Cooperative effect of gefitinib and fumitremorgin c on cell growth and chemosensitivity in estrogen receptor α negative fulvestrant-resistant MCF-7 cells

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Abstract. The selective ER downregulator, fulvestrant, is currently approved as a second line endocrine therapy after onset of resistance to prior antiestrogen therapy in post-menopausal breast cancer patients. Resistance to anti-hormonal therapies is common and, therefore, we anticipate that fulvestrant-resistance will occur as well. The current study was undertaken to investigate the underlying molecular changes after fulvestrant-resistance and find new therapeutic targets and agents for fulvestrant-resistant breast cancer cells. We developed a unique fulvestrant-resistant cell line (MCF-7/F), derived from MCF-7 estrogen receptor α (ERα)-positive human breast cancer cells, by culturing them in 1 μM fulvestrant containing medium for ~18 months. MCF-7/F cells became irreversibly ERα negative as withdrawal of fulvestrant did not alter the ERα-negative phenotype, determined by real-time PCR, Western blot analysis, and ERE-luciferase transfection assays. MCF-7/F cells grew in a hormone-independent manner. Interestingly, MCF-7/F cells over-expressed both epidermal growth factor receptor (EGFR) and breast cancer resistant protein (BCRP). Gefitinib, a specific EGFR tyrosine kinase inhibitor, preferentially inhibited the growth of MCF-7/F cells relative to MCF-7 cells by inhibiting both $MAPK^{44/42}$ and Akt phosphorylation. MCF-7/F cells became less sensitive to chemotherapeutic agents such as mitoxantrone. Moreover, fumitremorgin C, a specific BCRP inhibitor, significantly increased the efficacy of mitoxantrone in MCF-7/F cells. Gefitinib increased the inhibitory effect of mitoxantrone on cell growth. Similarly, fumitremorgin C increased the inhibitory effect of gefitinib on cell growth, suggesting that there is a bidirectional crosstalk between EGFR and BCRP. More importantly, these results provide a molecular basis for using gefitinib, BCRP inhibitors, and chemotherapeutic agents as combination therapy approaches in fulvestrant-resistant breast cancer.

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Introduction

Approximately two-thirds of breast cancer is estrogen receptor α (ERα) and/or progesterone receptor (PgR) positive. In ERα positive breast cancer, estrogens stimulate tumor growth by activating ERα transcription activity. Targeting the ERα with antiestrogen therapy has resulted in substantial advances in the treatment of breast cancer (1). Tamoxifen, a non-steroidal antiestrogen, has been used successfully for the treatment of all stages of ERα positive breast cancer (1), resulting in increased disease-free and overall survival (2). However, tamoxifen is a partial ERα agonist and as a result, has undesirable side-effects such as an increase in the incidence of endometrial cancer. In addition, most patients that initially respond to tamoxifen will eventually develop resistance during therapy. As a result of this knowledge, second line endocrine therapies blocking the ERα pathway via different mechanisms have now been established to inhibit the growth of breast tumors after tamoxifen failure. One of the approaches being advanced in the clinic is the use of the pure steroidal antiestrogen, fulvestrant (Faslodex®, ICI 182,780) (3,4), This compound has a high binding affinity for ERα, blocks ERα dimerization and DNA binding, and more intriguingly, targets ERα for degradation (5,6). As a result of this specific action on ERα, fulvestrant abolishes ERα-mediated gene expression and exhibits no known ERα agonist activity (7). In two randomized, controlled clinical trials (3,4) fulvestrant is as effective as anastrozole (Arimidex®), an aromatase inhibitor, for advanced breast cancer in postmenopausal women with disease which failed previous endocrine therapy. Fulvestrant is now approved in the United States as a second line endocrine therapy for the treatment of breast cancer that has progressed on prior antiestrogen therapy in postmenopausal women and provides clinical benefit of longer disease control in patients with

breast cancer resistant to tamoxifen. Although fulvestrant provides benefits with reduced side effects in tamoxifen-resistant disease, drug resistance to fulvestrant will eventually occur. Thus, new therapeutic targets must be identified to further control the growth of fulvestrant-resistant breast tumors.

To understand the mechanism of fulvestrant-resistance and explore potentially new therapeutic targets, several fulvestrant-resistant breast cancer cell models have been reported (8-11). Most of these resistant cells remain ERα positive (8-10) and are still responsive to estrogen in terms of expression of certain ERα regulated genes such as pS2 (9,10), cathepsin D (9), or PgR (8), or estrogen-stimulated growth *in vitro* (10) or *in vivo* (8,9). T47D-r, ZR-75-r and MCF-7-r are ERα/PgR negative (11). However, these three reportedly resistant cell models were not tested for their phenotype stability by withdrawal of fulvestrant from culture medium. In the present study, we established unique fulvestrant-resistant human breast cancer cells (MCF-7/F) that irreversibly lost ERα expression, overexpressed EGFR and breast cancer resistance protein (BCRP), and were less sensitive to chemotherapeutic agents such as mitoxantrone. Using this anti-hormone resistant cell model, we examined whether gefitinib and fumitremorgin C (FTC), a specific BCRP inhibitor (12), could be used as targeted combinations to improve chemosensitivity to cytotoxic drugs.

Materials and methods

Cell culture and materials. The MCF-7 cells used in this study (13) were cloned from MCF-7 ERα-positive human breast cancer cells originally obtained from Dr Dean Edwards, University of Texas San Antonio, TX (1985). MCF-7/F cells were derived from MCF-7 cells by originally growing the cells for more than 12 months in the presence of 1 μM fulvestrant (AstraZeneca Pharmaceuticals, Macclesfield, UK) in estrogen-free medium (phenol red-free minimal essential medium containing 5% calf serum treated three times with dextran-coated charcoal), then followed by culturing the cells in whole serum medium in the absence of fulvestrant for more than 12 months. 4-Hydroxytamoxifen, estradiol, mitoxantrone, doxorubicin and paclitaxel were purchased from Sigma-Aldrich (St. Louis, MO). FTC was purchased from Axxora, LLC (San Diego, CA). Gefitinib was a generous gift from AstraZeneca Pharmaceuticals. Raloxifene was a generous gift from Lilly Research Laboratories (Indianapolis, IN).

Western blot analysis. Whole-cell lysate was extracted in modified RIPA buffer (1X PBS, 0.1% SDS, 1% NP-40, 0.5% sodium deoxycholate, 1 mM EGTA, 2.5 mM EGTA, 10% glycerol, and 10 mM ß-glycerophosphate) with a protease inhibitor cocktail and a phosphatase inhibitor cocktail (Sigma-Aldrich). Protein concentration was measured with a Bio-Rad protein assay kit (Bio-Rad Laboratories, Hercules, CA). Antibodies against ERα, total EGFR, ß-actin and α-tubulin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies against phospho-EGFR^{Y1173}, phospho-Akt^{Ser467}, total Akt, phospho-MAPK$^{44/42}$ and total MAPK$^{44/42}$ were purchased from Cell Signaling (Boston, MA). Anti-BCRP antibody was purchased from Axxora, LLC. The appropriate secondary antibody conjugated to horseradish peroxidase (Santa Cruz Biotechnology) was used to visualize bands with an enhanced chemiluminescence (ECL) visualization kit (Amersham, Arlington Heights, IL).

Cell growth assays. Both MCF-7 and MCF-7/F cells were grown in estrogen-free medium for 4 days before the assay began. Ten thousand cells per well were seeded in 24-well plates. The cells were treated in estrogen-free medium containing different concentrations of different chemicals as shown in figures every other day for 7 days. The DNA content of cells was measured using a DNA quantitation kit (Bio-Rad Laboratories) with a Mithras LB 940 microlabel reader (Berthold Technologies, Oak Ridge, TN).

Transient transfection and luciferase assays. MCF-7 and MCF-7/F cells were cultured in the estrogen-free medium for 4 days. Five million cells were mixed with 1 μg of $VitA_2$-ERE_3-Luciferase ($VitA_2$ is vitellogenin A_2 and ERE_3 is three copies of estrogen response elements) (14) and 0.25 μg of pRL-TK (Promega, Madison, WI). pRL-TK served as an internal control for transfection efficiency. Cells were electroporated (950 μF, 320 V) with a Bio-Rad Gene Pulser II (Bio-Rad Laboratories). Luciferase activities were measured using a Dual-Luciferase® reporter assay system (Promega) with a Mithras LB 940 microlabel reader. The results are presented as relative light unit (RLU) = Firefly luciferase reading/Renilla luciferase reading.

Reverse transcription and real-time polymerase chain reaction. Reverse transcription and real-time PCR was performed as described previously (15). 18S rRNA primers and probe were purchased from Applied Biosystems (Foster City, CA). Sequences for ERα, ERß and EGFR primers and probes were published previously (16). ERα, ERß and EGFR primers and probes were synthesized by MegaBases (Evanston, IL).

Statistical analysis. All data are represented as the mean ± SD of three independent determinations in one representative experiment. Each experiment was repeated at least three times unless otherwise stated. The statistical significance ($p<0.05$) was determined with a One-way variance analysis followed by Tukey's test if there are more than 3 groups of conditions (GraphPad Prism 3.0, GraphPad Software Inc. San Diego, CA). Student's t-test was used to determine the significance at level $p<0.05$ when only two groups were compared. All statistical tests were 2-sided.

Results

Loss of ERα expression in MCF-7/F cells. To understand fulvestrant resistance and identify future therapeutic targets, we developed a fulvestrant-resistant breast cancer cell model (MCF-7/F) derived from MCF-7 cells as described in Materials and methods. MCF-7 parental cells are ERα positive. Estradiol stimulated growth and antiestrogens such as fulvestrant and raloxifene inhibited estradiol-stimulated growth in MCF-7 cells (Fig. 1). However, MCF-7/F cells grew in the absence of estradiol in a time-dependent manner (Fig. 1A) and grew independent of estrogen or antiestrogen treatment (Fig. 1B). To

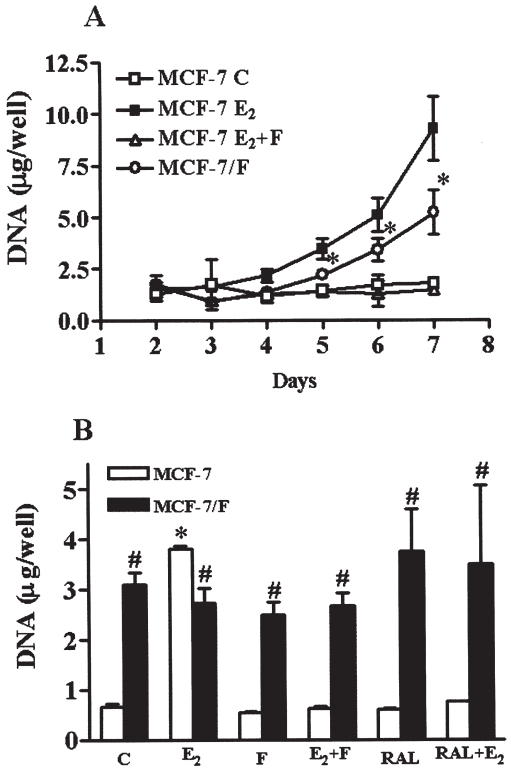

Figure 1. Characteristics of cell growth in MCF-7 and MCF-7/F cells. (A) Time-dependent growth of MCF-7 and MCF-7/F cells. MCF-7 cells were treated with ethanol (MCF-7 C), 1 nM estradiol (MCF-7 E_2), or combination of 1 nM E_2 + 1 μM fulvestrant (MCF-7 E_2+F). MCF-7/F cells were treated with 1 μM fulvestrant. *Compared with MCF-7 C, MCF-7 E_2, or MCF-7 E_2+F group, $p<0.05$. (B) Hormone-dependent growth of MCF-7 and MCF-7/F cells. Both MCF-7 and MCF-7/F cells were treated with ethanol (C), 1 nM E_2, 1 μM fulvestrant (F), 1 nM E_2 + 1 μM fulvestrant (E_2+F), 1 μM raloxifene (RAL), or 1 nM E_2 + 1 μM raloxifene (RAL+E_2). *Compared with any other treatment in MCF-7 cells, $p<0.05$. #Compared with the same treatment group in MCF-7 cells, $p<0.05$.

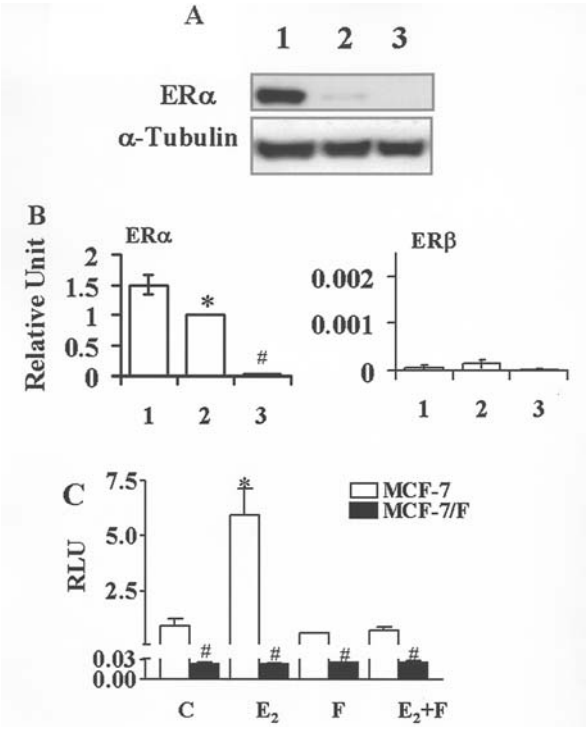

Figure 2. Loss of functional ER expression in MCF-7/F cells. (A) Western blot analysis of ERα protein levels in MCF-7 cells treated with ethanol (1) or 1 μM fulvestrant (2), and MCF-7/F cells treated with ethanol (3) for 24 h. Both cells were cultured in estrogen-free medium for 4 days before the experiment. α-Tubulin was used as the loading control. (B) Real-time PCR analysis of ERα (left panel) and ERß (right panel) mRNA expression in MCF-7 cells treated with ethanol (1) or 1 μM fulvestrant (2), and MCF-7/F cells treated with ethanol (3) for 24 h. Relative units are expressed as fold related to MCF-7 treated with 1 μM fulvestrant. *Compared with MCF-7 control, $p<0.05$. #Compared with MCF-7 control and 1 μM fulvestrant groups, $p<0.05$. ERß mRNA was almost undetectable in all groups. (C) Transient transfection and luciferase assays. The cells were transfected with $VitA_2$-ERE_3-luciferase and pRL-TK, followed by treatment with ethanol (C), 1 nM estradiol (E_2), 1 μM fulvestrant (F), or combination of 1 nM E_2 and 1 μM fulvestrant (E_2+F) for 24 h. *Compared with MCF-7 cells treated with C, F, or E_2+F, $p<0.05$. #Compared with MCF-7 cells with the same treatment, $p<0.05$.

determine how MCF-7/F cells became hormone-independent, we measured ERα expression by Western blot analysis, immunohistochemical staining, real-time PCR, and ER transcriptional activity by a transient transfection assay using a $VitA_2$-ERE_3-Luciferase reporter gene. Short-term treatment (24 h) with 1 μM fulvestrant dramatically decreased ERα protein level in MCF-7 cells (Fig. 2A, lane 2). However, ERα mRNA was detectable (66.7% of the ethanol control, Fig. 2B, left panel, lane 2). After withdrawal of fulvestrant for over one year, MCF-7/F cells remained ERα negative as determined by Western blot analysis (Fig. 2A, lane 3) and immunochemical staining (data not shown). ERα mRNA level was 120 times lower than that of MCF-7 control (Fig. 2B, left panel, lane 3). There was no significant increase in expression of ERß as measured by real-time PCR (Fig. 2B, right panel). Thus, we excluded a role for ERß in fulvestrant-resistance in MCF-7/F cells. In addition, ER transcriptional activity was very low and hormone-independent in MCF-7/F cells (Fig. 2C). These results demonstrated that there was no functional ER (α and ß) expressed in MCF-7/F cells after long-term withdrawal of fulvestrant. We conclude that MCF-7/F cells irreversibly lost ERα expression and became hormone-independent with resistance to fulvestrant.

Growth inhibition by gefitinib via inhibiting phosphorylation of MAPK$^{44/42}$ and Akt in MCF-7/F cells. It is well documented that EGFR expression inversely relates to ERα expression (17,18) and has been frequently reported that increased expression of EGFR accompanies antiestrogen resistance (10,11,19,20). Thus, it is not surprising that EGFR was highly expressed in MCF-7/F cells at both mRNA (Fig. 3B) and protein (Fig. 3A) levels. The EGFR gene was not amplified in MCF-7/F cells as determined by fluorescence *in situ* hybridization (data not shown). Overexpressed EGFR in MCF-7/F cells might be a potential target for the treatment of fulvestrant-resistant breast cancer.

To determine whether overexpressed EGFR had any effect on cell growth in MCF-7/F cells, we used an EGFR kinase specific inhibitor, gefitinib. Gefitinib had no significant effect on E_2-induced cell growth in MCF-7 cells. However, gefitinib inhibited cell growth in MCF-7/F cells in a concentration-dependent manner (Fig. 4A). These results indicate that the overexpressed EGFR has an important growth promoting effect in MCF-7/F cells and EGFR could be a potential target in breast cancer after failure on fulvestrant. To further investigate

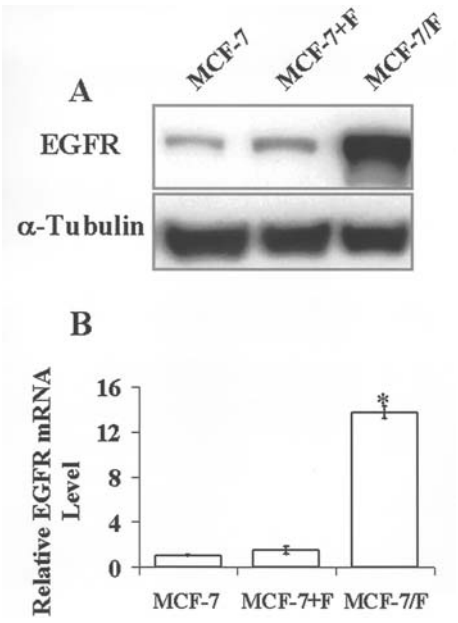

Figure 3. Expression of EGFR in MCF-7/F cells. (A) EGFR expression was measured by Western blot analysis. (B) Real-time PCR. MCF-7 cells were treated with ethanol control (MCF-7) or 1 μM fulvestrant (MCF-7+F) for 24 h. MCF-7/F cells were treated with ethanol for 24 h. *Compared to MCF-7 and MCF-7+F, $p<0.05$.

how gefitinib differentially inhibited the growth of MCF-7/F cells, we found that phosphorylated $EGFR^{Y1173}$ is barely detectable in MCF-7 cells (Fig. 4B). In contrast, MCF-7/F cells showed much higher phosphorylation of EGFR detected by anti-phospho-$EGFR^{Y1173}$ (Fig. 4B) and anti-phospho-$EGFR^{Y1068}$ (data not shown). Gefitinib decreased phosphorylation of EGFR in MCF-7/F cells. $MAPK^{44/42}$ and Akt are the two important downstream factors for EGFR signal transduction pathway (21,22). Although total protein levels of $MAPK^{44/42}$ and Akt were much lower, phosphorylation of both $MAPK^{44/42}$ and Akt were clearly increased in MCF-7/F cells (Fig. 4B), suggesting that the growth and survival of MCF-7/F cells might be predominantly dependent on $MAPK^{44/42}$ and Akt pathways. Gefitinib inhibited phosphorylation of $MAPK^{44/42}$ in both MCF-7 and MCF-7/F cells (Fig. 4B). However, gefitinib inhibited phosphorylation of Akt in a concentration-dependent manner only in MCF-7/F cells. U0126, an inhibitor of MEK (the upstream activator of $MAPK^{44/42}$), inhibited cell growth in a concentration-dependent manner in both MCF-7 and MCF-7/F cells but the inhibitory effect of U0126 at concentrations from 0.625 to 5 μM was significantly more apparent in MCF-7/F cells than parental MCF-7 cells (Fig. 4C). Similarly, MCF-7/F cells were more sensitive to Ly294002, an inhibitor of PI3K (one of the

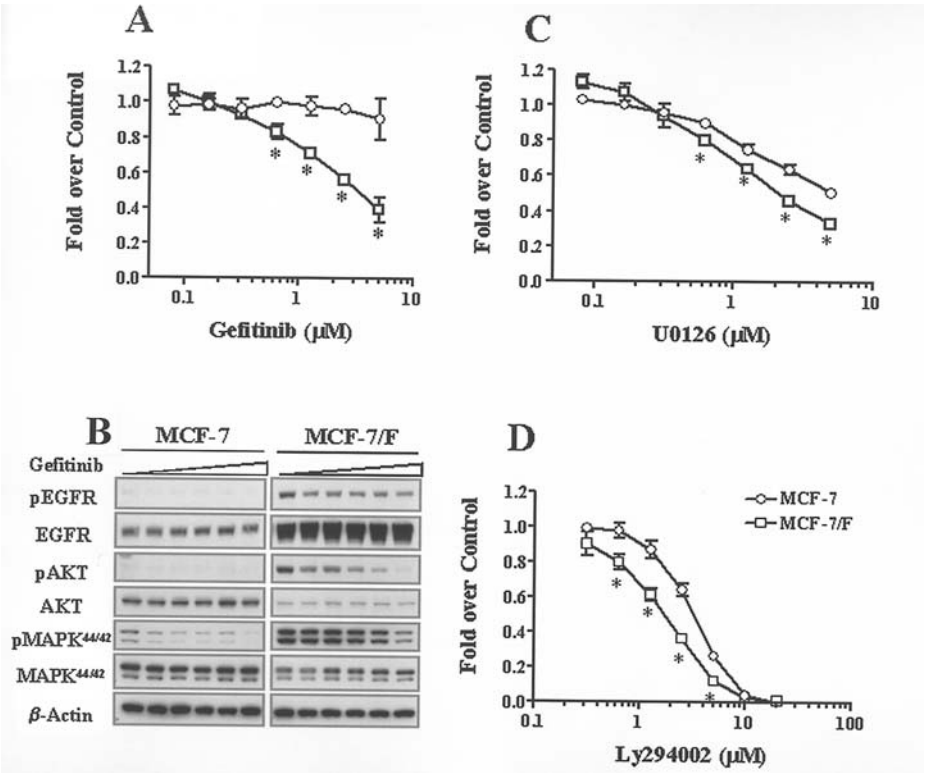

Figure 4. Effect of gefitinib on cell growth and EGFR signaling pathway in MCF-7 and MCF-7/F cells. (A) DNA assay to measure effect of gefitinib on cell growth. MCF-7 (○) and MCF-7/F (□) cells were treated with increasing concentrations (control, 0.08, 0.16, 0.31, 0.63, 1.25, 2.50, 5.00 μM) of gefitinib in the presence of $5x10^{-12}$ M of estradiol for 7 days. The results are presented as fold over $5x10^{-12}$ M of estradiol alone group (fold over control). *Compared to the same treatment group in MCF-7 cells, $p<0.05$. (B) Western blot analysis to determine the effect of gefitinib on EGFR signaling pathway. The cells were treated with increasing concentrations (control, 0.31, 0.63, 1.25, 2.50, 5.00 μM) of gefitinib in the presence of $5x10^{-12}$ M estradiol for 24 h. ß-actin was used as the internal loading control. (C) Concentration-dependent effect of U0126 on cell growth. MCF-7 and MCF-7/F cells were treated with increasing concentrations (control, 0.08, 0.16, 0.31, 0.63, 1.25, 2.50, or 5.00 μM) of U0126 in combination with $5x10^{-12}$ M estradiol for 7 days. The results are presented as fold over $5x10^{-12}$ M of estradiol alone group (fold over control). *Compared to the same treatment groups in MCF-7 cells, $p<0.05$. (D) Concentration-dependent effect of Ly294002 on cell growth. MCF-7 and MCF-7/F cells were treated with increasing concentrations (control, 0.31, 0.63, 1.25, 2.50, 5.00, 10.00, or 20.00 μM) of Ly294002 in combination with $5x10^{-12}$ M estradiol for 7 days. The results are presented as fold over $5x10^{-12}$ M of estradiol alone group (fold over control). *Compared to the same treatment groups in MCF-7 cells, $p<0.05$.

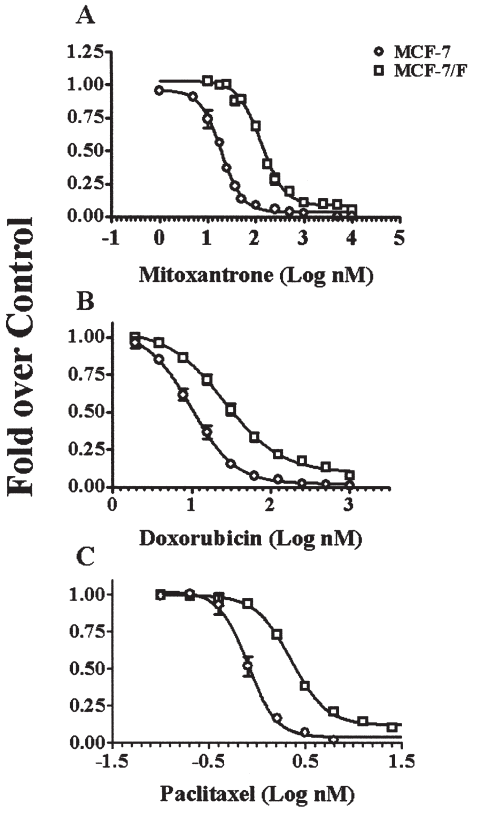

Figure 5. Decreased chemosensitivity in MCF-7/F cells. Sensitivity to chemotherapeutics such as (A) mitoxantrone, (B) doxorubicin, or (C) paclitaxel was determined by DNA assays. The cells were treated with increasing concentrations of mitoxantrone, doxorubicin, or paclitaxel in the presence of $5x10^{-12}$ M estradiol for 7 days. The data are presented as fold over $5x10^{-12}$ M of estradiol alone group (fold over control).

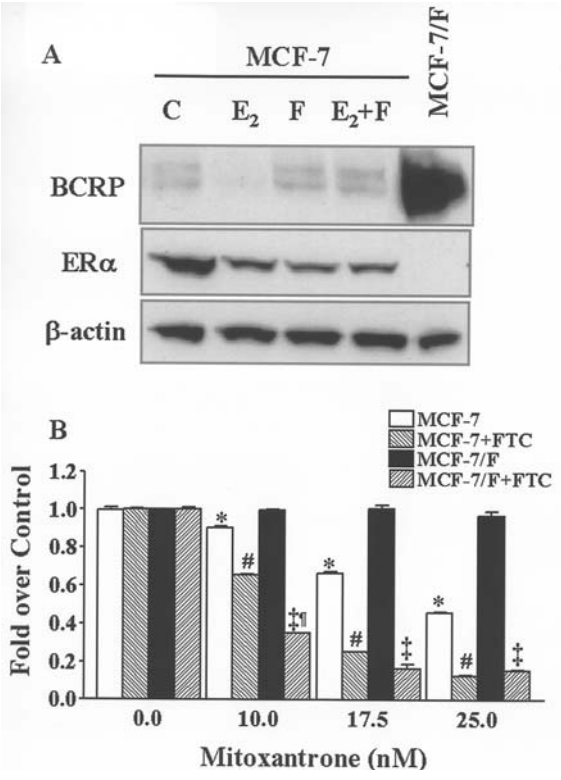

Figure 6. The effect of BCRP overexpression on chemosensitivity in MCF and MCF-7/F cells. (A) BCRP expression was determined by Western blot analysis. MCF-7 cells were treated with ethanol (C), $5x10^{-12}$ M estradiol (E_2), 1 μM fulvestrant (F) or combination of $5x10^{-12}$ M E_2 and 1 μM fulvestrant (E_2+F) for 24 h. MCF-7/F cells were treated with ethanol for 24 h. ß-actin was used as the loading control. (B) Inhibiting BCRP activity by 2.5 μM FTC enhanced the inhibitory effect of mitoxantrone on cell growth in MCF-7/F cells. The data are presented as fold of control (absence of mitoxantrone). *Compared to MCF-7 in the absence of mitoxantrone, $p<0.05$. #Compared to the same mitoxantrone treatment in the absence of FTC in MCF-7 cells, $p<0.05$. ‡Compared to the same mitoxantrone treatment without FTC in MCF-7/F cells, $p<0.05$. ¶Compared to the same mitoxantrone treatment in MCF-7+FTC, $p<0.05$.

upstream activators of Akt) (Fig. 4D). These results suggest that MAPK[44/42] and Akt pathways were more important for survival and growth in MCF-7/F cells, and gefitinib inhibited cell growth by inhibiting phosphorylation of MAPK[44/42] and Akt in MCF-7/F cells.

Chemotherapeutic insensitivity due to overexpression of BCRP in MCF-7/F cells. After ERα positive tumors fail fulvestrant and become ERα negative, cytotoxic chemotherapy is the treatment of choice. To quantify the impact of fulvestrant resistance on chemosensitivity we compared the effect of a number of chemotherapeutic agents on the growth of MCF-7 and MCF-7/F cells using a DNA assay. As shown in Fig. 5, the IC_{50} for mitoxantrone increased 6.8-fold ($p<0.05$) in MCF-7/F cells (135.1±7.1 nM) compared to MCF-7 cells (17.8±2.0 nM) (Fig. 5A), the IC_{50} for doxorubicin significantly ($p<0.05$) increased from 10.5±1.8 nM in MCF-7 to 29.6±4.6 nM in MCF-7/F cells (Fig. 5B), and the sensitivity to paclitaxel significantly decreased in MCF-7/F cells (IC_{50} = 2.3±0.04 nM) compared to MCF-7 cells (IC_{50} = 0.8±0.1 nM, $p<0.05$) (Fig. 5C).

There are three major multidrug-resistant genes, namely, p-glycoprotein (P-gp/ABCB1/MDR1/PGY1), multiple drug resistant protein (MRP/ABCC1) and breast cancer resistant protein (BCRP/ABCG2//ABCP/MXR) (23). We found that P-gp and MRP were expressed at similar levels in MCF-7 and MCF-7/F cells, but BCRP was overexpressed in MCF-7/F cells by a DNA microarray assay (unpublished data). Overexpression of BCRP was confirmed by Western blot analysis (Fig. 6A). To determine whether the observed decreased sensitivity to chemotherapeutics was due to overexpression of BCRP, we treated MCF-7 and MCF-7/F cells with different concentrations of mitoxantrone alone or in combination with 2.5 μM of FTC, a BCRP-specific inhibitor (12). FTC had no effect on cell growth in MCF-7 and MCF-7/F cells at a concentration as high as 10 μM (data not shown). The results in Fig. 6B show that mitoxantrone inhibited cell growth in MCF-7 cells in a concentration-dependent manner as demonstrated in more detail in Fig. 5A. Although MCF-7 cells expressed low levels of BCRP protein (Fig. 6A), 2.5 μM of FTC significantly enhanced the effect of mitoxantrone on inhibiting cell growth. In MCF-7/F cells, mitoxantrone had little effect on cell growth at 25.0 nM. Mitoxantrone, at concentrations of 17.5 and 25.0 nM, in combination with 2.5 μM of FTC inhibited cell growth to the same extent in MCF-7/F cells as observed in MCF-7 cells (Fig. 6B). Interestingly, 10.0 nM mitoxantrone with 2.5 μM FTC inhibited the growth of MCF-7/F cells significantly more than in

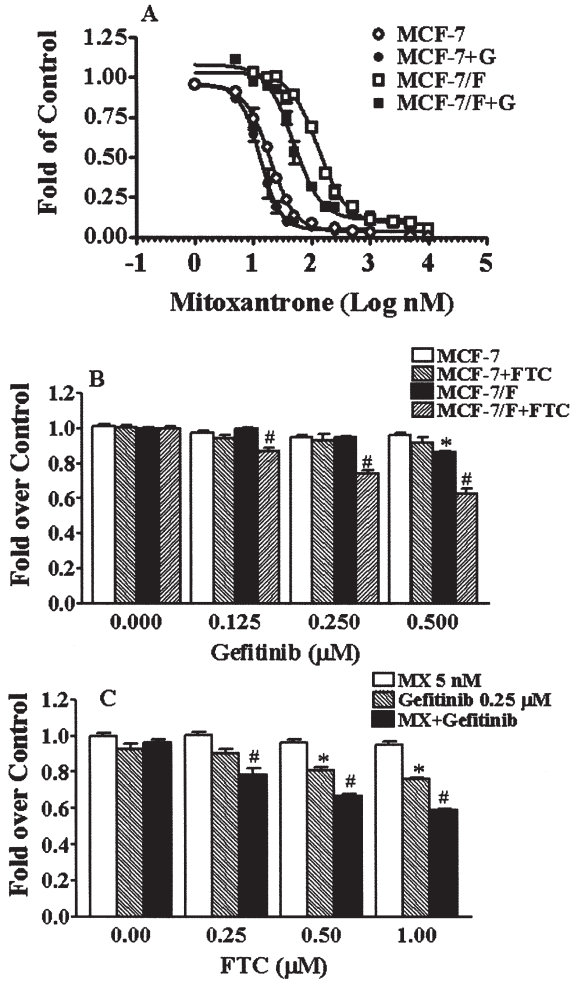

Figure 7. The cooperative effect of mitoxantrone, gefitinib and FTC on cell growth in MCF-7 and MCF-7/F cells. (A) Gefitinib augmented the inhibitory effect of mitoxantrone on cell growth in MCF-7/F cells. Both MCF-7 and MCF-7/F cells were treated with increasing concentrations of mitoxantrone (0.001-10 μM) plus $5x10^{-12}$ M estradiol in the absence or presence of 0.5 μM gefitinib for 7 days. The data are presented as fold over control [$5x10^{-12}$ M estradiol for MCF-7 (○) and MCF-7/F (□), $5x10^{-12}$ M estradiol + 0.5 μM gefitinib for MCF-7+G (●) and MCF-7/F+G (■)]. The results shown are from two separate experiments. (B) FTC enhanced the inhibitory effect of gefitinib on cell growth in MCF-7/F cells. Both MCF-7 and MCF-7/F cells were treated with different concentrations of gefitinib (as shown) plus $5x10^{-12}$ M estradiol with or without 2.5 μM FTC for 7 days. The results shown are from two independent experiments and presented as fold over control (groups in the absence of gefitinib). *Compared to MCF-7/F cells in the absence of gefitinib, $p<0.05$. #Compared to MCF-7/F cells treated with the same concentration of gefitinib in the absence of FTC, $p<0.05$. (C) The inhibitory effect of 5 nM mitoxantrone (MX 5 nM), 0.25 μM gefitinib (gefitinib 0.25 μM), or 5 nM mitoxantrone + 0.25 μM gefitinib (MX + gefitinib) was increased by low concentrations of FTC (0-1.00 μM) in MCF-7/F cells. *Compared to 0.25 μM gefitinib alone, $p<0.05$. #Compared to the other two treatments with the same concentration of FTC, $p<0.05$.

MCF-7 cells ($p<0.05$). Thus, we concluded that the decreased sensitivity to mitoxantrone in MCF-7/F cells is BCRP-mediated.

Cooperative effect of gefitinib, mitoxantrone, and FTC in MCF-7/F cells. It has recently been reported that gefitinib reverses BCRP-mediated drug resistance in BCRP-transfected MCF-7 cells (24), human epidermoid carcinoma A431 cells, human leukemia K562 cell, and human colon cancer HT-29 cells which endogenously express BCRP (25). There are no reports of a fulvestrant-resistant breast cancer cell model overexpressing endogenous BCRP. We investigated whether gefitinib affects chemosensitivity mediated by endogenously expressed BCRP in MCF-7/F cells. 0.5 μM gefitinib slightly decreased IC_{50} for mitoxantrone from 17.8±2.0 to 13.3±1.3 nM, ($p>0.05$) in MCF-7 cells (Fig. 7A). However, 0.5 μM gefitinib significantly shifted the concentration-dependent curve of mitoxantrone to the left, and IC_{50} for mitoxantrone changed from 135.1±7.1 to 48.0±6.5 nM ($p<0.05$) in MCF-7/F cells (Fig. 7A). Thus, gefitinib did enhance the inhibitory effect of mitoxantrone in MCF-7/F cells endogenously overexpressing BCRP, although it did not completely reverse BCRP-mediated chemoresistance.

Overexpression of BCRP not only causes chemoresistance, but is also reported to decrease gefitinib efficacy (26). We investigated whether blocking BCRP with FTC affects the inhibitory effect of gefitinib on cell growth in MCF-7/F cells. Fig. 7B shows that up to a 0.5-μM concentration gefitinib had no significant effect on cell growth in MCF-7 cells; 2.5 μM FTC did not affect the inhibitory effect of gefitinib in MCF-7 cells. Interestingly, gefitinib, with 2.5 μM FTC, inhibited cell growth significantly at a concentration as low as 0.125 μM in MCF-7/F cells, indicating that overexpression of BCRP decreased sensitivity to gefitinib treatment in MCF-7/F cells.

We further treated MCF-7 and MCF-7/F cells with a combination of very low concentrations of mitoxantrone (5 nM), gefitinib (0.25 μM), or mitoxantrone (5 nM) + gefitinib (0.25 μM) in the absence (0), or presence of low concentrations of FTC (0.25 μM, 0.50 μM, or 1.00 μM). None of the treatments had a significant effect on cell growth in MCF-7 cells (data not shown). As shown in Fig. 7C, treatment with 5 nM mitoxantrone, 0.25 μM gefitinib, or 5 nM mitoxantrone + 0.25 μM gefitinib did not significantly affect cell growth in the absence of FTC. However, FTC (0.25, 0.50 or 1.00 μM) significantly increased the efficacy of mitoxantrone + gefitinib.

Discussion

The goal of our study was to describe the therapeutic consequences of the prolonged treatment of ER-positive breast cancer cells with fulvestrant in a low-estrogen environment. We have found that the cells lose ER during antihormone therapy and become resistant to both antihormones and cytotoxic chemotherapy. The fulvestrant-resistant cells (MCF-7/F) were stable and remained ERα negative during the one year observation period following withdrawal of fulvestrant from culture medium. In addition, MCF-7/F cells overexpressed EGFR and BCRP. These targets were found to interact and could be modulated to improve sensitivity to cytotoxic chemotherapy.

We report the characterization of a unique fulvestrant-resistant ERα negative cell line (MCF-7/F) derived from the parental MCF-7 ERα-positive human breast cancer cells. Fulvestrant inhibited cell growth significantly at a concentration as low as 0.01 μM in MCF-7 cells (data not shown). In contrast, MCF-7/F cells grew in 1 μM fulvestrant-containing medium in the absence or presence of 1 nM E_2 (Fig. 1). However, fulvestrant did decrease the cell number at

a 10-μM concentration in MCF-7/F cells (data not shown), which might result from a non-specific cytotoxic effect with such a high concentration of the compound. Since MCF-7/F cells were ERα negative (Fig. 2A and B, left panel) with no significant change of expression level of ERß (Fig. 2B, right panel), it was not surprising that MCF-7/F cells exhibited cross-resistance to raloxifene (Fig. 1B) and 4-hydroxytamoxifen (data not shown). We also speculate that aromatase inhibitors would not have any effect on the growth of fulvestrant-resistant breast cancer because they are ERα negative and continue to grow in estrogen-free media. Our preliminary results (data not shown) did not support that DNA methylation or histone deacetylation played major roles in ERα negativity in MCF-7/F cells. However, the precise mechanism for the loss of ERα expression in MCF-7/F cells is still unclear.

EGFR expression inversely relates to ERα expression (17,18). It has been reported that increased expression of EGFR accompanies antiestrogen resistance (10,11,19,20). Gefitinib, an EGFR tyrosine kinase inhibitor, inhibits the growth of antiestrogen-resistant breast cancer (10,19), enhances the growth inhibitory effect of antiestrogens and prevents the development of antiestrogen resistance (27-29). Since MCF-7/F cells were ERα negative, we anticipated that MCF-7/F cells overexpressed EGFR (Fig. 3) and addressed the proposition that elevated EGFR might have an important role in cell survival and growth in MCF-7/F cells. Although elevated EGFR level is not a good predicator for response to gefitinib (30), gefitinib has been reported to inhibit the growth of tamoxifen-resistant and fulvestrant-resistant cell lines *in vitro* that exhibited enhanced EGFR signal transduction (10,19). We compared the inhibitory effect of gefitinib on cell growth in MCF-7 and MCF-7/F cells in the presence of estrogen. Although EGF stimulated the growth of MCF-7 cells in serum-free condition (data not shown), ERα signaling pathway might be the predominant cell growth promoting pathway in MCF-7 cells when estradiol is present. It was not surprising that gefitinib slightly, but not significantly ($p>0.05$), inhibited estrogen-induced growth in MCF-7 cells at a 5-μM concentration (Fig. 4A). However, gefitinib profoundly inhibited cell growth of MCF-7/F cells at a concentration as low as 0.625 μM (Fig. 4A). The results indicate that while cell growth and survival of MCF-7 cells largely rely on the ERα signal transduction pathway, MCF-7/F cells are at least partially dependent on the EGFR pathway. Enhanced activation of EGFR downstream factors $MAPK^{44/42}$ and Akt (Fig. 4B) made MCF-7/F cells more susceptible to the inhibitory effects of U0126 and Ly294002 (Fig. 4C and D), which further illustrate the important role of EGFR signaling in MCF-7/F cell growth. Gefitinib inhibited $MAPK^{44/42}$ phosphorylation in parental MCF-7 cells (Fig. 4B) without showing significant inhibition of cell growth (Fig. 4A), which is consistent with the lower sensitivity to U0126 in MCF-7 cells. We also studied the possible role of other ErbB family members in fulvestrant-resistance. The total and phosphorylated levels of ErbB2 were increased approximately 2-fold in MCF-7/F compared to MCF-7 cells (data not shown). However, we found that trastuzumab, a humanized monoclonal antibody specific for ErbB2, did not have a significant effect on MCF-7/F cell growth (data not shown). There were no significant changes in the expression of ErbB3 and ErbB4 determined by real-time PCR (data not shown). Overall our results with gefitinib support the hypothesis that growth of MCF-7/F cells occurs, at least in part, via EGFR with downstream $MAPK^{44/42}$ and Akt phosphorylation.

Cytotoxic chemotherapy is often the only alternative for patients with endocrine-resistant ERα-negative tumors. We examined the efficacy of chemotherapeutic drugs such as mitoxantrone, doxorubicin and paclitaxel in parental MCF-7 and MCF-7/F cells. Since chemotherapeutic agents affect rapidly growing cells, we controlled comparable cell growth rate in MCF-7 and MCF-7/F cells by using $5x10^{-12}$ M of estrogen in assay medium (data not shown). MCF-7/F cells were significantly less sensitive to mitoxantrone, doxorubicin, and paclitaxel (Fig. 5). Consistent with these chemosensitivity results, MCF-7/F cells overexpressed BCRP (Fig. 6). BCRP is a membrane-associated protein in the superfamily of ATP-binding cassette transporters. It effluxes a variety of anticancer drugs such as anthracyclines, anthracenediones, the camptothecins topotecan and SN-38, methotrexate, and flavopiridol (31-34), which results in chemoresistance. We found that estradiol induced BCRP expression at the mRNA level (data not shown) and inhibited BCRP expression at the protein level in MCF-7 cells (Fig. 6B). The effect of estradiol on BCRP expression is still controversial (35,36). To our knowledge MCF-7/F is the first antiestrogen-resistant breast cancer cell model overexpressing BCRP endogenously. To examine whether elevated BCRP plays a role in decreased chemosensitivity in MCF-7/F cells, we used a specific BCRP inhibitor, FTC, to block its activity (37). As shown in Fig. 6B, FTC significantly sensitized MCF-7/F as well as the parental MCF-7 cells to mitoxantrone treatment. This result indicates that the low level of BCRP is functional in terms of decreased chemosensitivity in the parental MCF-7 cells. Interestingly, FTC enhanced the inhibitory effect of 10.0 nM mitoxantrone significantly more in parental MCF-7 cells than in MCF-7/F cells. The reason for this could be based on the mechanism of action of mitoxantrone and the differing mechanisms for regulating apoptosis. Mitoxantrone can induce apoptosis via the mitochondria pathway (38), whereas bcl-2 is an antiapoptotic protein that inhibits mitochondria-mediated apoptosis. We observed that the expression of bcl-2 was stimulated by estrogen in MCF-7 cells, but the bcl-2 protein was not detected in MCF-7/F cells (data not shown). It is therefore possible that estrogen-induced bcl-2 expression might protect parental MCF-7 cells from the FTC-enhanced antitumor effect of mitoxantrone at 10.0 nM. It is known that high concentrations of mitoxantrone can decrease bcl-2 expression (39), and eventually overcome estrogen-induced bcl-2 expression. Thus, different survival mechanisms for the different cell lines could potentially explain the actions of FTC.

Interestingly enough, MCF-7/F cells also displayed a decreased sensitivity to paclitaxel (Fig. 5C) that has not been shown to be a substrate for BCRP to transport. FTC did not change the sensitivity to paclitaxel in MCF-7/F cells (data not shown). In contrast, FTC significantly enhanced the sensitivity to doxorubicin in MCF-7/F cells (data not shown). The mechanism by which MCF-7/F cells become insensitive to paclitaxel remains obscure. We compared the expression levels of the two other major multiple drug-resistant genes,

P-gp and MRP in MCF-7 cells and MCF-7/F cells. Both P-gp and MRP were not overexpressed in MCF-7/F cells (data not shown). ABCC2 (MRP2) was recently reported to mediate paclitaxel resistance (40). However, expression of ABCC2 was not increased significantly in MCF-7/F cells (data not shown).

There is increasing potential for combination therapy with targeted cancer therapy and cytotoxic chemotherapy in breast cancer. The combination of trastuzumab and chemotherapy has been shown to have a synergistic effect in cells overexpressing ErbB2 (41) *in vitro* and provide clinical benefits for patients with ErbB2 overexpressing breast tumors (42,43). Gefitinib has been shown to potentiate cytotoxic agents such as platinums, cisplatin, carboplatinum, taxanes, doxorubicin, and edatrexate against human skin, lung, and prostate cancer xenografts (44). However, gefitinib did not improve the efficacy of chemotherapeutics in non-small cell lung cancer in two phase III trials (45,46). For breast cancer, a phase I/II clinical trial is currently underway to determine the efficacy of gefitinib, trastuzumab, and docetaxel in combination in patients with ErbB2 overexpressing metastatic breast cancer (ClinicalTrials.gov identifier NCT00086957). Recently, gefitinib has been shown to reverse chemoresistance mediated by BCRP in skin cancer, colon cancer (25), lung cancer, and breast cancer cells (24) which exogenously overexpressed BCRP. Overexpression of BCRP protects cells from gefitinib-induced apoptosis in A431 cells (26), suggesting the possible role of BCRP in resistance to gefitinib. These reports collectively indicate an interaction between gefitinib and BCRP. However, the nature of the interaction is as yet not clear. It is known that gefitinib inhibits BCRP-mediated transport, but gefitinib is not a substrate for BCRP to transport (24,47). In addition, gefitinib has been shown to reverse BCRP-mediated chemoresistance in cells with undetectable levels of EGFR (24). These data suggest a direct effect of gefitinib on BCRP. Nevertheless, BCRP expression affects the efficacy of gefitinib (25) (Fig. 7B), which suggests a possible competitive binding of gefitinib to EGFR and BCRP. Moreover, the other tyrosine kinase inhibitors such as EKI-785, imatinib mesylate, and CI1033 also inhibit BCRP-mediated chemoresistance (48-50). Gefitinib, EKI-785, imatinib mesylate, and CI1033 are all small molecules that compete with ATP for ATP binding sites in receptor tyrosine kinases and inhibit receptor tyrosine kinase activity. Gefitinib has been shown to bind to other tyrosine kinases and even threonine-serine kinases with various affinities (51,52), Thus, it is plausible that the tyrosine kinase inhibitors have various binding affinities to the ATP binding domain in BCRP, block ATP binding, and inhibit BCRP activity.

We were surprised to find that gefitinib is not as effective in MCF-7/F cells as in other EGFR overexpressing cells (25,51). However, the explanation for our observation may involve multiple mechanisms for cancer cell survival. The MCF-7/F cell model is the only tumor cell model so far found to overexpress both endogenous EGFR and BCRP. Although gefitinib did not completely reverse insensitivity to mitoxantrone in MCF-7/F cells (Fig. 7A), inhibiting BCRP by FTC increased the sensitivity to gefitinib in MCF-7/F cells significantly (Fig. 7B). These results suggest that EGFR and BCRP may compete with each other for gefitinib. Additionally, inhibition of both EGFR and BCRP further enhanced the inhibitory effect of mitoxantrone on cell growth in MCF-7/F cells (Fig. 7C).

In conclusion, we have developed a stable ERα negative breast cancer cell model from ERα-positive MCF-7 cells that overexpress both EGFR and BCRP. We have demonstrated that targeting survival mechanisms and an efflux protein can enhance the effectiveness of cytotoxic chemotherapy. Application and translation of these principles to patient care may reduce side effects and enhance the efficacy of cytotoxic chemotherapy in antihormone refractory disease.

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