the GR were investigated by reverse transcription polymerase chain reaction and Western blotting respectively, in the ER positive MCF-7 breast cancer cell line. Expression in wild-type cells was compared to cells following short-term (7 day) oestrogen (1nM) and fulvestrant (100nM) treatment, and in cells with acquired resistance to fulvestrant and tamoxifen.

Results: Both fulvestrant treated and resistant MCF-7 cells exhibited increased GR mRNA and protein expression which was statistically significant in resistant cells at the protein (p=0.0345) but not mRNA level. Tamoxifen-resistant cells also exhibited increased GR protein expression.

Conclusion: These data demonstrate up-regulation of the GR during treatment with, and following acquisition of resistance to, the anti-hormone fulvestrant. This supports potential for increased expression of GR-regulated pro-survival genes in resistance, indicating a potential role for the GR in anti-hormone resistant breast cancer. Further research into this area is warranted to improve clinical outcomes.
BACKGROUND

Breast Cancer

Worldwide, breast cancer is the most common malignancy in females, and a significant health burden, accounting for 627,000 deaths in 2018. (1) Approximately 75% of breast cancers express or overexpress the ER isofrom designating them ER positive (2–4) and sensitive to the proliferative drive of oestrogens. (2) The central role played by oestrogen in breast cancer progression has denoted anti-oestrogens a mainstay in the therapeutic armamentarium against ER positive disease. However, the clinical utility of these agents is confounded by acquisition of resistance in approximately 40% of initially responsive adjuvant patients, (5) and 25% of all breast cancer cases. (6)

Availability of anti-oestrogens has nonetheless revolutionised disease prognosis. For example, the selective oestrogen receptor modulator (SERM) tamoxifen contributed to the nearly 50% annual reduction in breast cancer recurrence and 30% survival improvement observed in the last 20 years. (6) However, as a competitive inhibitor of the ER, (7) tamoxifen retains the potential to function as an agonist, (8) as observed in bone and endometrial tissue. (9) These experiences prompted development of newer endocrine agents, such as aromatase inhibitors, which interfere with oestrogen production by the aromatase enzyme, and selective oestrogen receptor down-regulators (SERDs) such as fulvestrant (Faslodex ®). Fulvestrant (Faslodex ®) is recommended by the National Institute for Health and Care Excellence (NICE) in post-menopausal women with locally advanced or relapsed metastatic ER positive breast cancer, who have previously been treated with aromatase inhibitors, or have developed treatment resistance. (10) In contrast to tamoxifen, fulvestrant is a pure ER antagonist, devoid of the adverse pharmacology and sequelae reflective of tamoxifen’s agonistic potential. (11) Mechanistically it exhibits widespread actions including inhibition of ER binding oestrogen and co-activator proteins, decreased nuclear translocation of ligand-bound ER, reduced capability of ER:ERE interactions, and inhibition of ER dimerisation by induction of receptor conformational changes. (8) It is also unique in that it enhances ER degradation. (8) Hence, by down-regulating the ER, fulvestrant abrogates transcription of oestrogen-regulated target genes by both inhibiting oestrogen-dependent signaling and preventing oestrogen-independent ER activation. (7,12)

Mechanisms of acquired anti-hormone resistance

Gradual loss of endocrine responsiveness occurs in the majority of patients treated with endocrine agents within 2–3 years. (4) Loss of ER expression has been postulated as a causative factor, although this appears unlikely given the majority of resistant tumours retain a functional ER, and demonstrate responsiveness to subsequent endocrine therapies. (13) An alternative hypothesised mechanism is interference mediated by other receptors. In particular, growth factor receptors such as human epidermal growth factor receptor 2 (HER2) have been extensively studied and as such established a precedent for receptor cross-talk in the induction of anti-hormone resistance. (14) However, since concomitant HER2 blockade with monoclonal antibodies fails to appease resistance development, (15) involvement of other receptors is indicated.

Recent studies have implicated nuclear steroid hormone receptors such as the glucocorticoid receptor (GR). Indeed, increased GR expression has been associated with anti-hormone resistance and tumour progression in prostate cancer, another predominantly hormone driven malignancy. (16) Similarly, in ER-negative breast cancer one study demonstrated that high GR expression is related to poor prognosis and increased relapse rates, (17) a significant finding given mechanistically fulvestrant generates an ER-negative cancer, particularly when resistance develops. (8) There is however a paucity of data investigating the role of the GR in ER-positive breast cancer which this study aims to address.

The GR in breast cancer

The anti-inflammatory properties of glucocorticoids (GCs), such as dexamethasone, position them an attractive and commonly used agent in breast cancer (18,19) which is potentially concerning if the GR is implicated in resistance as we hypothesise. However, this benefit appears to be cell-type-specific, (20) with anti-apoptotic functioning observed in mammary epithelial cells. (20,21) For example, dexamethasone treatment increases expression of the well-established anti-apoptotic, pro-proliferative gene serum-and-glucocorticoid-inducible-kinase-1 (SGK-1). (21) Significantly, SGK-1 overexpression has recently been cited as causal in anti-hormone resistance in prostate cancer (22) and found to abrogate apoptosis in mammary epithelial cells. (23) Similarly, dexamethasone induces up-regulation of the anti-apoptotic phosphatase enzyme MAPK phosphatase-1 (MKP-1). (24) Overexpression of MKP-1/DUSP-1 has been documented in breast and prostate carcinomas (24) and also ovarian cancer where it is associated with reduced progression free survival. (25) Such a pro-survival role for the GR in breast cancer is further supported by investigations of xenograft tumours of mice given systemic GCs, demonstrating increased mRNA expression of both SGK-1 and MKP-1, in addition to other anti-apoptotic genes. (24) Taken together, this evidence strongly implicates GR-regulated genes as mediators of breast tumour cell survival and suggests a role for the GR in the acquisition of endocrine resistance. This has particularly concerning implications regarding dexamethasone use in breast cancer patients.

Aims

The project evaluates the hypothesis that fulvestrant resistant ER-positive breast cancer cells display increased GR expression which theoretically would result in up-regulation of pro-survival genes, evidencing a clear role for the GR in the acquisition of anti-hormone resistance in breast cancer. The expression profile of the GR will be evaluated in ER-positive MCF-7 breast cancer cell line and compared between wild-type, oestrogen-treated, fulvestrant-treated, fulvestrant-resistant and in certain cases tamoxifen-resistant cells. This cell line was chosen as the ER-positive subtype is representative of approximately 70% of breast cancers seen clinically. (2-4)
METHODS

Cell culture and lysis

Oestrogen-receptor positive MCF-7 (AstraZeneca Pharmaceuticals, Macclesfield, UK) wild-type cells were grown and maintained in 5% Carbon dioxide, at 37°C, in red RPMI-1640 medium, containing Penicillin-streptomycin (10IU/ml-10µg/ml), Fungizone® (amphotericin B, 2.5µg/ml) and 5% foetal calf serum (FCS).

Cultured wild-type cells were incubated for 24 hours in phenol red-free RPMI containing L-glutamine (200mM), 5% FCS, and antibiotics. Cells were then incubated for 7 days in media containing either oestradiol (1nM in ethanol), fulvestrant (100nM in ethanol), or media alone, producing 7-day-oestrogen treated, 7-day-fulvestrant-treated and control/wild-type cells respectively. The influence of lengthened anti-hormone exposure, as experienced in clinical practice, was investigated using fulvestrant and tamoxifen resistant MCF-7 cells. Such cells were generated by culturing wild-type MCF-7 cells in white RPMI media containing inactivated FCS and fulvestrant (Faslodex®, 100nM) or 4-hydroxytamoxifen (100nM) respectively, for a minimum 6-month period.

Cultured cells were then lysed following three washes with phosphate buffered saline (PBS) by exposure to Halt Protease and Phosphatase Inhibitor Cocktail and ice-cold lysis buffer (Appendix A). Lysis product was transferred to eppendorfs, centrifuged (13,000 RPM, 4°C, 15 minutes), and stored at -20°C.

RNA isolation, amplification and detection

RNA samples were extracted from culture growing cells using a Tri® Reagent RNA Isolation Kit (Sigma-Aldrich, Gillingham, UK). Absorbance of samples (1 in 200 dilutions) was measured using a CECIL CE 2041 spectrophotometer (Cecil Instruments, UK). Absorbance of samples (1 in 200 dilutions) was measured using a CECIL CE 2041 spectrophotometer (Cecil Instruments, Cambridge, UK) at 260 and 280nm to determine total concentration.

The instability of isolated RNA demands it be reverse transcribed into relatively stable complementary DNA (cDNA) prior to amplification with Polymerase Chain Reaction (PCR). As such, 1µg of RNA sample and 11µl of RNA mastermix (Appendix B) were mixed in a sterile eppendorf and then denatured at 95°C for 5 minutes in a PCR thermal cycler (Techne TC-3000X, Bibby Scientific Ltd., Stone, UK). M-MLV reverse transcriptase and RNAase inhibitor (Fisher, UK) were added to the Eppendorf and the mixture reverse transcribed in the above machine (parameters in Appendix B) to form cDNA (stored at -20°C).

PCR was used to amplify small amounts of specific GR cDNA fragments using primer pairs (sequences in Appendix B). As with Western blotting, actin was used for control purposes. 0.5µl of cDNA from each treated cell sample was added to a PCR mastermix solution (Appendix B) to yield 4 samples: MCF-7 control (wild-type), MCF-7 oestrogen-treated, MCF-7 fulvestrant-treated and master-mix control (no cDNA), the latter to check for contamination. After centrifugation, samples were placed in a thermocycler at varying cycle numbers and parameters (Appendix B) determined by experimental optimisation. 7µl of each PCR product mixed with 5 µl of loading buffer (Appendix B) were loaded into the wells of a 2% agarose gel (Appendix B) containing RedSafe Nucleic Acid staining dye. The gel was run at 100V for 30 minutes using a Bio-Rad PowerPac. Once run, bands on the gel were visualised using G:Box (Syngene, Cambridge, UK) and GeneSys software (Syngene, Cambridge, UK).

Protein separation and expression

Protein contents of lysed cells were separated using Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE); see Appendix A for components of resolving gel, stacking gel and electrode running buffer. A total protein concentration of 20µg was analysed, mixed with an equal volume of loading buffer (Laemmli sample buffer [2x] with dithiothreitol [DTT]) (Appendix A). Once separated by SDS-PAGE, differential protein expression in the variably treated MCF-7 cell samples was assessed using Western blotting. Separated proteins were transferred from fragile gel medium to a solid nitrocellulose membrane by soaking in transfer buffer (Appendix A) and running at 100V for 1 hour. The nitrocellulose membrane was then submerged in Ponceau S stain for 30 seconds and washed in 1x Tris-buffered saline (TBS)-TWEEN 20 (Appendix A) prior to incubation with primary antibody specific to the protein of interest, in this case the GR and a actin control (New England Biolabs Ltd, UK) (Appendix C). A 1:1000 rabbit primary antibody dilution made in 1% Marvel milk solution made up in 1x TBS-Tween 20 was used. The membrane was subsequently incubated with secondary antibody (1:5000 IgG horseradish peroxidase [HRP] conjugated anti-rabbit antibody), specific for the Fc region of the primary antibody, to allow protein visualisation upon chemiluminescent exposure. 100µl of chemiluminescent detection reagents (Appendix A) were applied to the nitrocellulose membrane. The G:Box (Syngene, Cambridge, UK) and GeneSys software were used to visualise protein bands.

Statistical Analysis

Where applicable, densitometry using ImageJ quantification software (U.S. National Institutes of Health) was used to analyse PCR and western blot bands. Statistically significant differences were assessed using the student’s unpaired t-test and GraphPad software. Statistical significance was considered p≤0.05.
Fulvestrant treatment increases GR mRNA expression in MCF-7 cells. GR mRNA levels in MCF-7 breast cancer cell lines were compared by RT-PCR between cells given: no treatment (control/wild-type cells); 7-days of fulvestrant (100nM), or 7-days of oestrogen (1nM). Oestrogen-treated cells were included to provide the greatest differential for ER activity relative to fulvestrant. 7-day-fulvestrant-treatment visually up-regulated GR expression (Figure 1), though this was not statistically significant (Figure 2).

Increased GR mRNA expression is maintained in MCF-7 cells with acquired fulvestrant resistance. 7-days of fulvestrant therapy is insufficient to evoke acquisition of a fulvestrant resistant phenotype, given clinically resistance does not manifest until 2-3 years after therapy initiation. (7) GR involvement in anti-hormone resistance thus demands increased GR mRNA be sustained in fulvestrant-resistant cells. GR mRNA expression was up-regulated in fulvestrant-resistant versus wild-type MCF-7 cells (Figure 3). Difficulties obtaining PCR bands in this resistant cell sample precluded densitometry (n=1).

GR protein expression is increased in fulvestrant and tamoxifen resistant MCF-7 cells. GR mRNA alterations must translate to the protein level to confer biological significance. Thus, GR protein expression in wild-type (control) MCF-7 cells was compared to 7-day-fulvestrant-treated (Fas), tamoxifen-resistant (Tam-R) and fulvestrant-resistant (Fas-R) cells. GR protein (94kDa) was expressed in all control and treated cells, and unequivocally increased in fulvestrant-resistant, tamoxifen-resistant and 7-day-fulvestrant-treated cells relative to control, though the increase was most pronounced in anti-hormone resistant cell samples, evidenced by markedly denser bands in both fulvestrant and tamoxifen resistance (Figure 4). The increase in GR protein in fulvestrant-resistant cells was statistically significant (p=0.0345) compared to control (Figure 5), supporting a potential role for the GR in anti-hormone resistance.
DISCUSSION

Since 1977, breast cancer has been the most prevalent cancer in females within the United Kingdom with approximately 75% of cases ER-positive. (26) The efficacy of anti-hormone agents such as fulvestrant is limited by development of resistance in a large cohort of patients. (4) Numerous underlying mechanisms have been postulated but, as of yet, fail to fully explain this acquisition of resistance. (27) Accordingly, this study investigated the potential role of the GR in driving resistant cell growth in the ER positive MCF-7 breast cancer cell line, with the aim of better understanding the resistance process.

At the mRNA level, alterations in GR expression in both 7-day-fulvestrant treated and fulvestrant-resistant cells were not statistically significant. Considering up-regulation was significant in resistance at the protein level, this may reflect the small sample size (n=3) of the study. Alternatively, particularly regarding 7-day-treated cells, it implies that transcriptional alterations require longer to manifest, and so do not become apparent until later on in the resistance process. This is not wholly unreasonable, given clinical indications of acquired resistance are only apparent 2-3 years after commencement of therapy. (4, 28)

In the present study, a meaningful contribution of the GR in endocrine resistance was suggested by observation of potent and statistically significant increase in GR protein up-regulation in fulvestrant-resistance. Tamoxifen-resistant cells also exhibited an increase in GR protein, though this was not statistically significant and could be reflective of the small sample size or a non-genuine change. In the ER positive MCF-7 cell line, other authors have observed reduced GR levels when compared to expression in ER negative cells, (29) which has subsequently been shown to result from oestrogen suppression of GR. (30-32) Therefore, the GR up-regulation observed in fulvestrant-resistant cells in this study may in part be explained by the essentially ER negative status, conferred by fulvestrant-mediated down-regulation of ER. (8) Interestingly, high GR expression in ER negative disease is also linked to worse disease free survival. (14) Hence, fulvestrant degradation of ER plausibly accounts for both the observed increase in GR with anti-hormone therapy and the negative clinical implications of this in terms of resistance.

Notably, the phenomenon of anti-hormone-induced GR up-regulation is not unique to breast cancer. Similar patterns have been observed in other, also predominately hormone-driven malignancies, such as prostate cancer, (33) with pharmacological androgen receptor (AR) blockade circumventing AR repression of GR levels, resulting in GR up-regulation. (34)

Dexamethasone, a GC, is routinely used alongside anti-hormones in breast cancer as a potent analgesic, or to relieve cancer-associated inflammatory symptoms. (20) Hence, implication of GR-mediated GC-induced transcription of survival genes in breast cancer has concerning implications surrounding the clinical worth of adjuvant GCs. (35, 36) Speculatively, if anti-hormone resistance is indeed stimulated by the GR, current practice of administering exogenous agonists such as dexamethasone, whilst beneficial in the short-term, could be having an overall detrimental impact. Hence, there is an urgent need for a prospective randomised controlled clinical trial investigating the effect of concomitant use of, and pre-treatment with, GCs.

The importance of ameliorating treatment-induced side effects cannot be downplayed. However, given the uncertainty of GCs in breast cancer, perhaps a different and safer approach should be employed, for example non-steroidal anti-inflammatory drugs. (37) Alternatively, concomitant use of anti-hormones with a GR antagonist, such as mifepristone, may show promise in delaying or preventing resistance, (38) though this needs further investigation.

Furthermore, in experimental models of prostate cancer, the GR gene product SGK-1 was inhibited, resulting in reversal of resistance to androgen therapy. (34) Indeed, this may be superior to systemically administered GR inhibitors which would inevitably have widespread undesirable effects.

The overriding limitation of this study is the small number of experimental repeats performed which may mean observed changes have arisen due to random chance. Furthermore, breast cancer is clinically heterogeneous, with combinations of expressed receptors varying greatly and co-expression (or lack of) of notable significance. This study investigated only one cell line, MCF-7, which restricts the generalisability of conclusions drawn from the data. Future studies utilising other cell lines would thus be of value. Investigation of other hormonal therapies is also warranted, and particularly tamoxifen given its widespread use. Similarly, the widespread use of dexamethasone as an adjuvant therapy compels investigation into the biological changes this may be evoking.

In conclusion, the present study demonstrates significantly increased GR protein in both fulvestrant-resistant and tamoxifen-resistant MCF-7 cells, supporting the hypothesis that GR signalling may have a role in driving anti-hormone resistance in breast cancer. Given the implications of these data, and the scale of anti-hormone resistance in breast cancer, further confirmatory studies are necessitated.
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PMid:23693080

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PMid:21818591

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PMid:24016618 PMCid:PMC3860283
APPENDIX A  

SOLUTIONS

Lysis Buffer (100mL), pH 7.6

- Tris base (50mM) 0.61g
- EGTA (5mM) 0.19g
- NaCl (150mM) 0.87g
- Triton (1%) 1ml

2X Laemmli Sample Buffer (10mL)

- 10% sodium dodecyl sulphate (SDS) 4ml
- 20% glycerol 2ml
- Tris buffer (0.5M, pH 6.8) 2.4ml
- Distilled water 1.6ml
- Bromophenol blue 1mg
- Dithiothreitol (DTT) 15.4mg

7.5% Acrylamide Resolving Gel (10mL)

- Distilled water 4.8ml
- Tris buffer (1.5M, pH 8.8) 2.5ml
- 30% Acrylamide solution 2.5ml
- 10% Sodium dodecyl sulphate (SDS) 0.1ml
- 10% Ammonium persulphate (APS) 0.1ml
- Tetramethylethylenediamine (TEMED) 6µl

4% Acrylamide Stacking Gel (10mL)

- Distilled water 6.1ml
- Tris buffer (2.5M, pH 6.8) 2.5ml
- 30% Acrylamide solution 1.3ml
- 10% Sodium dodecyl sulphate (SDS) 0.1ml
- 10% Ammonium persulphate (APS) 0.1ml
- Tetramethylethylenediamine (TEMED) 10µl

1X Running Buffer (1L)

- Tris base (0.25M) 3.02g
- Glycine (1.92M) 14.4g
- 0.1% sodium dodecyl sulphate (SDS) 1.0g

Transfer Buffer (1L)

- Tris Base (0.25M) 3.03g
- Glycine (1.92M) 14.4g
- 20% Methanol 200ml
- Distilled Water 800ml
1X TBS-Tween (1L)
- Tris base: 1.21g
- NaCl: 5.8g
- HCl (5M, pH 7.6): 1.5ml
- Tween 20: 0.5ml

Ponceau S stain: 0.1% (w/v) in 5% acetic acid

5% Milk: 1.5g Marvel skimmed milk powder in 30mL 1X TBS-Tween

1% Milk: 0.5g Marvel skimmed milk powder in 50mL 1X TBS-Tween

Detection Reagents (Pierce and Warriner Ltd., Chester, UK)
- SuperSignal West Pico Chemiluminescent Substrate
- SuperSignal West Dura Chemiluminescent Substrate
- SuperSignal West Femto Chemiluminescent Substrate

Reverse transcription PCR Mastermix Solutions
- 5µl Deoxyribonucleotide Triphosphates (dNTP) (2.5mM)
- 4µl 5X PCR Buffer
- 2µl Random Hexamers (100µM)

Reverse Transcription Cycling Parameters
- 22°C, 10 minutes (annealing)
- 42°C, 42 minutes (chain elongation)
- 95°C, 5 minutes (denaturing)

PCR Mastermix Solution
- 18.75µl sterile RNA/DNAase free water
- 2.5µl 10X PCR buffer (Fisher Scientific, Loughborough, UK)
- 2µl deoxyribonucleotide triphosphates (dNTP) (2.5mM stock)
- 0.625µl of each primer (20µM stock)
- 0.75µl MgCl (50mM stock)
- 0.2µl Taq DNA Polymerase (Fisher Scientific, Loughborough, UK)

PCR Primer Sequences
B-actin
Forward: 5’ GGA GCA ATG ATC TTG ATC TT
Reverse: 5’ TCC TGA GGT ACG GGT CCT TCC
GR
Forward: 5’TCT GAA CTT CCC TGG TCG AA
Reverse: 5’ GTG GTC CTG TTG TTG CTG TT

**PCR Cycling Parameters**

1st Cycle
- 95°C, 2 minutes (denaturation)
- 55°C, 1 minute (annealing)
- 72°C, 5 minutes (chain elongation)

*Intermediate cycles*
- 94°C, 30 seconds
- 55°C, 1 minute
- 72°C, 1 minute

*Final Cycle*
- 94°C, 1 minute
- 55°C, 1 minute
- 60°C, 10 minutes

**2% PCR agarose gel (50ml)**
- Agarose 2g
- 1X Tris acetate-EDTA buffer (TAE) 100ml
- RedSafe Nucleic Acid Staining Solution 5µL

**DNA loading buffer**
- 40% sucrose 4g
- Bromophenol blue (0.25%) 25mg
- Sterile pure nuclease free water 10ml
## APPENDIX C

### ANTIBODIES

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Animal origin species</th>
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<tbody>
<tr>
<td>GR</td>
<td>Rabbit</td>
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<tr>
<td>β-actin</td>
<td>Rabbit</td>
</tr>
</tbody>
</table>

### Primary Antibody Solutions (Dilution of 1 in 1000 in 1% milk)

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Primary Antibodies visualised</th>
</tr>
</thead>
<tbody>
<tr>
<td>2(^*) Anti-rabbit</td>
<td>GR</td>
</tr>
</tbody>
</table>

### Secondary Antibody Solutions (Dilution of 1 in 5000 in 1xTBS-TWEEN-20)