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ORIGINAL ARTICLE

BRCA1 modulates sensitivity to 5F-203 by regulating xenobiotic stress-inducible protein levels and EROD activity

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Abstract
Purpose We have investigated the effects of BRCA1 over-expression and knockdown on 5F-203-induced gene expression and cytotoxicity in human breast cancer cells. 5F-203 is a chemotherapeutic prodrug that both induces a p450 enzyme, CYP1A1, and is metabolically activated by CYP1A1.
Methods We used several molecular biological techniques to confirm our findings. BRCA1 regulates sensitivity to 5F-203 by regulating the expression of CYP1A1 mRNA and its EROD activity. XRE-Luc reporter assays, semi-quantitative RT-PCR, Western blot analysis, EROD activity measurements, gene knockdown and MTT cell survival assays were used for this study.
Results Our results show that the ability of 5F-203 treatments to increase CYP1A1 mRNA level and CYP1A1 enzymatic activity (EROD activity) are affected by BRCA1 protein levels. In addition, the ability of 5F-203 treatments to induce proteins, P53 and P53 target genes such as P21, is significantly decreased in BRCA1 knockdown cells, suggesting that BRCA1-related effects could at least partially explain why BRCA1 knockdown increases resistance to 5F-203-mediated cytotoxicity. We also observed altered expression of the two major transcription factors (AhR and ARNT) that affect CYP1A1 expression when BRCA1 protein levels are altered.
Conclusion BRCA1 is an important protein, which affects 5F-203-mediated cytotoxicity. Our findings are potentially clinically significant; they suggest that those patients most likely to respond to this new prodrug will have tumors containing normal amounts of BRCA1.

Keywords BRCA1 · 5F-203 · AhR · CYP1A1 · P53 · EROD activity

Abbreviations

AhR	Aryl hydrocarbon receptor
BRCA1	Breast cancer susceptibility gene-1
DMEM	Dulbecco's modified Eagles' medium
DMSO	Dimethyl sulfoxide
FBS	Fetal bovine serum
SEM	Standard error of mean

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Introduction

The tumors of cancer patients are often treated with chemotherapy without prior knowledge of whether the tumors are relatively sensitive or resistant to the toxic agents being used. Thus, diagnostics that are able to better match chemotherapies and patient tumor sensitivities for existing as well as for emerging, potentially more selective antitumor agents that have fewer or less severe side effects are needed to improve treatment outcomes and patient's quality of life. Tests that are able to identify which tumors are potentially sensitive or resistant to one new class of promising candidate antitumor agents, amino-phenyl benzothiazole and its derivatives (such as DF-203, 5F-203), have not yet been developed. The benzothiazole drugs represent a new class of chemotherapeutic agents whose mechanism of action includes the ability to induce the expression of an enzyme, a p450 isoform, CYP1A1, which accelerates the bioactivation of these drugs [3, 4]. 5F-203 and DF-203 have potent antitumor properties in a subset of the NCI collection of 60 breast, ovarian, and renal cancer cell lines often used to screen for anticancer drugs [3]. 5F-203 showed potent and selective antitumor properties and its l-Lysylamide form, a prodrug called Phortress, is currently being tested in Phase I clinical studies in the UK [4].

DF-203 induces its own bioactivation by binding to and activating the aryl hydrocarbon receptor (AhR) transcription factor [9]. AhR is a well-studied ligand-activated transcription factor belonging to the basic helix-loop-helix/Per-ARNT-Sim family of proteins [10]. It mediates the toxic effects of several chemical carcinogens, including polycyclic and halogenated aromatic hydrocarbons and is activated by multiple and diverse ligands, including dietary compounds, natural and synthetic flavonoids, natural products and pharmaceuticals [8]. Prior to ligand binding, AhR exists in the cytoplasm in a complex with heat shock protein 90 [16], the co-chaperone P23 [14] and the immunophilin homolog XAP2 [6]. After ligand binding, including by 5F-203, AhR moves to the nucleus, dissociates from the chaperone complex and forms a heterodimer with the ARNT (aryl-hydrocarbon receptor nuclear translocator) transcription factor. This heterodimer binds to xenobiotic response elements (XREs) in the promoter and enhancer regions of target genes to regulate their transcription. CYP1A1, one of these AhR-responsive genes, plays a critical role in the anticancer activity of some pharmaceuticals by biotransforming them from prodrugs into active drugs [7, 19]. Several inhibitors of the human AhR have been discovered, including two protein inhibitors (HAhRR, a human protein [20], and FAhRR, a fish protein [11]) and alpha-naphthaflavone (α-NF), a chemical inhibitor [18].

Women with germ-line BRCA1 mutations have an increased risk for breast and ovarian cancer, although the functions of BRCA1 in tumor suppression are not completely understood. The known functions of BRCA1 include the regulation of cell proliferation, cell cycle progress and DNA repair and transcription [17]. The BRCA1 effects on transcription include effects on several xenobiotic stress-inducible genes, including CYP1A1 [12, 1]. Since BRCA1 protein levels can influence the ability of certain xenobiotics to induce the expression of CYP1A1, we hypothesized that BRCA1 protein levels would also affect the ability of the amino-phenyl benzothiazole family of pharmaceutical, includes 5F-203 and DF-203, to induce CYP1A1 gene expression. If validated, BRCA1 protein levels might be a useful diagnostic tool for predicting whether tumors will be clinically responsive to these prodrugs.

Materials and methods

Semi-quantitative reverse transcription (RT)-PCR analysis

Semi-quantitative RT-PCR was performed as previously described [12, 1]. In brief, cDNA templates were prepared from 1 μg of total RNA with SuperScript II reverse transcriptase and oligo (dT) primers (Life Technologies, Inc.). Then, cDNA templates were-PCR-amplified with dNTPs, specific primers for CYP1A1 [12] and Taq DNA polymerase (Perkin-Elmer, Norwalk, CT, USA). *β*-Actin was used as the loading control. PCR products were quantified from photographs taken after gel eletectrophoresis and staining with ethidium bromide.

Cell culture and chemicals

MCF-7 and T47D (human breast cancer cell lines, ER+) were obtained from ATCC (American Type Culture Collection, Manasass, VA, USA) and maintained in Dulbecco's modified Eagles' medium (DMEM) containing 5% fetal bovine serum (FBS), 100 unit per ml penicillin and 100 mg per ml streptomycin at 37°C in a humid atmosphere of 5% CO_2. All cell culture reagents were purchased from BioWhittaker, Inc. (Walkersville, MD, USA); [2-(4-amino-3-methylphenyl)-5-flurobenxothiazole] (5F-203, NSC 703786) and its non-fluorinated parent compound [2-(4-amino-3-methylphenyl) benzothiazole] (DF-203, NSC 674495) were obtained from the Drug Synthesis and Chemistry Branch, Developmental Therapeutics Program, Division of Cancer Treatment and Diagnosis, National Cancer Institute. A stock solution was prepared in DMSO at 100 mM and α-naphthoflavone (α-NF) was purchased from Sigma and dissolved in DMSO to make a 1 mM stock solution.

Reporter gene assay

Cells (5×10^4 cells/well) were seeded in 24-well plates and transfected with reporter plasmids (125 ng/well) and various expression plasmids (125 ng/well, unless indicated otherwise in the figure legends) using Lipofectamine Plus (Invtrogen). After being transfected for 24 h, the cells were treated with 1 μM of 5F-203 for an additional 24 h and then lysed in a reporter assay lysis buffer (Promega). Luciferase activity was measured using a luminometer according to the manufacturer's instructions. Luciferase activity was normalized for relative transfection efficiency by measuring β-galactosidase activity of a co-transfected reporter plasmid as described [12, 13]. Reporter gene constructs, p(XRE.1A1)-Luc and p(Cyp1a1)-Luc [12], a BRCA1 expression plasmid (pCDNA3-BRCA1) [1], a human AhRR expression plasmid (pCDNA3-HAhRR) [20] and a fish AhRR expression plasmid (pCDNA3-FAhRR) [11] were used.

BRCA1 knockdown

We used chemically synthesized BRCA1-siRNAs purchased from Dharmacon [1, 13].

BRCA1 mutants

The constructs used for this study: wtBRCA1 and BRCA1 mutants [T300G, C5365G, 5677insA and 5382insC], have been described previously [13].

Ethoxyreorufin-O-deethylation activity

CYP1A1 enzymatic activity was measured using a CYP1A1 ethoxyreorufin-O-deethylation (EROD) activity kit (IKZUS environment Co.) according to the manual provided with the kit. Briefly, cells transfected with siRNA (control vs. BRCA1) for 72 h or plasmid DNAs (empty vector, pCDNA3-wtBRCA1 or BRCA1 mutant expression vectors) for 24 h were treated with various doses (0, 0.01, 0.1 and 1 μM) of 5F-203 for an additional 24 h. Then, the medium was removed, the cells washed twice with warm PBS and reaction buffers, containing 5 μM of 7-ethoxyresorufin and 10 μM of dicumarol, and added to each well. After incubation for 30 min at 37°C, fluorescence was measured every 10 min for 60 min at 37°C with an Ultra 384 fluoremetry (Tecan, Switzerland) using 535 nm excitation and 590 nm emission filter as previously described [5]. A standard curve was constructed using resorufin.

Western blot analysis

Cells pretreated with siRNAs (control or BRCA1) were incubated with 1 μM of 5F-203 and lysed in a buffer containing [20 mM *Tris*-HCl (pH 8.0), 0.5 M NaCl, 1 mM EDTA, 1 mM EGTA, 0.25% Triton X-100, 1 mM DTT and 2 μM PMSF] and then centrifuged to yield whole cell lysates. A weight of 40 μg of protein was subjected to SDS-PAGE and transferred to a PVDF membrane (Millipore). The membrane was incubated with specific antibodies

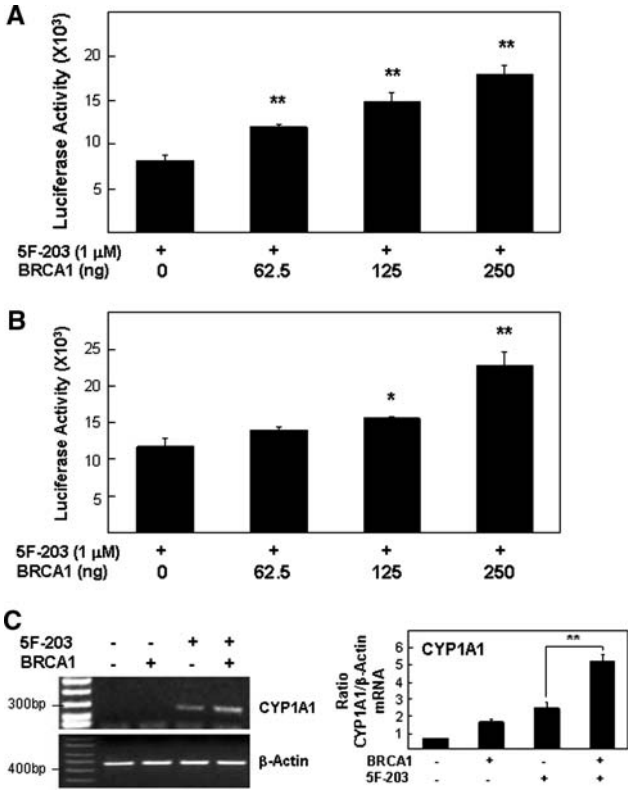

Fig. 1 BRCA1 over-expression enhances the ability of 5F-203 to induce expression from XRE-containing reporter plasmids. Cells (MCF-7) transfected with a constant amount (125 ng) of (**a**) p(XRE.1A1)-Luc or (**b**) rat p(Cyp1a1)-Luc reporter were co-transfected for 24 h with increasing doses of wtBRCA1 expression vector (62.5, 125, 250 ng per well) and then treated with 1 μM of 5F-203 for an additional 24 h. A pSV-β-gal expression plasmid (125 ng per well) was included in each transfection to normalize for transfection efficiency. Luciferase reporter activities were measured as described in "Materials and methods" and are presented as means ± SEMs of $N = 4$ wells. The values presented have been corrected for the background values (DMSO-treated), 2,170, 2,651, 3,202 and 3,788, respectively for the increasing doses of BRCA1 (**a**). The BRCA1 expression vector (62.5, 125, 250 ng) significantly enhanced 5F-23-induced p(XRE.1A1-Luc) reporter activity ($P < 0.005$ for comparison of cells transfected withy empty vector vs. BRCA1 expression vector). Background values for (**b**) were 4,603, 5,221, 7,829 and 8,416. Similar results were found in three independent experiments. The BRCA1expression vector (125 and 250 ng) significantly enhanced 5F-203-induced Cyp1a1-Lucreporter activity ($P < 0.05$ or 0.005 for comparisons of cells transfected with pCDNA3 control vector vs. pCDNA3-BRCA1). **c** Semi-quantitative RT-PCR. Cells transfected with empty vector or the wtBRCA1 expression vector were treated with DMSO or 1 μM of 5F-203 for 24 h and then harvested for total RNA isolation. cDNA template preparation and semi-quantitative RT-PCR were performed as described in "Materials and methods". A bar graph (*in the right panel*) gives the results of triplicate RT-PCR measurements

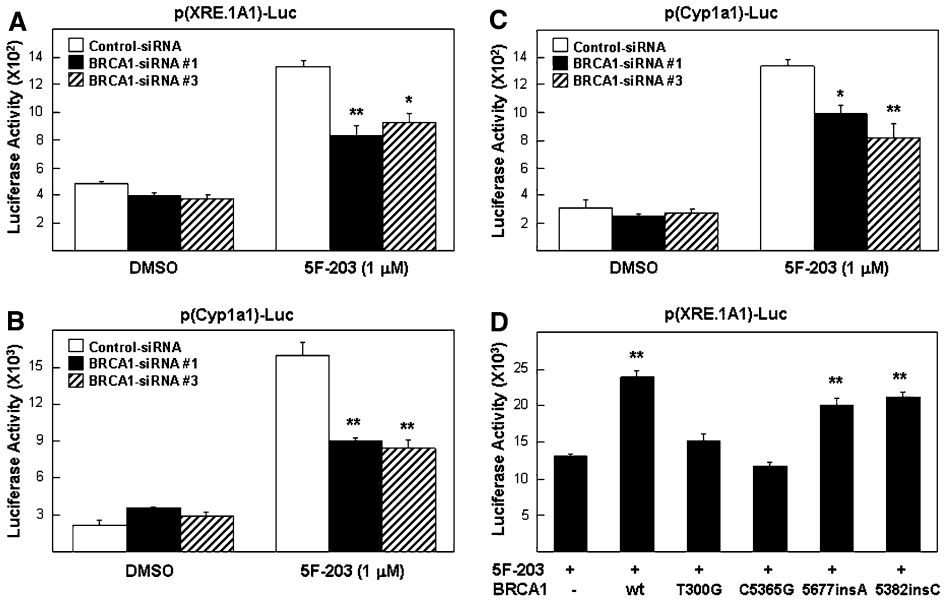

Fig. 2 BRCA1 protein levels and protein alteration can modulate the ability of 5F-203 to induce XRE reporter plasmid activity. BRCA1 knockdown reduced the 5F-203 inducibility of XRE-containing reporter plasmids. MCF-7 (**a**, **b**) or T47D (**c**) cells transfected with BRCA1-specific siRNA (or control-siRNA) for 72 h were re-seeded for reporter gene assays. The reseeded cells were transfected with either (**a**) p(XRE.1A1)-Luc or (**b**, **c**) p(Cyp1a1)-Luc for 24 h and then treated with 1 μM of 5F-203 for 24 h. Luciferase activity ($\times 10^3$) was determined after normalizing transfection efficiency and is presented ($\times 10^3$) as means ± SEMs of values from four wells. Similar results were obtained in three independent experiments. BRCA1 knockdown significantly reduced 5F-203-induced XRE reporter activity ($P < 0.05$ for comparisons between control-siRNA vs. BRCA1-siRNA). We used two BRCA1-siRNAs designed from two different DNA sequence regions of BRCA1 to eliminate any possible artifacts resulting from choosing a non-specific siRNA. **d** Wt and mutant BRCA1 differentially affect the ability of 5F-203 to mediate expression from XRE-containing reporter plasmids. Cells co-transfected with expression vectors, encoding either wt or mutant BRCA1 and reporter plasmids, p(XRE.1A1)-Luc, for 24 h were then treated with 1 μM of 5F-203 for an additional 24 h. Normalization and measurements of luciferase activity were performed as described in the legends to Fig. 1. The BRCA1 mutants are described in "Materials and methods". The values in (**d**) were corrected for the background values (treated with DMSO), 1,147, 1,838, 987, 877, 821 and 1,068, respectively, for the each BRCA1 expression vectors. We repeated similar experiments in three independent experiments. Wild-type and two mutants BRCA1 (5677insA and 5382insC) over-expression significantly enhanced 5F-203-induced XRE reporter activity ($P < 0.005$ for comparisons between empty vector vs. BRCA1 expression vector). No significant differences were found in control vs. two BRCA1 mutants (T300G and C5365G)

against BRCA1 (C-20, Santa Cruz Biotechnology, Inc.), AhR (H-211, Santa Cruz Biotechnology), ARNT (H-172, Santa Cruz Biotechnology), P53 (V7064, Biomeda, Foster City, CA, USA), P21 (H-164, Santa Cruz Biotechnology), Cyp1A1 (Affinity BioReagents, Golden, CO, USA) or PLAB/NAG-1/MIC-1 [2] and then washed and incubated with horseradish peroxidase-conjugated secondary antibodies. Anti-β-actin antibody (Sigma) was used as a loading control. These antibody complexes were visualized by enhanced chemiluminescence (ECL; Santa Cruz Biotechnology) using X-ray films (ImageTek).

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay

Cells growing in microtitre 96-well plates were pretreated with siRNAs (control or BRCA1) and then treated with various doses of 5F-203. After 72 h, the cells were incubated with an MTT solution for 4 h. Then supernatants were removed, the remaining cells attached to the plate were dissolved in DMSO and absorbance at 590 nm was measured as previously described [1].

Statistics

The two-tailed Student's test was used for statistical comparisons and analysis. The symbols (*) and (**) in the figures indicate $P < 0.05$ and $P < 0.005$, respectively.

Results

BRCA1 protein levels affect the ability of 5F-203 to induce CYP1A1 gene expression

Exposure to TCDD, a xenobiotic stressor, induces the expression of multiple xenobiotic stress-responsive genes, including CYP1A1, in breast and many other types of cancer cells. We recently reported that BRCA1 over-expression enhances the ability of TCDD to induce CYP1A1 gene

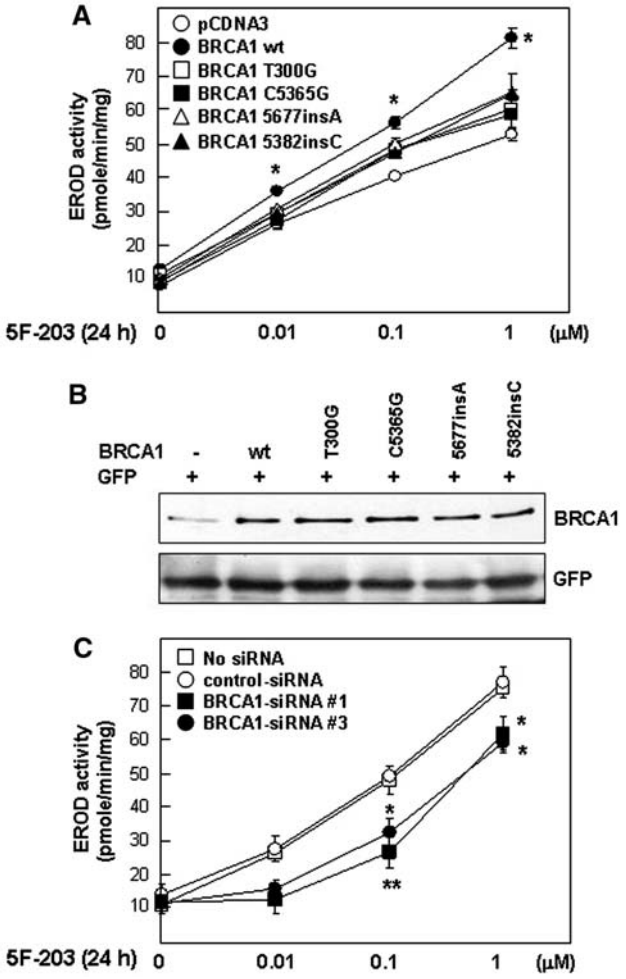

Fig. 3 BRCA1 protein levels and sequences regulate the magnitude of 5F-203's ability to induce CYP1A1-encoded EROD activity. **a** Cells (MCF-7) pretreated with empty vector, wtBRCA1, or four different BRCA1 mutants (T300G, C5365G, 5677insA, 5382insC) for 24 h were treated with various doses of 5F-203 for an additional 24 h and EROD activity was measured. WtBRCA1 over-expression significantly increased EROD activity induced by 5F-203 ($P < 0.05$ for comparisons between pCDNA3 empty vector vs. wtBRCA1). The two BRCA1 mutants significantly increased EROD activity only at the does of 0.1 μM (both 5677insA and 5382insC) or 1 μM (only 5382insC) ($P < 0.05$, compared to control empty vector). **b** To measure the amounts of BRCA1 protein synthesized from the various expression vectors, Western blots were performed using anti-BRCA1 antibodies. **c** Cells transfected with siRNA (control vs. BRCA1) for 72 h were treated with various doses of 5F-203 for 24 h when EROD activity was measured as described in "Materials and methods". BRCA1 knockdown significantly decreased EROD activity following 5F-203 treatment ($P < 0.05$ or $P < 0.005$ for comparisons between control-siRNA vs BRCA1-siRNA transfected cells)

expression and that BRCA1 knockdown reduces, but does not eliminate, this induction in both MCF-7 and T47D cells [12]. In this study, we report experiments testing the hypothesis that BRCA1 protein levels also regulate the ability of the prodrug 5F-203 to induce CYP1A1 gene expression. This hypothesis is suggested by observations showing that both 5F-203 and TCDD are AhR agonists and that CYP1A1 is an AhR target gene. Our first test of this hypothesis examined the effect of BRCA1 over-expression on two different CYP1A1 promoter–reporter plasmids in two different cell lines (MCF-7 and T47D). One of the reporter plasmids (XRE.1A1-Luc) contains 3 XRE repeats of the minimal CYP1A1 promoter and the other (Cyp1a1-Luc) contains a single copy of a larger part of the rat Cyp1a1 promoter sequence (to −1195 bp upstream from UAG translation start signal) [12]. MCF-7 cells were co-transfected with a fixed amount of the reporter plasmids and increasing doses of a BRCA1 expression vector. We found that exogenous BRCA1 over-expression enhanced the ability of 5F-203 to XRE.1A1-Luc reporter activity in MCF-7 cells (Fig. 1a). Over-expression of BRCA1 also increased expression from the other CYP1A1 reporter, Cyp1a1-Luc, after 5F-203 treatment of MCF-7 cells (Fig. 1b). When BRCA1 was over-expressed in a second cell line, T47D, similar dose-dependent increases in reporter activity were found for both reporter plasmids (data not shown). These reporter plasmid expression results suggest that BRCA1 over-expression might also increase the ability of 5F-203 treatments to induce genomic CYP1A1 gene expression. Indeed, semi-quantitative RT-PCR measurements show that over-expression of BRCA1 increases the ability of 5F-203 treatments to increase endogenous CYP1A1 mRNA levels in MCF-7 cells (Fig. 1c).

Although our data in Fig. 1, showing that above-normal amounts of BRCA1 act as a positive regulator of CYP1A1, increasing the capacity of breast cancer cell lines to induce CYP1A1 gene expression in response to a fixed amount of 5F-203, suggests that normal amounts of BRCA1 protein function might also function as a positive regulator, or a limiting factor, for the responses of these cells to 5F-203 treatments, this is not necessarily so. That is, the effects documented in Fig. 1 might be an artifact, occurring only under BRCA1 over-expression conditions and that normal BRCA1 protein levels do not limit or regulate the robust response of these cells to 5F-203 treatments (i.e., the left-most columns in Fig 1a, b and c). Our hypothesis that normal BRCA1 protein levels regulate the ability of the breast epithelial cells to induce CYP1A1 gene expression, in response to 5F-203 treatment, predicts that reduced BRCA1 protein levels should reduce the ability of 5F-203 to induce CYP1A1 expression, whether measured by the activity of XRE-containing reporter plasmids or by the levels of endogenous CYP1A1 mRNA, protein and/or enzymatic activity. As expected, knockdown of endogenous BRCA1 by siRNAs significantly reduced the ability of 5F-203 to induce expression from both CYP1A1 promoter–reporter plasmids and in two human breast cancer cell lines (MCF-7 and T47D) (Fig. 2a, b, c). This reduced capacity to induce reporter activity was produced by transfecting cells with

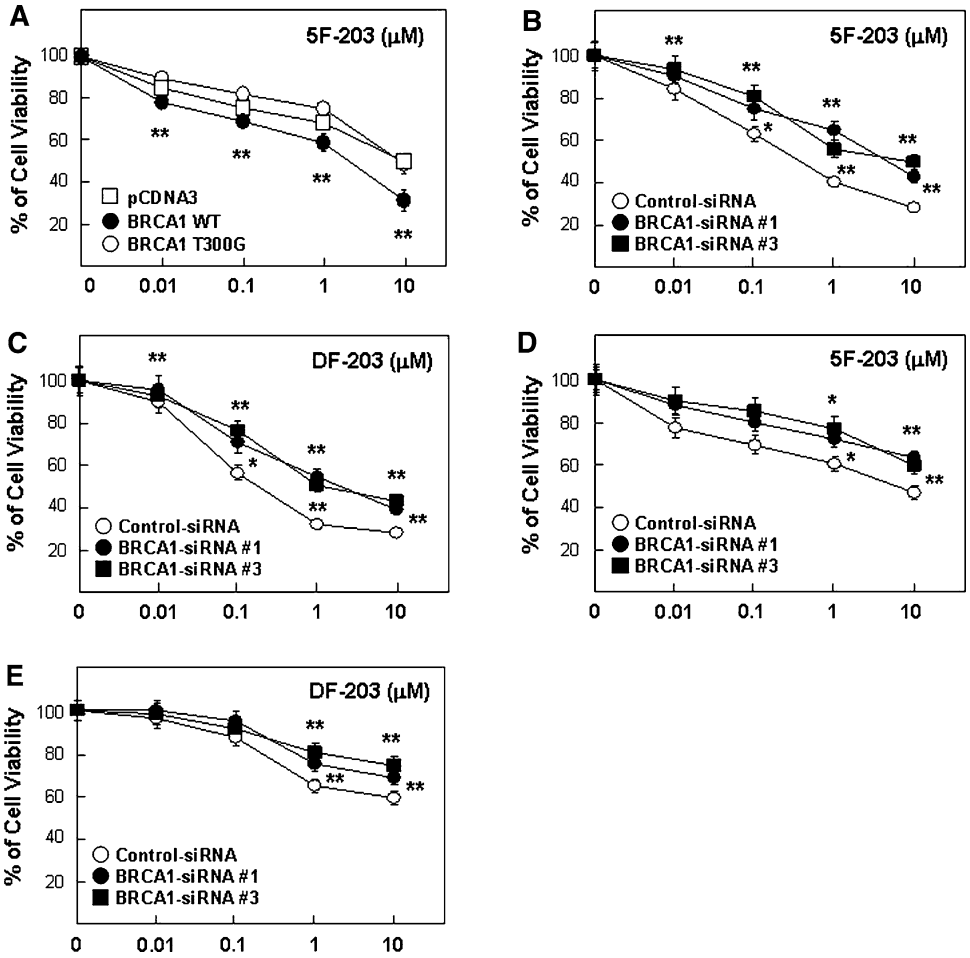

Fig. 4 (MCF-7) BRCA1 modulates the cytotoxicity of 5F-203 or DF-203. **a** Cells transiently transfected with empty vector, wtBRCA1, or the T300G mutant for 24 h were treated with various doses of 5F-203 for 72 h and cell survival measured by MTT assays (see "Materials and methods"). Wild-type BRCA1 over-expression significantly increased sensitivity to 5F-203 ($P < 0.005$ for comparisons between pCDNA3 control vs. wtBRCA1 transfected cells). MCF-7 (**b**, **c**) or T47D (**d**, **e**) cells transfected with siRNAs (control vs. BRCA1) for 72 h were seeded in 96 well plates and treated with various doses of 5F-203 (**b, d**) or DF-203 (**c, e**) for an additional 72 h plus fresh siRNAs (control vs. BRCA1) and assayed for cell viability by MTT assays. For each drug dose, $N = 20$ replicate wells were treated, pooled and averaged. Values are mean ± SEMs of cell viability (expressed relative to the 0 dose as 100%). The viability of cells treated with control-siRNA vs. BRCA1-siRNA was significantly different ($P < 0.005$ or $P < 0.05$, two-tailed *t*-test) at each dose of 5F-203

two different BRCA1-siRNAs (#1 and #3), specific for two different coding regions of BRCA1 [12].

Additional evidence consistent with the hypothesis that BRCA1 protein levels normally regulate CYP1A1 gene expression (and 5F-203 activation) comes from experiments using mutant BRCA1 proteins. In these experiments, cell transfected with BRCA1 expression vectors encoding mutant BRCA1 proteins (5677G and 5382insC, containing mutations associated with increased breast cancer risk) also had a smaller, but statistically significant reporter-plasmid response to 5F-203 treatments than the control cells, which over-expressed wtBRCA1 protein (Fig. 2d). In contrast, two other BRCA1 mutants, T300G and C5365G, could not enhance the reporter activity induced by 5F-203. These results suggest that the intracellular amounts of either of the fully functional and some mutant BRCA1 proteins regulate the response of specific genes in breast cancer epithelial cells, e.g. CYP1A1, to 5F-203 treatments.

BRCA1 protein levels affect the ability of 5F-203 to increase endogenous CYP1A1 encoded enzymatic activity

Although the results presented this far, on reporter-plasmid expression and endogenous CYP1A1 mRNA levels, are consistent with the hypothesis that reduced wtBRCA1 protein/activity levels in breast cancer cells could result in reduced activation of the prodrug 5F-203, this is not necessarily so. That is, the critical component for 5F-203 activation, the amount of activity of the CYP1A1 encoded enzyme, EROD, has not yet been examined under any of the experimental conditions described thus far. Our hypothesis predicts that over-expression of wtBRCA1 will have a

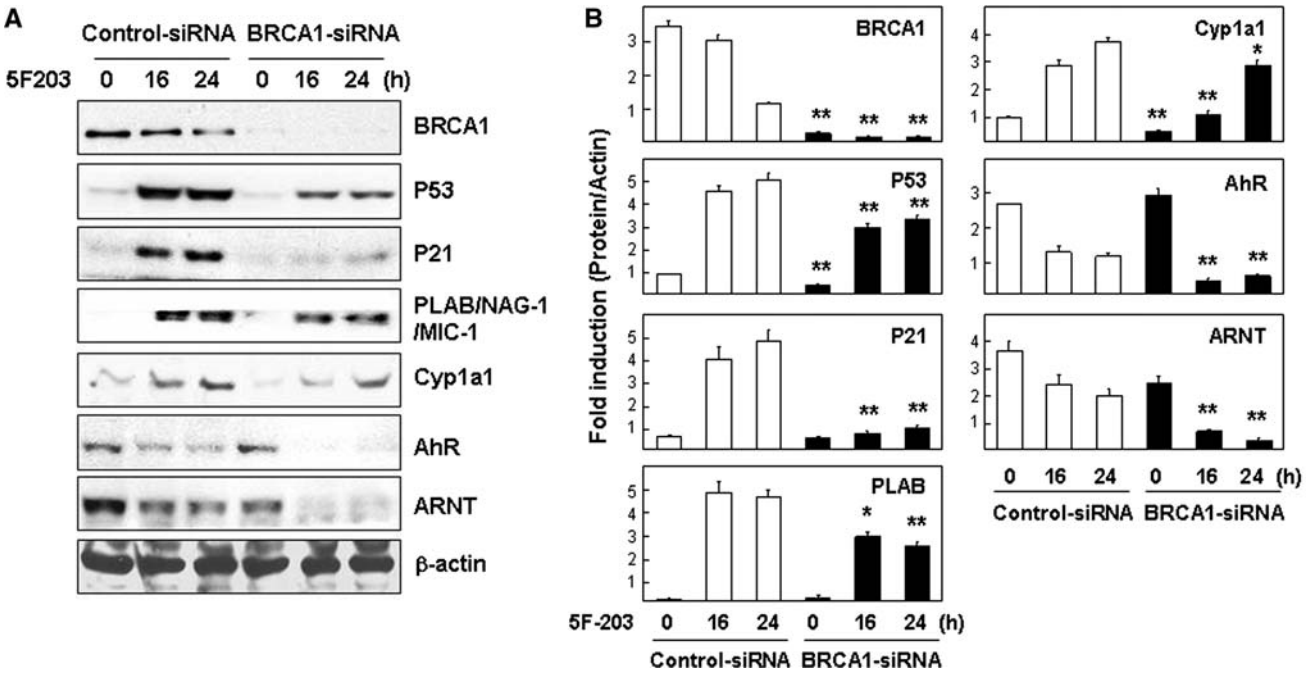

Fig. 5 BRCA1 knockdown reduces the ability of 5F-203 treatments to induce the expression of various proteins. **a** MCF-7 cells transfected with siRNAs (control vs. BRCA1) for 72 h were then treated with 1 μM of 5F-203 for 16 or 24 h and harvested for Western blot analysis. The relative amounts of P53, P21, PLAB/NAG-1/MIC-1, CYP1A1, AhR and ARNT were determined by using specific antibodies as described in "Materials and methods". **b** The results from (**a**) and two additional independent experiments were quantitated and displayed as bar graphs showing the means ± S.E. The Western blots analysis (**a**) is representative of the triplicate experiments. Reduced P53, P21, PLAB/NAG-1/MIG-1, CYP1A1, AhR and ARNT expression levels following 5F-203 treatment in BRCA1 knockdown cells (vs. control-siRNA-transfected cells) were significantly different than the control value ($P < 0.005$)

greater effect on 5F-203-induced EROD activity than over-expressing mutant BRCA1 proteins. In this test using MCF-7 cells, BRCA1 and BRCA1 mutants were over-expressed for 24 h before various doses (0, 0.01, 0.1 and 1 μM) of 5F-203 were given for an additional 24 h when EROD activity was measured (Fig. 3a). Dose-dependent increases in EROD activity were found under all the conditions tested. However, cells with wtBRCA1 over-expression were more responsive to increasing 5F-203 doses than cells over-expressing any of the mutants tested, or control empty vector (Fig. 3a). Western blot analysis found similar amounts of BRCA1 protein in each of the transfected cultures (Fig. 3b), indicating that the observed differences in enzymatic activities are not merely the result of differences in transfection efficiencies or exogenous protein levels. Finally, these results predict and we indeed found that pre-treating breast epithelial cells for 24 h with either of the two BRCA1 siRNAs significantly reduced the capacity of the siRNA-treated cells to accumulate EROD enzymatic activity after treatments with various doses of 5F-203 (Fig. 3c).

BRCA1 modulates the cytotoxicity of 5F-203 and its ability to induce gene expression

Next, we determined if differences in EROD activity correlate with different sensitivities to 5F-203. First, we examined the EROD activity levels in MCF-7 cells transiently transfected with various expression vectors, either an empty vector or vectors encoding wtBRCA1 or the T300G mutant protein and incubated with various doses of 5F-203 (0–10 μM) for 72 h. In this experiment wtBRCA1 over-expression significantly increased cytotoxicity compared to either the empty control vector or the T300G mutant encoding vector (Fig. 4a). We then investigated the role of endogenous BRCA1 protein levels on 5F-203 (or DF-203)-induced cytotoxicity by reducing endogenous BRCA1 protein level in MCF-7 (Fig. 4b, C) or T47D (Fig. 4d, e) by treating them with siRNA for 72 h before exposing them to various doses of 5F-203 (or DF-203; 0–10 μM) for an additional 72 h. MTT cell survival assays showed that BRCA1 knockdown reduced the cytotoxicity of 5F-203 (Fig. 4b, d). Similar results were obtained when an analogue of 5F-203, DF-203, was used (Fig. 4c, e). Next, total lysates from knockdown cells treated with 5F-203 were used for Western blot analysis to determine whether BRCA1 knockdown affected the ability of 5F-203 to induce the accumulation of various proteins in addition to CYP1A1. A previous study showed that 5F-203 treatments could increase P53 gene expression at both the mRNA and protein levels in MCF-7 cells [15]. We confirmed that 5F-203 treatment increased P53 protein levels and in addition found that the amounts of two P53 target

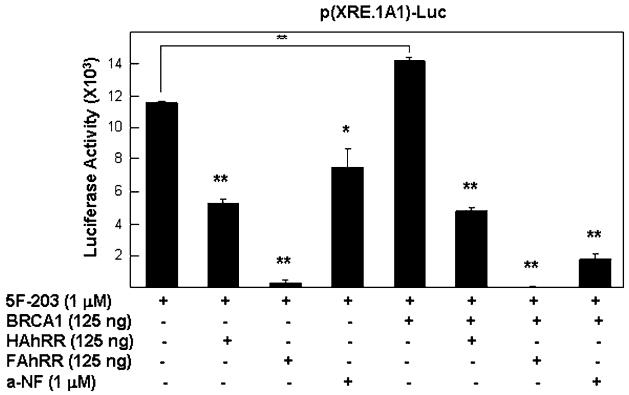

Fig. 6 The BRCA1 effect on 5F-203-induced reporter activity requires AhR. Cells (MCF-7) were cotransfected with a reporter plasmid, p(XRE.1A1)-Luc, and either a BRCA1 expression vector or the empty BRCA1 expression vector. These cotransfected cells were treated in three different ways: no additional transfection or simultaneously transfected with a second expression vector, encoding AhR inhibiting proteins (HAhRR or FAhRR) for 24 h. All cells were then treated with 1 μM of 5F-203 for an additional 24 h and reporter activity was measured. Alternatively, cells transfected with p(XRE.1A1)-Luc and the BRCA1 expression plasmid were treated with 1 μM of α-NF together with 5F-203. In all cases, luciferase activity was measured as described in the Fig. 1 legend. All three AhR inhibitors significantly and to a similar extent inhibited 5F-203-induced gene expression in cells transfected with empty vector or wtBRCA1 ($P < 0.005$ for comparisons of cells with or without AhR inhibitors). The values presented have been corrected for the background values, 2,363, 1,316, 864, 2,272, 3,190, 3,096, 2,165 and 5,042, respectively, for the DMSO-treated cells

gene products, p21 and PLAB/NAG-1/MCI-1 [2], were also significantly increased by 5F-203 treatments and that these increases were significantly less in BRCA1 knockdown cells (Fig. 5). To determine whether BRCA1 knockdown affected 5F-203-mediated AhR signaling, we determined the amounts of three key xenobiotic stress response proteins, AhR, ARNT and CYP1A1, on Western blot analysis. We found that 5F-203 induced significantly less amounts of all these three proteins in BRCA1 knockdown cells following 5F-203 treatments compared to the control (Fig. 5a). A graph quantifies these results (Fig. 5b).

The data in Fig. 1 is consistent with our previous study suggesting that BRCA1 regulation of CYP1A1 gene expression occurs, at least in part, at the mRNA level. Since the CYP1A1 gene is transcribed primarily by the AhR/ARNT heterodimeric transcription factor, we hypothesized that AhR inhibitors would reduce the ability of 5F-203 to induce CYP1A1 gene expression, independent of the BRCA1 protein levels. Thus, we treated cells containing either normal endogenous BRCA1 protein levels or increased BRCA1 protein levels with protein or chemical inhibitors of AhR. In this experiment, all cells were cotransfected with the XRE reporter plasmid and either the BRCA1 expression vector or the empty expression vector. In addition, endogenous AhR activity was blocked in these cells in three different ways: the simultaneous addition of a chemical inhibitor or the simultaneous cotransfection with expression vectors encoding HAhRR or FAhRR. The prodrug 5F-203 was added to all cells 24 h later and after an additional 24 h reporter activity was measured. All three AhR inhibitors reduced the ability of 5F-203 to increase expression from the CYP1A1 promoter–reporter (Fig. 6). The inhibitory effect of FAhRR is much greater than either of the other AhR inhibitors, at least under the conditions used in this experiment. Thus, FAhRR can essentially completely eliminate the ability of 5F-203 to induce reporter activity, even when BRCA1 is over-expressed, suggesting that BRCA1's ability to enhance reporter-plasmid expression requires AhR.

Discussion

In this study, we found that altered BRCA1 protein levels, either over-expression of exogenous BRCA1 or knockdown of endogenous BRCA1, affect the ability of the prodrug 5F-203 to induce CYP1A1 gene expression and the encoded enzymatic activity (EROD activity). This is the first report demonstrating that BRCA1 can regulate the CYP1A1-encoded enzymatic activity (EROD activity). These results provide at least a partial mechanistic explanation for our second major finding that BRCA1 protein levels can modulate the cytotoxicity of 5F-203 towards human mammary epithelial cells. Thus, our finding that BRCA1 knockdown in two cell lines, MCF-7 or T47D, reduces 5F-203 (or DF-203) cytotoxicity might be at least partially explained by reduced activation of these prodrugs, either by CYP1A1 or other enzymes induced by 5F-203 (or DF-203) exposure. Our results also suggest a second possible mechanism relating BRCA1 protein levels to 5F-203 cytotoxicity. This possibility is suggested by the previous finding that 5F-203 treatments induce cell cycle arrest and apoptosis in MCF-7 cells [15]. This cell cycle arrest and apoptosis was associated with an effect of 5F-203 on P53 expression [15]. P53 becomes activated following 5F-203 treatment and increases expression of P53 target genes, P21 and PLAB/NAG-1/MIC-1. In this study, we found that BRCA1 knockdown resulted in a reduced ability of 5F-203 treatment to induce the expression of P53 and two P53 target genes, P21 and PLAB/NAG-1/MIC-1. PLAB/NAG-1/MIC-1 is a member of transforming growth factor (TGF)-β superfamily and is known to have proapoptotic and antitumor activity [2]. Consequently, the reduced induction of P53 and its target genes following 5F-203 treatment of MCF-7 BRCA1 knockdown cells may reduce P53-induced apoptosis and cell cycle arrest, possibly contributing to the reduced cytotoxicity of 5F-203 observed under these conditions. In addition, we found that the induced expression of

AhR/ARNT and CYP1A1 proteins are significantly reduced in BRCA1 knockdown cells following 5F-203 treatment. These results suggest reduced biotransformation of 5F-203 in BRCA1 knockdown cells.

In conclusion, our findings suggest that a normal amount of wtBRCA1 protein is required for maintaining the cytotoxicity of 5F-203 towards breast cancer cells and that two BRCA1-dependent mechanisms may contribute to this regulation. BRCA1 facilitates the activation of 5F-203 via its role in the induction of CYP1A1 enzymatic activity and plays a role in p53-dependent apoptosis and cell cycle arrest. Consequently, sporadic breast cancer cells containing decreased levels or activity of BRCA1 protein (due to hypermethylation of promoter sequences, haplo deficiency or other reasons) may not respond to these drugs as well as tumor cell containing normal amounts of wtBRCA1 protein. Nevertheless, 5F-203, a rationally designed novel antitumor agent may have potential clinical utility in many settings.

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References

1. Bae I, Fan S, Meng Q, Rih JK, Kim HJ, Kang HJ, Xu J, Goldberg LD, Jaiswal AK, Rosen EM (2004) BRCA1 induces antioxidant gene expression and resistance to oxidative stress. Cancer Res 64:7893–7909
2. Baek SJ, Kim KS, Nixon JB, Wilson LC, Eling TE (2001) Cyclooxygenase inhibitors regulate the expression of a TGF-beta superfamily member that has proapoptotic and antitumorigenic activities. Mol Pharmacol 59:901–908
3. Bradshaw TD, Bibby MC, Double JA, Fichtner I, Cooper PA, Alley MC, Donohue S, Stinson SF, Tomaszewjski JE, Sausville EA, Stevens MF (2002) Preclinical evaluation of amino acid prodrugs of novel antitumor 2-(4-amino-3-methylphenyl) benzothiazoles. Mol Cancer Ther 1:239–246
4. Bradshaw TD, Shi DF, Schultz RJ, Paull KD, Kelland L, Wilson A, Garner C, Fiebig HH, Wrigley S, Stevens MF (1998) Influence of 2-(4-aminophenyl)benzothiazoles on growth of human ovarian carcinoma cells in vitro and in vivo. Br J Cancer 78:421–429
5. Burke MD, Mayer RT (1974) Ethoxyresorufin: direct fluorimetric assay of a microsomal O-dealkylation, which is preferentially inducible by 3-methylcholanthrene. Drug Metab Dispos 2:583–588
6. Carver LA, Bradfield CA (1997) Ligand-dependent interaction of the aryl hydrocarbon receptor with a novel immunophilin homolog in vivo. J Biol Chem 272:11452–11456
7. Chua MS, Kashiyama E, Bradshaw TD, Stinson SF, Brantley E, Sausville EA, Stevens MF (2000) Role of Cyp1A1 in modulation of antitumor properties of the novel agent 2-(4-amino-3-methylphenyl)-benzothiazole (DF 203, NSC 674495) in human breast cancer cells. Cancer Res 60:5196–5203
8. Denison MS, Nagy SR (2003) Activation of the aryl hydrocarbon receptor by structurally diverse exogenous and endogenous chemicals. Annu Rev Pharmacol Toxicol 43:309–334 Review
9. Hose C, Rivera MI, Sausville EA, Monks A (2001) Induction of cytochrome P450 1A1 and cytochrome P450 1B1 by 2-(amino-3-methylphenyl) benzothiazole (BZ) in 60 human tumor cell lines: correlation with BZ toxicity. Proc Am Assoc Cancer Res 42:511
10. Hankinson O (1995) The aryl hydrocarbon receptor complex. Annu Rev Pharmacol Toxicol 35:307–340 Review
11. Karchner SI, Franks DG, Powell WH, Hahn ME (2002) Regulatory interactions among three members of the vertebrate aryl hydrocarbon receptor family: AHR repressor, AHR1, and AHR2. J Biol Chem 277:6949–6959
12. Kang HJ, Kim HJ, Kim SK, Barouki R, Cho CH, Khanna KK, Rosen EM, Bae I (2006) BRCA1 modulates xenobiotic stress-inducible gene expression by interacting with ARNT in human breast cancer cells. J Biol Chem 281:14654–14662
13. Kang HJ, Kim HJ, Rih JK, Mattson TL, Kim KW, Cho CH, Isaacs JS, Bae I (2006) BRCA1 plays a role in the hypoxic response by regulating HIF-1alpha stability and by modulating vascular endothelial growth factor expression. J Biol Chem 281:13047–13056
14. Kazlauskas A, Sundstrom S, Poellinger L, Pongratz I (2001) The hsp90 chaperone complex regulates intracellular localization of the dioxin receptor. Mol Cell Biol 21:2594–2607
15. Monks A, Harris E, Hose C, Connelly J, Sausville EA (2003) Genotoxic profiling of MCF-7 breast cancer cell line elucidates gene expression modifications underlying toxicity of the anticancer drug 2-(4-amino-3-methylphenyl)-5-fluorobenzothiazole. Mol Pharmacol 63:766–772
16. Perdew GH, Bradfield CA (1996) Mapping the 90 kDa heat shock protein binding region of the Ah receptor. Biochem Mol Biol Int 39:589–593
17. Rosen EM, Fan S, Pestell RG, Goldberg LD (2003) BRCA1 gene in breast cancer. J Cell Physiol 196:19–41 Review
18. Swanson HI, Perdew GH (1993) Half-life of aryl hydrocarbon receptor in Hepa 1 cells: evidence for ligand-dependent alterations in cytosolic receptor levels. Arch Biochem Biophys 302:167–174
19. Trapani V, Patel V, Leong CO, Ciolino HP, Yeh GC, Hose C, Trepel JB, Stevens MF, Sausville EA, Loaiza-Perez AI (2003) DNA damage and cell cycle arrest induced by 2-(4-amino-3-methylphenyl)-5-fluorobenzothiazole (5F 203, NSC 703786) is attenuated in aryl hydrocarbon receptor deficient MCF-7 cells. Br J Cancer 88:599–605
20. Watanabe T, Imoto I, Kosugi Y, Fukuda Y, Mimura J, Fujii Y, Isaka K, Takayama M, Sato A, Inazawa J (2001) Human arylhydrocarbon receptor repressor (AHRR) gene: genomic structure and analysis of polymorphism in endometriosis. J Hum Genet 46:342–346