Neoadjuvant endocrine therapy in breast cancer up-regulates the cytotoxic drug pump *ABCG2*/BCRP, and may lead to resistance to subsequent chemotherapy

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Conflicts of interest

The authors have no conflicts of interest to declare.

Compliance with Ethical Standards

MicroAbstract

ABCG2/BCRP, expression of which is commonly associated with chemoresistance, was found to be significant up-regulated in tumour cells after neoadjuvant endocrine therapy in three separate cohorts of primary breast cancer patients. Endocrine-induced up-regulation of ABCG2/BCRP *in vitro* was associated with resistance to subsequent treatment with chemotherapy.

ABSTRACT

Introduction: Neoadjuvant treatments for primary breast cancer are becoming more common, however little is known about how these impact on response to subsequent adjuvant therapies. Conveniently, neoadjuvant therapy provides opportunities to consider this question, by studying therapy-induced expression changes using comparisons between pre- and post-treatment samples. These data are relatively lacking in the context of neoadjuvant endocrine therapy, as opposed to the more common neoadjuvant chemotherapy. Here, we investigate the relevance of expression of the xenobiotic transporter *ABCG2*/BCRP, a gene/protein associated with chemoresistance, in the context of neoadjuvant endocrine therapy and particularly with reference to subsequent chemotherapy treatment.

Materials and methods: *ABCG2*/BCRP expression was assessed by immunohistochemistry or by expression arrays in matched patient samples pre- and post-neoadjuvant endocrine therapy. Cell culture was used to model the impact of endocrine therapy induced changes in *ABCG2*/BCRP on subsequent chemotherapy response, using Western blots, qPCR, survival assays and cell cycle analyses.

Results: *ABCG2*/BCRP was commonly and significantly up-regulated in breast cancers after treatment with neoadjuvant endocrine therapy in three separate cohorts encompassing a total of 200 patients. Treatment with the endocrine therapeutic tamoxifen similarly induced *ABCG2*/BCRP up-regulation in a relevant model cell line, the estrogen receptor positive line T47D. Critically, this up-regulation was associated with significantly increased chemoresistance to subsequent treatment with epirubicin, an anthracycline commonly used in breast cancer adjuvant chemotherapy.

Conclusion: Our data suggest that NAET may induce poor responses to adjuvant chemotherapy, and therefore that clinical outcomes following this treatment sequence warrant further study.

Introduction

Use of neoadjuvant therapies for treatment of primary breast cancer is becoming more frequent for at least two reasons. First, these therapies can down-stage tumours, thereby enabling increase rates of breast conserving surgery as opposed to mastectomy[1]. Secondly, they can provide opportunities to assess tumour responses to specific therapeutics using longitudinal imaging and clinical assessments, therefore allowing switching to potentially more effective treatment regimens if initial responses are deemed inadequate[2]. Chemotherapy is the most common neoadjuvant approach, however neoadjuvant endocrine therapy (NAET) is preferred in some patients with ER-positive disease[3], and is particularly accepted in patients who are elderly, frail or have problematic co-morbidities and therefore extended non-surgical management may be desirable[4]. Increased consideration of NAET in selected patients has recently been recommended[5, 6] on account of similar overall response rates to neoadjuvant chemotherapy[3] with lower toxicity[7], although it should be noted that complete pathological responses are far rarer with NAET. It has also been suggested that clinical or molecular responses to NAET should be integrated with other factors to stratify patients to appropriate adjuvant treatments such as chemotherapy[8, 9].

Use of neoadjuvant therapies provides powerful opportunities to assess molecular responses of cancers to specific therapies by comparison between matched pre-treatment diagnostic samples and post-treatment resection samples. These comparisons are quite prevalent in the context of neoadjuvant chemotherapy, both at the level of individual genes or at the transcriptome level[10-12], but many fewer studies are available for NAET, with only a handful of transcriptome-wide investigations[13-17]. A focus of many of these studies has been identification of potential induced mechanisms of resistance to the given neoadjuvant therapy. Little attention has been given to how molecular changes resulting from these neoadjuvant therapies might impact on response to subsequent adjuvant treatments. This question may have growing importance if response to NAET is to be used to stratify patients for assignment to adjuvant chemotherapy[8, 9].

We have an interest in roles of xenobiotic drug pumps in resistance to cancer therapies, and have previously shown that expression of Breast Cancer Resistance Protein (BCRP), encoded by the *ABCG2* gene, can be associated with poor survival after chemotherapy in breast cancer[18]. In this new work, we were interested to assess whether NAET impacted on expression of *ABCG2/*BCRP, and whether this could have implications for subsequent adjuvant chemotherapy responses.

Material and methods

Patient selection, ethical approval and immunohistochemistry

Ethical approval for use of patient samples and anonymised data and for the consent process used was obtained from Leeds (East) Research Ethics Committee (reference 06/Q1206/180). Informed consent was obtained when appropriate. Primary breast cancer patients treated with neoadjuvant endocrine therapy (NAET) at Leeds Teaching Hospitals NHS Trust from 2005-2013 were identified. Criteria for inclusion in our study were NAET duration of 1 month to 1 year, NAET alone without combination therapy (for example, excluding individuals on the NEO-EXCEL trial who additionally received celecoxib), a diagnosis of invasive ductal or lobular carcinoma, Allred score for estrogen receptor expression of 7 or 8, no change in NAET regime during treatment, and lack of HER2 overexpression. In addition, we required that tissue before NAET (diagnostic biopsies) and after NAET (resection) was available. This identified a cohort of 51 patients. Relevant clinico-pathological data are outlined in Table 1. Tissues were sectioned at 5μm onto SuperFrost Plus slides (Menzel-Glaser, Braunschweig, Germany). Matched biopsy and resection samples were placed on the same single slide therefore subsequent staining/analysis conditions for the pairs were identical and relative expression between them was directly comparable. Immunohistochemistry was performed and quantified exactly as described previously[18]. In brief, sections were dewaxed and rehydrated and further antigen retrieval was not necessary. Endogenous peroxidase activity was blocked using H_2O_2. BCRP staining was performed with clone BXP-21 (Abcam, Cambridge, UK), which has been used and validated in clinical breast tissue previously by us and others[18, 19], at 1:50 for 16h at 4°C. BCRP staining was visualised using Envision reagents (Dako, Gostrup, Denmark) and sections were counterstained in Mayer's haematoxylin. Sections were digitally scanned using Scanscope XT and were analysed using Imagescope (Aperio, Vista, USA). Staining was assessed under guidance of breast histopathologists (AMH and ETV). Positive (brown) staining was quantified in tumour cells only by weighted histoscores using a semi-automated protocol, validated extensively previously[18]. In brief, tumour epithelial regions were manually marked on digital images and positive staining was quantified within these using the positive pixel count algorithm in three intensity ranges to ape manual scoring (counts of <100 defined as weakly positive, 100 to <175 as moderate, and <=175 as strong). Percentages of total pixels categorized into each intensity band were used to determine automated histoscores: (1x% weakly positive pixels)+(2x% moderate)+(3x%strong).

Cell culture and drug treatments

T47D cells were obtained originally from the European Collection of Animal Cell Cultures. Cell line identity was confirmed (STR profiles, Leeds Genomics Service) and cells were consistently negative for mycoplasma (MycoAlert Mycoplasma detection assay, Lonza, Basal, Switzerland). Cells were cultured in DMEM supplemented with 10% foetal calf serum and 1% penicillin/streptomycin (reagents from Thermo Fisher, Waltham, USA). Cells were treated with final concentrations of $1\mu M$ or $5\mu M$ tamoxifen (Sigma, Poole, UK) or appropriate amounts of ethanol (the vehicle for tamoxifen), namely 0.1% (v/v) or 0.5% (v/v) ethanol and incubated as normal.

Harvesting protein and RNA

Cells were harvested using trypsin and divided, with three-quarters of the cells to be used for protein extraction and the remainder used for RNA extraction. Cells were washed with phosphate buffered saline (PBS). For protein extraction, cells were washed again with PBS before being lysed for fifteen minutes on ice in RIPA buffer (10mM Tris-HCl, 140mM NaCl, 0.1% SDS, 1% Triton X-100, 0.1% sodium deoxycholate) supplemented with the chelating agents EDTA and EGTA and the protease inhibitor PMSF (Sigma, Poole, UK). RNA extraction was performed using Promega's (Madison, USA) RNA extraction kit using the manufacturer's instructions. In brief, cells were lysed in BL buffer supplemented with thio-glycerol. Isopropanol was added and the sample added to a spin column. Samples were washed and treated with DNase I before being eluted in nuclease-free water.

Expression analyses (SDS-PAGE and western blotting; qPCR)

SDS-PAGE and transfers were performed as described previously[20] using 4-12% Bis-Tris gels, PVDF membrane, and other reagents from Thermo Fisher (Waltham, USA). After transfer, membranes were blocked in 1 or 5% milk in TBS-T. Antibodies were diluted in 1% milk/TBS-T. Primary antibodies (anti-beta-actin, 1:10000, Sigma, Poole, UK) or anti-BCRP (clone BXP-21, 1:250, Abcam, Cambridge, UK) were incubated overnight at 4°C followed by washing with TBS-T then incubation with the secondary antibody (anti-mouse HRP conjugate, 1:2000, Abcam, Cambridge, UK) for 1h. Blots were visualised using SuperSignal West Pico (Thermo Fisher, Waltham, USA) and Bio-Rad Gel Doc Imaging system, and quantified using Image Lab software (version 5.2.1). Reverse transcription of RNA was performed using the GoScript Reverse Transcription System (Promega, Madison, USA) according to the manufacturer's instructions. In brief, total RNA was diluted in nuclease-free water before incubation with random primers at 70°C for 5min. A reaction buffer mixture including reverse transcriptase and nucleotides was then added and the reactions were

placed in a controlled temperature heat block at 42°C to allow reverse transcription to occur. Quantification of mRNA levels of the control (beta-actin) and the gene of interest (*ABCG2*) was performed using the GoTaq qPCR Master Mix kit (Promega, Madison, USA) according to the manufacturer's instructions. In brief, a total volume of 10μl was plated in a 96-well plate containing diluted cDNA, CXR Reference Dye, GoTaq qPCR Master Mix and forward and reverse primers for each gene of interest (actin: 5'-TTCTACAATGAGCTGCGTGTG-3' and 5'-GGGGTGTTGAAGGTCTCAAA-3'; ABCG2: 5'-CAGGTGGAGGCAAATCTTCGT-3' and 5'-ACACACCACGGATAAACTGA-3'). Reactions were performed in the ABI 7500 qPCR machine (Thermo Fisher, Waltham, USA).

Cell viability assay (MTT)

After 15 days of treatment with tamoxifen, fresh media was placed on the cells for 24h. Cells were then trypsinised and plated into 96 well plates (Corning, New York, USA). Cells were left for another 24h before being treated with 2.6μM or 10μM epirubicin (Sigma, Poole, UK) or water (the vehicle for epirubicin), and incubated as normal for a further 24h. MTT assays (Thermo Fisher, Waltham, USA) were then performed as previously described[21], with readings taken using the Mithras LB 940 Multimode Microplate Reader (Berthold Technologies, Harpenden, UK).

Cell cycle analysis

After pre-treatment with tamoxifen, cells were given fresh media for 24h. Cells were then fixed in 70% ethanol for 2h. Cells were washed then resuspended in PBS before addition of propidium iodide (Sigma, Poole, UK) at 0.02mg/ml and RNase A (Thermo Fisher, Waltham, USA) at 0.4mg/ml. After incubation for 20min at room temperature in the dark, cell cycle status was analysed using the Attune Acoustic Focusing Cytometer (Thermo Fisher, Waltham, USA) and accompanying Attune Cytometric Software Version 2.1.

Analysis of publicly available gene expression datasets

Microarray data from related breast cancer studies looking at primary tumours or cell lines were downloaded from NCBI Gene Expression Omnibus (www.ncbi.nlm.nih.gov/geo/). Primary tumour datasets looking at the effects of 2 weeks and 3 months of letrozole from Edinburgh (GSE20181) and 2 weeks of an unspecified aromatase inhibitor in Houston (GSE87411) were considered, along with a dataset of breast tumours that were untreated between diagnosis and surgery, again from Edinburgh (GSE76728). Gene expression data on MCF7 breast cancer cell line following tamoxifen-treatment (GSE21618) and long term

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estrogen deprivation (GSE20361) were also considered. Normalised pre-processed data was used in all cases.

Results

ABCG2/BCRP is up-regulated post-NAET in breast cancer patients

We were interested to study changes in expression patterns in primary breast tumours induced by NAET. Since we have previously studied xenobiotic drug pumps that are known to impact on outcomes in breast cancer[18, 20], we focused on one of these pumps - *ABCG2*/BCRP. We identified a cohort of fifty-one breast cancer patients treated with NAET in Leeds and for whom tumour tissue was available from both pre-NAET (diagnostic biopsies) and post-NAET (resection tissue). Clinico-pathological details of these patients are shown in Table 1. We detected BCRP using immunohistochemistry and quantified expression objectively using an automated scoring system, as previously described[18]. Representative staining in a matched pair of pre- and post-NAET samples is shown in Fig 1A, while expression levels in all samples are shown in Fig 1B, with lines indicating the matched samples from individual patients for comparison. Expression was up-regulated after NAET in 48/51 (94%) cases and up-regulation was significant overall (p<0.0001; Fig 1B). To confirm whether *ABCG2* was increased in other breast cancer cohorts following endocrine treatment, we examined published microarray mRNA expression datasets of matched samples from breast cancer patients, taken before and after aromatase inhibitor treatment. Levels of *ABCG2* was found to be up-regulated after NAET in the majority of patients in two cohorts from Edinburgh and Houston[14, 15] after two weeks and three months, and up-regulation was significant (p<0.01 in all cases, paired Wilcoxon). By way of a negative control, *ABCG2* expression was not significantly changed (p=0.12) in a cohort of 37 breast tumours that did not receive treatment between diagnosis and surgery[22] (Fig 1C), where the interval ranged from 13 to 53 days (mean 27.5 days).

ABCG2/BCRP is up-regulated by tamoxifen in estrogen receptor positive breast cancer cell lines

Having determined that *ABCG2*/BCRP is up-regulated post-NAET in patients, we were interested to investigate whether this could be reproduced *in vitro* using a breast cancer cell line, and if so, what the implication of this might be. Therefore, we treated the ER-positive cell line T47D with the endocrine therapeutic tamoxifen and examined BCRP/*ABCG2* expression using Western blots and/or qPCR. BCRP was up-regulated as quickly as 7h after

treatment with1μM tamoxifen (Fig 2A), and this up-regulation increased with up to 3 days of continuous treatment (Fig 2B). Significant and dose-dependent up-regulation of both transcript (*ABCG2*) and protein (BCRP) was evident after 15 days of continuous tamoxifen treatment at 1 and 5μM (Fig 2C). Using publically available datasets[23, 24], we have also shown up-regulation of *ABCG2* in response to 1μM tamoxifen treatment and in response to estrogen (E2) withdrawal in another ER-positive cell line, MCF7 (Fig 2D), suggesting that our findings are not limited to T47D cells. Physiological intra-tumoural concentrations of tamoxifen have been estimated as between 0.5 to 2μM[25-27], therefore these doses used *in vitro* are within an appropriate range.

Tamoxifen pre-treatment leads to resistance to subsequent chemotherapy in vitro

Increased *ABCG2*/BCRP expression can be associated with resistance to many standard cytotoxic chemotherapeutics[28, 29], so we were next interested to assess whether tamoxifen-induced changes in *ABCG2*/BCRP expression would have an impact on subsequent chemo-response. Therefore, we again treated cells with control or two different doses of tamoxifen (1 or 5μM) for fifteen days, before removing tamoxifen and treating with control or two different doses of epirubicin (2.6 or 10μM), an anthracycline chemotherapeutic drug frequently used in breast cancer treatment and known to be an *AGCG2* substrate [30]. Relative cell survival was determined using MTT assays (Fig 3A). In cells without tamoxifen pre-treatment (black bars), epirubicin treatment caused a dose-dependent reduction in cell survival of up to 76%. Pre-treatment with either dose of tamoxifen increased cell survival from epirubicin, most notably the higher tamoxifen dose giving significant protection from 10μM epirubicin (p<0.05) with increased survival by more than 2-fold.

One explanation for the chemoresistance shown by cells pre-treated with tamoxifen would be that tamoxifen-induced exit from cell cycle was providing protection from the effects of epirubicin, which at least in part targets cells undergoing DNA replication. In order to support or refute this hypothesis, we next examined the influence of 15 days of tamoxifen treatment on the cell cycle in T47D cells using propidium iodide staining and flow-cytometry (Fig 3B). There were no significant alterations in the cell cycle profile of tamoxifen pre-treated cells, providing no support for the hypothesis that cell cycle changes were responsible for the increased chemoresistance. In this context, we believe our data support a direct functional role for BCRP up-regulation in chemoresistance after endocrine treatment.

Discussion

Here, we present the first study to address the influence of NAET on *ABCG2*/BCRP expression in clinical cohorts of breast cancers. A number of previous studies have assessed the impact of estrogens on *ABCG2*/BCRP expression in breast cancer cell lines[31-33]. Unfortunately, published data are conflicting, and there is no clear consensus concerning whether estrogens stimulate or repress expression across a range of cell lines. For example, it has been reported that treatment with estradiol repressed BCRP expression in ER-positive breast cancer cells lines, including T47D as used in our study[31], findings that are compatible with our observations that blocking estradiol function with tamoxifen resulted in *ABCG2*/BCRP up-regulation. However, by contrast, others have shown estradiol to induce transcriptional up-regulation of *ABCG2* at one of its promoters in T47D cells as well as other ER-positive cancer lines[32], and the anti-estrogen toremifene to repress both ABCG2 mRNA and BCRP expressions in ER-positive MCF7 cells[33]. This literature likely reflects the complexity of *ABCG2*/BCRP regulation in different cell types and under different culture conditions[34]. Nevertheless, our clinical observation that BCRP up-regulation in 48/51 patients after NAET is highly consistent and statistically significant in our Leeds cohort, and in all other available relevant clinical datasets (Fig 1). The up-regulation of BCRP we observe after tamoxifen treatment in T47D cells (Fig 2) is compatible with all studies in this cell line that have looked at endogenous protein, and similar increases in *ABCG2* were observed for tamoxifen treatment or E2 withdrawal in MCF7 cells.

A concerning conclusion from these observations is that NAET-induced up-regulation of *ABCG2*/BCRP could potentially reduce the efficacy of adjuvant chemotherapy treatment in breast cancer patients. We have attempted to model this effect using T47D cells, in which *ABCG2*/BCRP is up-regulated after initial treatment with tamoxifen. We find clear evidence that pre-treatment with tamoxifen protects the cells from chemotherapy, and this appears to be independent of any cell cycle effects (Fig 3). Unfortunately, the hypothesis that patients who received NAET respond relatively poorly to adjuvant chemotherapy is not easy to test using existing clinical data since treatment with NAET followed by adjuvant chemotherapy is currently an uncommon clinical pathway. From our initial Leeds cohort of 51 patients in which we examined BCRP expression pre- and post-NAET, only 6 patients received adjuvant chemotherapy. From the Edinburgh cohort of 55, only 2 received adjuvant chemotherapy. Therefore, outcome data on a large enough number of patients are not available to evaluate the effect of NAET on adjuvant chemotherapy. Furthermore, it is not clear what represents a suitable comparator group to allow relative assessment of chemotherapy response, since patients who receive NAET are typically deemed suitable for

this therapy for specific clinical reasons that likely mean they are not usefully comparable to other groups. A formal randomised clinical trial may be the only way to allow a robust assessment of whether NAET negatively influences responses to adjuvant chemotherapy; such a trial is unlikely and even if it took place outcomes would not be known for many years as these ER-positive cancers overall have good prognoses and recurrences tend to be late. However, the potential for NAET to impair responses to chemotherapy may well be worth noting for the future, particularly in the context that it has been suggested that response to NAET could be a useful tool to stratify patients to adjuvant chemotherapy[8, 9], and some have combined endocrine therapy with chemotherapy in the neoadjuvant context[35]. Our results suggest that analyses of outcomes with these approaches in the future is warranted.

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| Characteristic	Categories	No. of patients (%) n=51
Age	Median: 67 years old (range 40-95 years old)	
Histological type	IDC	38 (74.5)
	ILC	7 (13.7)
	IDC/ILC	7 (13.7)
Grade	1	15 (29.4)
	2	30 (58.8)
	3	6 (11.8)
Stage (pre-NAET)	T1	17 (33.3)
	T2	26 (51)
	T3	5 (9.8)
	T4	3 (5.9)
Stage (post-NAET)	T1	19 (37.3)
	T2	25 (49)
	T3	7 (13.7)
Tumour size change	Increase or no change	22 (43.1)
	Decrease	29 (56.9)
Lymphovascular invasion	Positive	11 (21.6)
Receptor status (4/8 as cut-off for positive expression)	ER	51 (100)
	PR	37 (78.7)
NAET duration	Median: 90 days (range 30-362 days)	
NAET regimen	Anastrozole	24 (47.1)
	Letrozole	20 (39.2)
	Tamoxifen	7 (13.7)
Axillary metastasis	Positive	21 (41.2)
Surgery	breast conserving	22 (43.1)
	Mastectomy	29 (56.9)

Table 1. Clinico-pathological characteristics of the breast cancer patients
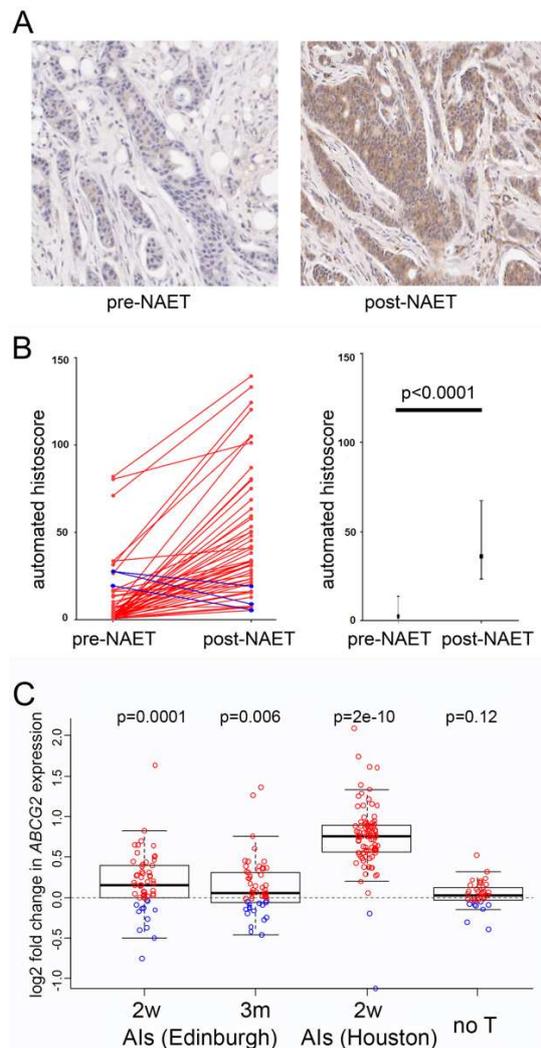
Fig 1 BCRP expression is increased in patients post-NAET. 51 matched pre- and post-NAET breast tumour samples were stained for BCRP using immunohistochemistry and expression was quantified using automated histoscores. (A) Representative images of a matched pre- and post-NAET tissues showing BCRP expression (brown). (B, left) Individual scores for pre- and post-NAET tissues with lines connecting the matched samples. Red lines indicate increases in BCRP expression and blue lines indicate decreases. (B, right) Median histoscore values with interquartile range (significance assessed using the Wilcoxon signed rank test). (C) Expression of *ABCG2* (the transcript encoding BCRP) was assessed using expression microarrays in pre-NAET samples and matched post-NAET breast cancer samples in two separate cohorts of patients. In the Edinburgh cohort (n=55), patients were treated with letrozole and change in expression assessed after 2 weeks (2w) and 3 months (3m). In the Houston cohort (n=94), patients were treated with aromatase inhibitors (no information as to which) and change in expression was assessed after 2 weeks (2w). Expression was also assessed in matched diagnostic biopsy and surgical excision samples from breast cancer patients who received no intervening treatment ("no T"; n=37). Expression in later samples is shown relative to the first as log2 fold change, with red and blue indicating up- or down-regulation respectively. Median change in expression is shown by the black line, boxes show the upper and lower quartiles, and whiskers 1.5x the interquartile range. Significance of changes in expression was assessed using paired Wilcoxon tests.
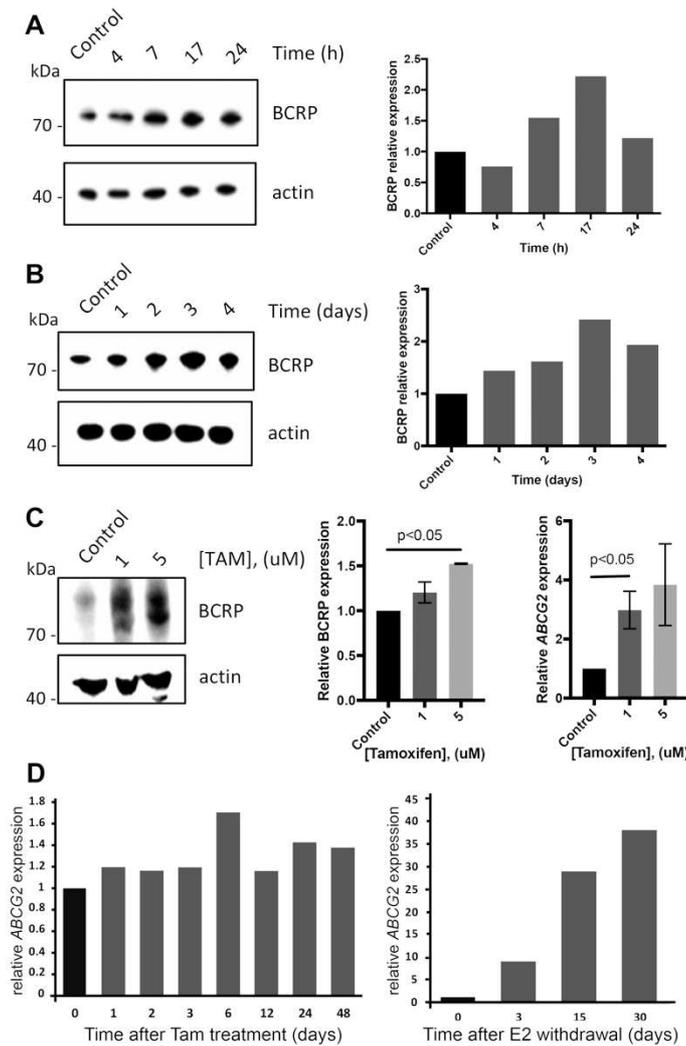
Fig 2 *ABCG2*/BCRP is up-regulated by tamoxifen in ER-positive breast cancer cells.
(A-C) T47D cells were treated with 1μM or 5μM tamoxifen or vehicle control (100% ethanol)
for the indicated lengths of time (vehicle control at the longest timepoint) before analysis for
BCRP protein expression (western blot and densitometry) or *ABCG2* transcript expression
(qPCR). (A) BCRP expression was determined after a single dose of 1μM tamoxifen. A
representative western blot is shown on the left and the accompanying histogram depicting
relative BCRP expression on the right. (B) BCRP expression was determined after a single
dose of 1μM tamoxifen. A representative western blot is shown on the left and the
accompanying histogram depicting relative BCRP expression on the right. (C) Cells were
treated daily with either 1μM or 5μM tamoxifen for fifteen days. The left plot shows a
representative western blot. The middle plot shows densitometry of relative BCRP
expression (independent experiments n=2, +/- SEM). The right plot shows relative ABCG2
expression (independent experiments n=3, +/- SEM). (D) MCF7 cells were treated with 1μM
tamoxifen or were deprived of estrogen and gene expression was assessed at various
timepoints using expression arrays[23, 24]. ABCG2 expression is shown relative to untreated;
the left plot shows effect of tamoxifen treatment while the right plot shows effect of estrogen
deprivation at the timepoints indicated.
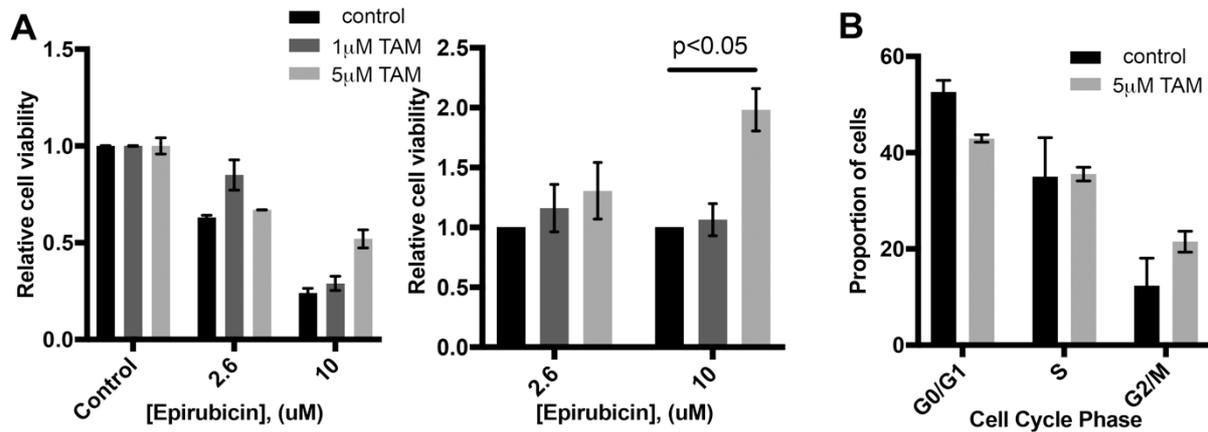
Fig 3 Pre-treatment with tamoxifen induces relative chemoresistance. T47D cells were treated with either tamoxifen (1μM or 5μM) or vehicle control (100% ethanol) daily for fifteen days. (A) Tamoxifen or control was then removed, and cells were treated with 2.6μM or 10μM epirubicin or vehicle control (water) for 24h before relative survival was determined using MTT assays. The left panel shows a representative graph of the effects of epirubicin on viability of cells following pre-treatment with TAM. Error bars show the standard deviation of three technical repeats. The right panel shows 2 biological repeats (+/- SEM) of the experiment depicted in the left panel, normalising the tamoxifen pre-treated data to the control-pre-treated sample for each dose of epirubicin, to allow focus on the differences between tamoxifen pre-treated vs without tamoxifen pre-treatment. (B) After 15 days of pre-treatment with 5μM tamoxifen or vehicle control, cells were stained with propidium iodide and analysed with by flow cytometry to determine proportions of cells in each of the three cell cycle stages. Error bars represent SEM of two biological repeats.