Moderate Increase in Mdr1a/1b Expression Causes In vivo Resistance to Doxorubicin in a Mouse Model for Hereditary Breast Cancer


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Abstract

We have found previously that acquired doxorubicin resistance in a genetically engineered mouse model for BRCA1-related breast cancer was associated with increased expression of the mouse multidrug resistance (Mdr1) genes, which encode the drug efflux transporter ATP-binding cassette B1/P-glycoprotein (P-gp). Here, we show that even moderate increases of Mdr1 expression (as low as 5-fold) are sufficient to cause doxorubicin resistance. These moderately elevated tumor P-gp levels are below those found in some normal tissues, such as the gut. The resistant phenotype could be completely reversed by the third-generation P-gp inhibitor tariquidar, which provides a useful strategy to circumvent this type of acquired doxorubicin resistance. The presence of MDR1A in drug-resistant tumors with a moderate increase in Mdr1a transcripts could be shown with a newly generated chicken antibody against a mouse P-gp peptide. Our data show the usefulness of realistic preclinical models to characterize levels of Mdr1 gene expression that are sufficient to cause resistance.

Introduction

The anthracycline doxorubicin is frequently used in standard adjuvant, neoadjuvant, or palliative chemotherapy regimens for breast cancer patients (1). Doxorubicin increases the levels of (double-stranded) DNA breaks resulting in cell death. Spontaneous mammary tumors deficient in the error-free homology-directed repair of DNA damage are especially sensitive to this drug (2). However, successful chemotherapy of breast cancer is hampered by the development of multidrug resistance. As underlying cause, a range of different mechanisms has been identified, including alterations in drug target, drug accumulation/metabolism, DNA repair, or cell death pathways (3–6). For several of these mechanisms, their relevance to resistance in real tumors is sparse (7–10). One of the best-characterized mechanisms of multidrug resistance in vitro is drug efflux by ATP-binding cassette (ABC) transporters such as ABCB1/MDR1/P-glycoprotein (P-gp), ABCG2/BCRP, or ABCC1/MRP1 (3). P-gp is present in the apical membrane of many different normal cells and its substrate specificity is broad (11, 12). Nevertheless, whether P-gp is relevant for clinical multidrug resistance of breast cancers is still debated (13–15). One issue that feeds the argument is the difficulty to detect P-gp in situ using formalin-fixed tumor samples. Another issue is that inhibitors of P-gp have only yielded a modest benefit in the clinic. First-generation, nonspecific inhibitors, such as verapamil and cyclosporine A, did not reach clinically effective plasma levels necessary for P-gp inhibition. Second-generation inhibitors, such as PSC 833 (valspodar), were more potent and selective, but their pharmacokinetic interactions with drugs often necessitated reductions of doses of the anticancer drugs. A more selective third-generation P-gp inhibitor is tariquidar (XR9576, Avant; refs. 17). In several studies, no clinically relevant interactions with pharmacokinetics of agents such as doxorubicin, vincristine, paclitaxel, or vinorelbine were detected, although (moderate) increases in chemotherapy side effects were observed in patients (18).

We have recently investigated anticancer drug responses in a genetically engineered mouse model for hereditary breast cancer (K1-tcre;Bra11/11;p53+/−; refs. 2, 19). Mammary tumors that spontaneously develop in this model mimic key features of breast cancer 1 (BRCA1)-associated mammary carcinomas in humans. An advantage of this mouse model is that these features as well as the individual tumor gene expression profiles are preserved on orthotopic grafting of small tumor fragments into syngeneic mice. Because BRCA1 is essential for the repair of double-stranded DNA breaks by homologous recombination, it is not surprising that the Brca1−/−/p53−/− tumors tested were sensitive to the DNA-interacting drugs cisplatin, doxorubicin (2), topotecan,3 and carboplatin or the poly(ADP-ribose) polymerase inhibitor AZD2281 (20, 21). Nevertheless, tumors could not be eradicated and eventually acquired resistance to each of the drugs tested, with the exception of cisplatin and carboplatin. A major mechanism of acquired doxorubicin and AZD2281 resistance in these tumors was increased expression of the Mdr1a and Mdr1b (in short Mdr1) genes, which encode the murine drug-transporting P-gp. High Mdr1 expression was associated with increased washout of the P-gp substrate 99mTc-sestamibi and cross-resistance to the taxane docetaxel (2). Intriguingly, we found only low (5-fold) increases of Mdr1 transcripts in some tumors and wondered whether these were sufficient to explain the resistant phenotype.

Here we report that a 5-fold increase above the average Mdr1a and Mdr1b transcript levels of untreated tumors is sufficient to cause doxorubicin resistance. Such amounts are far below...
transcript quantities found in normal tissues such as the gut (Mdr1a) or kidney and adrenals (Mdr1b). In several tumors showing a moderate increase in Mdr1 gene expression, P-gp levels could be detected with a newly generated polyclonal antibody.

Materials and Methods

Animals, generation of mammary tumors, and orthotopic transplantations into syngeneic wild-type mice. Doxorubicin-sensitive and doxorubicin-resistant Brca1<sup>-/-</sup>/p53<sup>-/-</sup> mammary tumors were generated in K14cre;Brca1<sup>F/F</sup>;<p53<sup>F/F</sup> mice, genotyped, and orthotopically transplanted as described (2, 22). Starting 2 weeks after tumor grafting, the onset of tumor growth was checked at least three times a week. Mammary tumor size was determined by caliper measurements (length and width in mm) and tumor volume (in mm<sup>3</sup>) was calculated using the following formula: 0.5 × length × width<sup>2</sup>. Animals were euthanized with CO<sub>2</sub> when the tumor volume reached 1,500 mm<sup>3</sup>. In addition to sterile collection of multiple tumor pieces for grafting experiments, tumor samples were snap frozen in liquid nitrogen and fixed in 4% formalin. All experimental procedures on animals were approved by the Animal Ethics Committee of the Netherlands Cancer Institute.

Drugs. Doxorubicin (Adriblastina; Pharmacia Netherlands) was diluted to 1 mg/ml in saline (Braun). Tariquidar (Avantad) was diluted in 5% glucose such that the final volume administered by i.p. injection was 10 μl/g of body weight.

Treatment of mammary tumor-bearing animals. When mammary tumors reached a size of ~200 mm<sup>2</sup>, 5 mg doxorubicin was given i.v. on day 0. Controls were left untreated. To avoid accumulating toxicity of repeated drug injections, an additional treatment was not given after the recovery time of 7 days when the tumor responded to the treatment (tumor size <50% of the original volume, partial response). In this case, treatment was resumed once the tumor relapsed to its original size (100%). For tumors with a volume ≥50% after the recovery time, an additional treatment with the same dose as mentioned above was given.

Multiplex ligation-dependent probe amplification analysis. Total RNA and DNA were isolated from snap frozen tumor samples with Trizol (Invitrogen) according to the manufacturer’s protocol. Multiplex ligation-dependent probe amplification (MLPA) reactions on DNA samples were done as described<sup>4</sup> and the employed oligos are indicated in Supplementary Table S1. The integrity of RNA was verified by denaturing gel electrophoresis. For RNA quantification, reverse transcription-MLPA analysis comprising reverse transcription, hybridization, ligation, PCR amplification, and fragment analysis by capillary electrophoresis was done as described (2). To detect hypoxanthine phosphoribosyltransferase 1 (Hprt1), we used CAGGTGACGAAACT as reverse transcription primer (5’-3’) and TCCCTATGGACTGATTATGGACAGGACTGAAAG, ACTTGCTCGAGATGTTGCACTCA as target-specific sequences for the ligation reaction. For β<sub>j</sub>-microglobulin, CTGGTTCGATATTTGTTATG (5’-3’) was used in the reverse transcription reaction and CATGTGGATACATCATCAGTATGCTGGTAA and GATTCTTATGGACTGAACTTACACATCTCCATTAATCTG for the ligation step. 5’-Rapid amplification of cDNA ends. Total RNA was extracted from four doxorubicin-resistant tumors and four untreated tumors. RNA was quantified, and a standard 5’-rapid amplification of cDNA ends (5’-RACE) protocol was done to determine the transcriptional start site of Mdr1a and Mdr1b transcripts. The Generacer kit was obtained from Invitrogen, and 5’-RACE were done as per the manufacturer’s instructions. Primers were designed to be within coding regions near the ATG start site. RATE products were cloned into TOPO TA vector (Invitrogen) and transformed into bacteria. Multiple clones (at least 20 for each 5’-RACE experiment) were sequenced to build a repertoire of transcribed mRNA sequences expressed in each tumor. RACE primers (5’-3’); gene-specific primer Mdr1a: MseMdr1a JM5: CTCTCTTTACGGTGCTCTCATGTCGTC, nested primer Mdr1a: MseMdr1a JM6: AAGTCTTACAGCTACGGTGCTCTTAC, gene-specific primer Mdr1b: MseMdr1b JM7: GAACTTGAATATTTGTCGATCCCTTTTGCCC, nested primer Mdr1b: MseMdr1b nestm JM8: TCGTGTCTCTCTTACGTTCTCCTCAACTC. To set up the procedure, we used the vibinlastine-selected mouse macrophage cell lines J774.L and J774.L described by Greenberger and colleagues (23) and kindly provided by C.P.H. Yang and Dr. Susan B. Horowitz (Albert Einstein College of Medicine).

Western blot analysis. Tumor samples were homogenized in 10 mM L KCl, 1.5 mM MgCl<sub>2</sub>, 10 mM Tris-HCl (pH 7.4), and 0.5% (w/v) SDS supplemented with Complete protease inhibitors (Roche). DNA was sheared by sonication, and samples containing 50 μg protein were fractionated by SDS-PAGE on a 7.5% Tris-glycine gel and then transferred onto a nitrocellulose membrane by electroblotting. After blotting, the membranes were blocked for at least 1 h in TBS (10 mM Tris, 100 mM NaCl) containing 5% milk powder and 0.05% Tween followed by an overnight incubation at 4°C with the C219 antibody at 12 μg/ml. Immunoreactivity was visualized with horseradish peroxidase-conjugated rabbit anti-mouse immunoglobulins (DakoCytomation; diluted 1:5,000) followed by enhanced chemiluminescence detection.

L59509 antibody. The sequences for mouse Mdr1a and Mdr1b genes were analyzed by the algorithm of Hopp and Woods to determine potential epitopes for synthesis and antibody production. The peptides were then BLASTed against the Swiss-Prot database to determine uniqueness and to help predict the specificity of the resulting antibodies. Peptide KMGGKSKKEKEKPAVSV was selected and synthesized, and chicken polyclonal antisera were generated at Lifespan Biosciences. To allow for peptide conjugation to the carrier protein, a cysteine residue was added to the NH₂ terminus of the peptides. The 77-day yolk were subjected to peptide affinity purification, and the resulting antisera were then used as primary antibody, termed L59509.

Immunocytochemistry. LLC-PK1 and its corresponding P-gp-transfected lines grown on slides were fixed with −20°C acetone for 40 s and incubated in serum-free protein block (DakoCytomation) for 1 h. The cells were then incubated overnight with PBS containing 1% bovine serum albumin and a primary antibody, which was either L59509 or C219 (Calbiochem) at a final protein concentration of 0.26 or 2.9 μg/ml, respectively. Slides were washed with PBS followed by subsequent incubation with a biotin-coupled appropriate secondary antibody for 1 h at a dilution of 1:1,000 (v/v) and a streptavidin-horseradish peroxidase conjugate (Vector Laboratories) for 30 min at room temperature. Immunoreactions were visualized with diaminobenzidine (Sigma), and the sections were counterstained with hematoxylin.

Immunofluorescence on tissue sections. Freshly cut 4-μm-thick frozen sections of mouse tumor tissue were fixed with −20°C acetone for 10 min. Sections were then blocked for avidin/biotin (DakoCytomation) for 10 min and in serum-free protein block (DakoCytomation). For C219 immunofluorescence, additional blocking steps were introduced to remove nonspecific interactions with mouse-on-mouse block (M.O.M kit; Vector Laboratories) according to the manufacturer’s instructions. The tissue sections were then incubated overnight at 4°C with primary antibodies (0.26 μg/ml for the L59509 antibody or 11.6 μg/ml for the C219 antibody). Slides were washed with PBS followed by subsequent incubation with a biotin-coupled appropriate secondary antibody for 1 h at a dilution of 1:1,000 (v/v) and a streptavidin-horseradish peroxidase conjugate (Vector Laboratories) for 30 min at room temperature. Immunoreactions were visualized with diaminobenzidine (Sigma), and the sections were counterstained with 4,6-diamidino-2-phenylindole fluorescence in the tissue sections was analyzed with a Leica confocal AOBS fluorescence microscope.

Results

Doxorubicin resistance of Brca1<sup>-/-</sup>/p53<sup>-/-</sup> tumors with a moderate increase of Mdr1a/Mdr1b expression can be reversed by the P-gp inhibitor tariquidar. In addition to

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1 http://www.mrc-holland.com
published ratios of resistant tumors versus matched samples taken before treatment (2), we determined Mdr1 gene expression levels in doxorubicin-sensitive and doxorubicin-resistant mouse mammary tumors in comparison with selected normal tissues by reverse transcription-MLPA. Actinß, Hprt1, and β2-microglobulin were used as reference and relative Mdr1 transcript levels were compared with those of the liver, gut, adrenals, and kidney, which are known to express P-gp. As shown in Fig. 1, the average level of Mdr1 RNA in untreated tumors was comparable with that of the spleen. Intriguingly, tumor 5 (T5), which has a 1.9- and 2.0-fold higher RNA level than the average Mdr1a and Mdr1b of untreated tumors, showed a stable disease in response to doxorubicin, whereas the other tumors usually shrank in response to treatment (2). In the resistant tumors, at least 2-fold increased Mdr1 RNA levels above the Mdr1 average of untreated tumors were detected in 11 of 13 tumors (Fig. 1; Supplementary Table S2). Mdr1a transcript levels varied between those reached in the liver (T6doxres and T8doxres, 2- to 3-fold above the average of untreated tumors), duodenum (T5doxres, T7doxres, and T*19doxres, 5- to 6-fold above the average of untreated tumors), and ileum and large intestine (T*4doxres, T*20doxres, T*22doxres, T*23doxres2, and T*23doxres3, up to 36-fold above the average of untreated tumors). Regarding Mdr1b transcripts, only in two tumors (T*23doxres2 and T*23doxres4) levels between those found in the kidney or adrenal glands were measured (31- to 52-fold above the average of untreated tumors), but none reached those found in the adrenal gland. In summary, only one doxorubicin-resistant tumor (T*22doxres) had higher Mdr1 RNA levels than some normal tissues.

On orthotopic transplantation into syngeneic animals, doxorubicin-resistant tumors kept their resistance phenotype or showed only a slight delay in growth in the presence of doxorubicin (ref. 2; Fig. 2), whereas the primary sensitive tumors shrank substantially.

Figure 1. Comparison of Mdr1 mRNA levels with those found in normal tissues. Reverse transcription-MLPA analysis of the ratios of Mdr1a and Mdr1b expression in normal tissues (yellow), doxorubicin-resistant tumors (red), and samples from the corresponding untreated tumors (blue). The sum of actinß, Hprt1, and β2-microglobulin (β2M) expression was used as reference. Green dotted lines, average Mdr1a/(actinß+Hprt1+β2M) and Mdr1b/(actinß+Hprt1+β2M) ratios (0.19 and 0.072) found in the untreated tumors. Columns, mean ratio of three reactions; bars, SD.
To determine the importance of P-gp in doxorubicin-resistant tumors, we pretreated the mice with the P-gp inhibitor tariquidar (17). When 10 mg tariquidar/kg i.v. was given 15 min before doxorubicin, animals could only tolerate four to five subsequent treatments of the MTD of doxorubicin (5 mg/kg i.v., minimally 7 days recovery time). This is less than what can be administered when doxorubicin is given as a single agent (at least eight cycles are usually tolerated). Histologic analysis of sacrificed animals after repeated doxorubicin + tariquidar doses revealed bone marrow depletion as the most likely cause of toxicity (data not shown). After the initial injection on day 0 (volume ~ 200 mm³), additional treatments were given 7 days later in case the tumor size was >50% of the size on day 0. In case the tumor regressed to a size below 50%, we resumed treatment when the tumor relapsed to 100%. As shown in Fig. 2, P-gp inhibition successfully reversed doxorubicin resistance in three individual tumors in which we found increased Mdr1 mRNA levels. T*23doxres1, in which no Mdr1 increase was observed (Fig. 1), showed only a small benefit of the pretreatment with tariquidar. Despite the fact that tumors became sensitive again to doxorubicin, they were not eradicated by the tariquidar-doxorubicin combination.

Up-regulation of Mdr1 is not caused by gene amplification in most tumors and does not correlate with differential promoter usage of Mdr1a or Mdr1b. Amplification of MDR1 or its rodent homologues has been observed in many multidrug-resistant cell lines (reviewed in ref. 4). In contrast, we found no increase in the DNA content of 12 of 13 doxorubicin-resistant tumors relative to samples taken before tumor treatment (Supplementary Fig. S1). Only for T*23doxres4 a 1.8-fold increase was identified. However, a small increase in Mdr1 copy number might be difficult to detect in crude tumor samples. We therefore enriched tumor cells from two doxorubicin-resistant tumors (T*23doxres2 and T7doxres) by removing dead cells, endothelial, fibroblastic, and hematopoietic (Lin+) cells after tumor dissociation. MLPA analysis of these Lin- cells did not detect any Mdr1 amplification (Supplementary Fig. S2). These results suggest that increased transcription of Mdr1 rather than gene amplification drives the increased Mdr1 expression in most of the tumors.

Figure 2. Response of doxorubicin-resistant tumors to doxorubicin combined with tariquidar. Fragments of doxorubicin-resistant tumors with different increases of Mdr1 transcripts (T*23doxres2, T*23doxres3, and T7doxres) or without detectable Mdr1 mRNA alterations (T*23doxres1) were transplanted orthotopically into syngeneic mice and left untreated (dark blue) or treated with 10 mg tariquidar/kg i.v. (turquoise), 5 mg doxorubicin/kg i.v. (pink), or 10 mg tariquidar/kg i.v. followed 15 min later by 5 mg doxorubicin/kg i.v. (green, orange, and brown). In case tumors did not shrink <50%, animals were retreated after a recovery time of 7 d. Days on which animals were retreated are indicated (open circles, triangles, or squares). Where error bars (SD) are given, the data points represent the mean of three individual animals. Due to the variability of relapsing tumors after tariquidar-doxorubicin combinations, the three individual curves are presented for T*23doxres2, T*23doxres3, and T7doxres.
Two types of transcriptional activation of the human MDR1 gene have been reported in human tumor samples and cell lines: DNA rearrangements linking the MDR1 gene to a strong promoter (24, 25) and activation of a "distal" promoter, 100 kb upstream of the MDR1 gene (26, 27). In mouse cell lines, Mdr1 gene activation may also occur by (defective) retrovirus insertion (28) or by DNA rearrangements proposed to result from unequal sister chromatid exchange mediated by LINE-1 repetitive elements (29). To test whether any of these mechanisms apply in our resistant tumors, we mapped the start of Mdr1a and Mdr1b transcripts using a 5'-RACE protocol, which allows cloning of 5'-cap-linked sequences. The results obtained for the capped transcripts of four individual doxorubicin-resistant tumors and their matched doxorubicin-sensitive controls are presented in Fig. 3. Both for Mdr1a and Mdr1b we find a range of transcripts starting at a proximal promoter, 132 to 186 nucleotides upstream of the ATG in the case of Mdr1a and 116 to 186 nucleotides for Mdr1b. These start sites are often coincident with transcriptional start positions found previously for Mdr1a (30, 31) and Mdr1b (32, 33) in cell lines and normal tissues. In our model, there is no obvious difference between the exact start sites of resistant and sensitive tumors.

For Mdr1a, we also find transcripts starting far upstream of the gene. We find these in four individual tumors, all starting at position 8567101 bp of chromosome 5.5 Remarkably, in this limited analysis of a small number of tumors, we see no correlation between the use of this distal promoter and Mdr1a up-regulation. In two drug-sensitive tumors (T*20 and T*22) and one drug-resistant tumor without detectable Mdr1a up-regulation (T*23dooxres4), we find a transcript from the distal promoter, whereas among the three tumors with Mdr1a up-regulation we only see a distal promoter transcript in T4dooxres. Even from this limited set of tumors, it is clear that the up-regulation of Mdr1a is not specifically associated with activation of a distal promoter in our mouse mammary tumors, in contrast to what was observed in human tumors/cell lines (26, 34). A distal promoter for Mdr1a, not identical to the one identified here, has only been found in one set of vinblastine-selected mouse macrophage cell lines (30).

Detection of MDR1A with a new mouse-specific antibody.

We determined the amount of P-gp by Western blotting and/or immunofluorescence with a range of mouse- and human-specific antibodies such as C219 (Calbiochem), 265F4, 4E3, Ab36743, and SPM137 (all from Abcam), NH211 (35), 15D3 (36), and UIC2 (Chemicon). Of the antibodies tested, the C219 monoclonal antibody also bound nonspecifically to other proteins, as indicated by blotting using C219 (Supplementary Fig. S3), although this antibody bound less than with the C219 antibody (Supplementary Table S3): all tumors with high levels of Mdr1a transcripts (T4dooxres, T*22dooxres, and T23*dooxres2) were positive. Of tumors with moderate increase in Mdr1a, T5dooxres, T7dooxres, T19dooxres, T20dooxres, and T23dooxres3 showed positive staining, whereas all other doxorubicin-resistant tumors were negative. Figure 4C shows representative examples of T*22dooxres, T23*dooxres2, and T5dooxres with corresponding controls. These results show that P-gp is indeed expressed in tumors with a moderate transcriptional up-regulation of Mdr1.

Discussion

In this study, we show that moderate increases in the expression of Mdr1 genes encoding P-gp are sufficient to cause acquired doxorubicin resistance in a realistic mouse model for BRCA1-associated breast cancer. In several resistant tumors, Mdr1 mRNA levels were only 5- to 13-fold above the average of Mdr1a or Mdr1b in untreated tumors. The levels in the resistant tumors are comparable with those found in the liver or duodenum of wild-type mice and we rarely found tumor levels exceeding those normally found in the large intestine (Mdr1a) or kidneys (Mdr1b). In normal tissues, reduced doxorubicin sensitivity has also P-gp-independent causes such as lack of proliferation or intact DNA repair mechanisms. In the investigated tumors, we are confident that these low levels of P-gp cause doxorubicin resistance, as resistance could be reversed by the third-generation P-gp inhibitor tariquidar. We conclude that in our tumor model P-gp plays a pivotal role in causing drug resistance even in tumors with only a 5-fold increase in Mdr1a and Mdr1b mRNA. Our inability to eradicate tumors by the tariquidar-doxorubicin combination might be due to dormancy of residual tumor-initiating cells or other mechanisms (44). Thus far, it has been difficult to show a role for P-gp in drug-resistant breast cancer in vivo. As an example, a meta-analysis of 31 breast cancer trials found P-gp expression in 41% of the tumors and associated a 3-fold chemotherapy response reduction with the presence of P-gp (45). Nevertheless, such studies need to be taken with a grain of salt due to huge differences in the definition of what

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5 http://www.ensembl.org/Mus_musculus

To improve the detection of mouse P-gp in situ, we raised a new chicken polyclonal antibody using the peptide KMGGKSSKKEKKEPKAVSV, which corresponds to amino acids 17 to 35 of mouse MDR1A (accession no. NP_035206). This NH2-terminal sequence has a high homology to mouse MDR1B (18 of 19) and human MDR1 (16 of 19). In our search for suitable peptides, we avoided extracellular loops due to their increased risk of yielding conformation-specific antibody binding. Moreover, we chose KMGKSSKKEKKEPKAVSV to avoid cross-reaction with MDR3/ABCB4, as this complicates the use of C219, which reacts with the core sequence VQEALD also present in ABCB4 (41, 42). When we tested the resulting affinity-purified polyclonal LS9509 antibodies on the transfected LLC-PK1 cells, only MDR1A could be detected by immunocytochemistry, whereas MDR1B and human MDR1 were not identified (Fig. 4A). Unfortunately, this antibody did not yield a positive result on Western blot (data not shown). To evaluate the suitability of LS9509 for immunostaining, we compared brain sections of Mdr1 wild-type animals with those derived from Mdr1−/− animals (43). As illustrated in Fig. 4B, LS9509 stained brain capillaries in wild-type but not in knockout animals. When we probed our panel of doxorubicin-resistant tumors with LS9509, we found that more tumors yielded plasma membrane staining than with the C219 antibody (Supplementary Table S3): all tumors with high levels of Mdr1a transcripts (T4dooxres, T*22dooxres, and T23*dooxres2) were positive. Of tumors with moderate increase in Mdr1a, T5dooxres, T7dooxres, T19dooxres, T20dooxres, and T23dooxres3 showed positive staining, whereas all other doxorubicin-resistant tumors were negative. Figure 4C shows representative examples of T*22dooxres, T23*dooxres2, and T5dooxres with corresponding controls. These results show that P-gp is indeed expressed in tumors with a moderate transcriptional up-regulation of Mdr1.
Figure 3. Mdr1 5′-RACE of doxorubicin-resistant and matched doxorubicin-sensitive tumors. A, Mdr1a products aligned to the sequence of Mdr1a. Numbers (in nucleotides; nt) indicate that each tumor (sensitive or resistant) expresses transcripts of various 5′-untranslated region lengths. The long transcripts match to regions containing canonical splice sites far upstream of the ATG start site, indicating that a distal transcriptional start site is used. All tumors (doxorubicin-resistant and doxorubicin-sensitive) express transcripts that use either proximal transcription start sites or a mixture of proximal and distal transcriptional start sites. Levels of Mdr1a RNA derived from these tumors are normalized to actinβ, Hprt1, and β2M.

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B, Mdr1b 5′-RACE products aligned to the sequence of Mdr1b. Numbers (in nucleotides) indicate that each tumor expresses transcripts of various 5′-untranslated region lengths. All tumors (doxorubicin-resistant and doxorubicin-sensitive) express Mdr1b transcripts that use a proximal transcription start site. Levels of Mdr1b RNA derived from these tumors are normalized to actinβ, Hprt1, and β2M.

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constitutes a “P-gp positive” tumor. More recently, in a phase II clinical trial, tariquidar showed limited ability to restore drug sensitivity in 17 women with stage III to IV breast cancer who progressed or had stable disease on anthracycline or taxane therapy (46). Only the patient with the greatest increase in $^{99m}$Tc-Sepamibi uptake, who also showed inducible P-gp expression, responded partially to tariquidar-containing therapy. Clearly, careful patient selection is necessary to increase potential benefit from P-gp-inhibiting therapy. In vivo imaging using the P-gp substrate $^{99m}$Tc-Sepamibi is one approach, and we also found increased sestamibi washout in our doxorubicin-resistant tumor model (2, 47). However, this tracer is not completely P-gp-specific and other drug efflux pumps (e.g., MRP1; ref. 48) that are not inhibited by P-gp-specific transport blockers could give a false-positive result.

Another approach is in situ detection with P-gp-specific antibodies. Our attempts to generate a more sensitive antibody for staining mouse P-gp were partially successful: the new chicken-derived LS9509 antibody detects only mouse MDR1A in situ. Still, eight doxorubicin-resistant tumors, with at least 5-fold increased Mdr1a transcript levels relative to untreated tumors, were scored P-gp positive, whereas C219 detected P-gp only in one of these.

Figure 4. Analysis of doxorubicin-sensitive and doxorubicin-resistant tumors with the chicken polyclonal LS9509 antibody. A, LLC-PK1 polar porcine kidney cells transfected with mouse Mdr1a, Mdr1b, or human MDR1 probed with LS9509 (brown). B, brain sections of wild-type (wt) or Mdr1a/b-/- animals tested with the LS9509 antibody (white, columns 1 and 3; green, columns 2 and 4). Nuclei were stained with 4',6-diamidino-2-phenylindole (blue, columns 2 and 4). C, OCT snap-frozen tumor slides of untreated or doxorubicin-resistant tumors incubated with LS9509 (white, columns 1 and 3; green, columns 2 and 4). Nuclei were stained with 4',6-diamidino-2-phenylindole (blue, columns 2 and 4). All incubations were done with LS9509 antibody at a final concentration of 0.26 μg/mL.
tumors. Possibly, the use of the mouse-on-mouse block for C219 is responsible for its reduced sensitivity. Although it remains possible that other human-specific antibodies that lack mouse cross-reactivity do a better job on human samples, it may be useful to generate more sensitive tools to detect P-gp in tumor sections. Besides new antibodies that are sensitive for both immunohistochemistry and immunoblotting, signal amplification via a proximity ligation assay (49) is another option that could be tested and optimized in our model.

Although our model mimics key features of BRCA1-associated breast cancer, it does not represent the full heterogeneity of treatment responses observed in breast cancer. We did not observe intrinsic doxorubicin resistance of drug-naive tumors, although stable or progressive disease is often found in response to anthracycline-containing chemotherapy in humans. To what extent increased P-gp expression contributes to primary chemotherapy resistance in human breast cancer patients remains questionable. The poor outcome of tartrixidarpretreatment in such patients (46) does not favor a key role for P-gp. An advantage of our mouse model is that we can breed in Mdr1-null alleles (43) to identify Mdr1-independent doxorubicin resistance mechanisms.

Future studies with Mdr1−/−;Brca1−/−; p53−/− tumors should reveal novel drug resistance mechanisms that may also be involved in human breast cancers where P-gp inhibition is not beneficial.

Disclosure of Potential Conflicts of Interest
A.O.H. Nygren is an employee of MRC-Holland, which markets the MLPA tests used in this article. The other authors disclosed no potential conflicts of interest.

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