

Sensitivity and Acquired Resistance of BRCA1;p53-Deficient Mouse Mammary Tumors to the Topoisomerase I Inhibitor Topotecan

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Abstract

There is no tailored therapy yet for human basal-like mammary carcinomas. However, BRCA1 dysfunction is frequently present in these malignancies, compromising homology-directed DNA repair. This defect may serve as the tumor's Achilles heel and make the tumor hypersensitive to DNA breaks. We have evaluated this putative synthetic lethality in a genetically engineered mouse model for BRCA1-associated breast cancer, using the topoisomerase I (Top1) poison topotecan as monotherapy and in combination with poly(ADP-ribose) polymerase inhibition by olaparib. All 20 tumors tested were topotecan sensitive, but response heterogeneity was substantial. Although topotecan increased mouse survival, all tumors eventually acquired resistance. As mechanisms of *in vivo* resistance, we identified overexpression of *Abcg2/Bcrp* and markedly reduced protein levels of the drug target Top1 (without altered mRNA levels). Tumor-specific genetic ablation of *Abcg2* significantly increased overall survival of topotecan-treated animals ($P < 0.001$), confirming the *in vivo* relevance of ABCG2 for topotecan resistance in a novel approach. Despite the lack of ABCG2, a putative tumor-initiating cell marker, none of the 11 *Abcg2*^{-/-};*Brca1*^{-/-};*p53*^{-/-} tumors were eradicated, not even by the combination topotecan-olaparib. We find that olaparib substantially increases topotecan toxicity in this model, and we suggest that this might also happen in humans. *Cancer Res*; 70(4): 1700–10. ©2010 AACR.

Introduction

Topotecan is used to treat ovarian, cervical, and small cell lung cancer patients. Topotecan inhibits topoisomerase I (Top1)-mediated religation of ssDNA breaks (SSB). When drug-stabilized covalent Top1-DNA complexes cause stalling of the replication machinery, SSBs are converted into double-stranded DNA breaks (DSB). Such replication-mediated DSBs seem to be the primary cytotoxic mechanism of Top1 inhibitors in dividing cells (1). Cell lines defective in proper DSB repair pathways show an increased sensitivity to Top1 poisons (reviewed in ref. 1). An example is a defect in BRCA1

(2), which is required for error-free repair of DSB by homologous recombination (HR). HR is frequently impaired in basal-like breast cancer, and BRCA1 dysfunction is one of the causes of this defect (3–5). About 15% of clinical patients are diagnosed with basal-like breast cancer, which is usually negative for ERBB2 and hormone receptors (“triple negative”; refs. 6, 7). Patients with this disease face a poor prognosis, as there is no targeted therapy available (6, 7). Topotecan is not applied in the clinic to breast cancer patients; hence, we have investigated in a mouse model whether the use of this Top1 inhibitor is an alternative therapeutic approach for patients with basal-like breast cancer. We used the *K14cre;Brca1*^{F/F};*p53*^{F/F} genetically engineered mouse model (GEMM), in which *Brca1*^{-/-};*p53*^{-/-} mammary adenocarcinomas arise, that recapitulate key features of human BRCA1-associated breast cancer (invasive ductal carcinoma not otherwise specified) and can be transplanted orthotopically into syngeneic mice without loss of their genomic profile, morphology, or sensitivity to drug (8, 9). In this model inhibition of SSB repair by the poly(ADP-ribose) polymerase (PARP) inhibitor olaparib (AZD2281; KU-0059436) induces DNA damage resulting in synthetic lethality of *Brca1*^{-/-};*p53*^{-/-} tumor cells and a strong increase in overall survival (10). Remarkable responses to olaparib treatment with only few adverse effects were also observed in a phase I clinical trial with patients that carry *BRCA1* or *BRCA2* mutations (11). Because in our model olaparib treatment alone did not result in tumor eradication (10), we have tested whether increasing the induction of SSBs

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by a Top1 poison would enhance antitumoral efficacy of this PARP inhibitor.

Here we show that topotecan alone or in combination with olaparib may be another therapeutic option for breast cancer patients who carry cancers with HR defects. However, we find that *Brca1*^{-/-}; *p53*^{-/-} tumors relapse and eventually acquire resistance. Of the various mechanisms reported in cultured cells to cause resistance to Top1 inhibitors (1, 12, 13), we identified only two in our model: an increased expression of the drug efflux transporter ABCG2 and reduced protein levels of the drug target Top1. We confirmed the *in vivo* relevance of ABCG2 for topotecan resistance through orthotopic transplantation of *Abcg2*^{-/-}; *Brca1*^{-/-}; *p53*^{-/-} tumors into syngeneic wild-type mice. The generation of spontaneous mammary tumors deficient for a drug efflux transporter that might protect tumor-initiating cells from chemotherapy (14, 15) is novel and cannot be achieved in humans. Our data strongly suggest that ABCG2 is dispensable for the survival of tumor-initiating cells in this model.

Materials and Methods

Animals, generation of (Abcg2-deficient) mammary tumors, and orthotopic transplantation of tumor fragments into syngeneic wild-type mice. *Brca1*^{-/-}; *p53*^{-/-} mammary tumors were generated and genotyped as described (8). To generate *Abcg2*^{-/-}; *Brca1*^{-/-}; *p53*^{-/-} mammary tumors, animals carrying *Abcg2*-deleted alleles (16) were bred with *K14cre*; *Brca1*^{F/F}; *p53*^{F/F} mice to homozygosity (*Abcg2*^{-/-}; *K14cre*; *Brca1*^{F/F}; *p53*^{F/F}) on a mixed 129/Ola and FVB/N genetic background. *Abcg2* genotypes were confirmed by PCR with specific primers (forward: 5'-CTTCTCCATTATCAGCCTCG-3') for wild-type (reverse: 5'-GGAGCAAAGCTGCTATTGGC-3') and deleted alleles (reverse: 5'-CAGTCGATGGATCCACTTAGG-3'). Orthotopic transplantations, mammary tumor measurements, and sampling were performed as explained previously (9). All experimental procedures were approved by the Animal Ethics Committee of the Netherlands Cancer Institute.

Drugs and treatment of tumor-bearing animals. Topotecan was dissolved in glucose (5%, w/v) to yield a solution of 0.4 mg/mL (of active compound) and administered at 10 μ L/g of body weight by i.p. injection. When tumors reached a size of \sim 200 mm³, 4 mg/kg topotecan were administered i.p. daily for 4 (Fig. 1A) or 5 (Fig. 1B) consecutive days or on days 0 to 4 and 14 to 18 (Figs. 1C, 3A and 4A). Tumors that responded [volume, <50% 14 d after the first topotecan injection of the first treatment course (Fig. 1A, B) or second course (Figs. 1C, 3A and 4A)] were left untreated until they relapsed to 100%. If tumors did not respond (volume remaining, \geq 50%), treatments were continued after a recovery time of 9 d after the last treatment. Olaparib and tariquidar were applied as reported (10, 17). In topotecan-olaparib combination-treated animals (Fig. 6B and C), the topotecan dose was lowered 8-fold to 0.5 mg/kg and administered on days 0 to 4, 14 to 18, and 28 to 32, whereas 50 mg/kg olaparib were injected i.p. daily for 42 d and, when combined with topotecan, administered 15 min after topotecan injection. Tariquidar (2 mg/kg;

Fig. 6C) was administered i.p. every second day during this 42-d period.

Genome-wide expression profiling. RNA extraction, amplification, microarray hybridization, data processing, and statistical analyses were performed as described (9). Two-color duplicate hybridizations on mouse microarrays (NKI Central Microarray Facility) were used, containing 38,784 70-mer probes representing 23,527 genes and 35,172 gene transcripts (MEEBO, Illumina BV). The microarray data reported in this article have been deposited in the Array Express database of the European Bioinformatics Institute (Cambridge, United Kingdom; accession no. E-NCMF-28).

Multiplex ligation-dependent probe amplification analysis. (Reverse Transcriptase-)Multiplex ligation-dependent probe amplification (MLPA) analyses on tumor DNA or RNA were carried out as before (9, 17).

Immunohistologic analysis. Immunohistochemical stainings were performed as described (10). ABCG2 was probed with the rat anti-mouse monoclonal (BXP53) from Abcam (ab24115, 1:400) and detected with a biotinylated rabbit anti-rat secondary antibody (Dakocytomation, E0468, 1:100).

Tumor protein extraction and Top1 immunoblotting. Snap-frozen tumor samples were ground into a fine powder by brief in a liquid nitrogen cooled mortar and transferred into 1.5-mL Eppendorf vials containing 20 μ L 1 \times Lämmli lysis buffer/mg tumor sample. Lysates were spun 5 min at 20,800 \times g to obtain the soluble protein fraction. The supernatants were boiled for 5 min at 100°C, and equal amounts of protein were size-fractionated on an 8% SDS-polyacrylamide gel by electrophoresis and transferred onto a nitrocellulose membrane (Protran BA83, Whatman). After a 2 \times 15-min block with Blotto (5% w/v milkpowder in 1 \times TBS-0.1% Tween 20), both anti-Top1 (rabbit polyclonal, 1:1,000; Topogen, 2012-2) and anti-Lamin A/C (rabbit polyclonal, 1:1,000; Santa Cruz, sc-20681) antibodies were used as probes and visualized by enhanced chemoluminescence after incubation with horseradish peroxidase-conjugated swine anti-rabbit IgG (1:10,000; Dakocytomation, P0217). Top1 band intensities were quantified digitally (ImageJ, NIH) in scanned pictures of linearly exposed films and normalized to Lamin A/C.

Results

***Brca1*^{-/-}; *p53*^{-/-} mammary tumors respond to the maximum tolerated dose of topotecan but relapse and eventually acquire resistance.** We established the maximum tolerated dose (MTD) of topotecan in 12- to 14-week-old female *K14cre*; *Brca1*^{F/F}; *p53*^{F/F} mice to maximize antitumoral drug efficacy and thereby mimicking a clinically relevant dose (Supplementary Fig. S1A). Topotecan (4 mg/kg) i.p. on 4 consecutive days was tolerated and resulted in 10% to 20% weight loss. When two animals carrying "spontaneous" mammary tumors (\sim 200 mm³ in volume) were treated with this dose, tumors shrank (Fig. 1A, T1 and T2). Despite this good response, tumors were not eradicated and relapsed. Once the tumors grew back to a volume of \sim 200 mm³ (100%) or showed progressive growth after a recovery period of 14 days after the first topotecan injection, treatments were resumed.

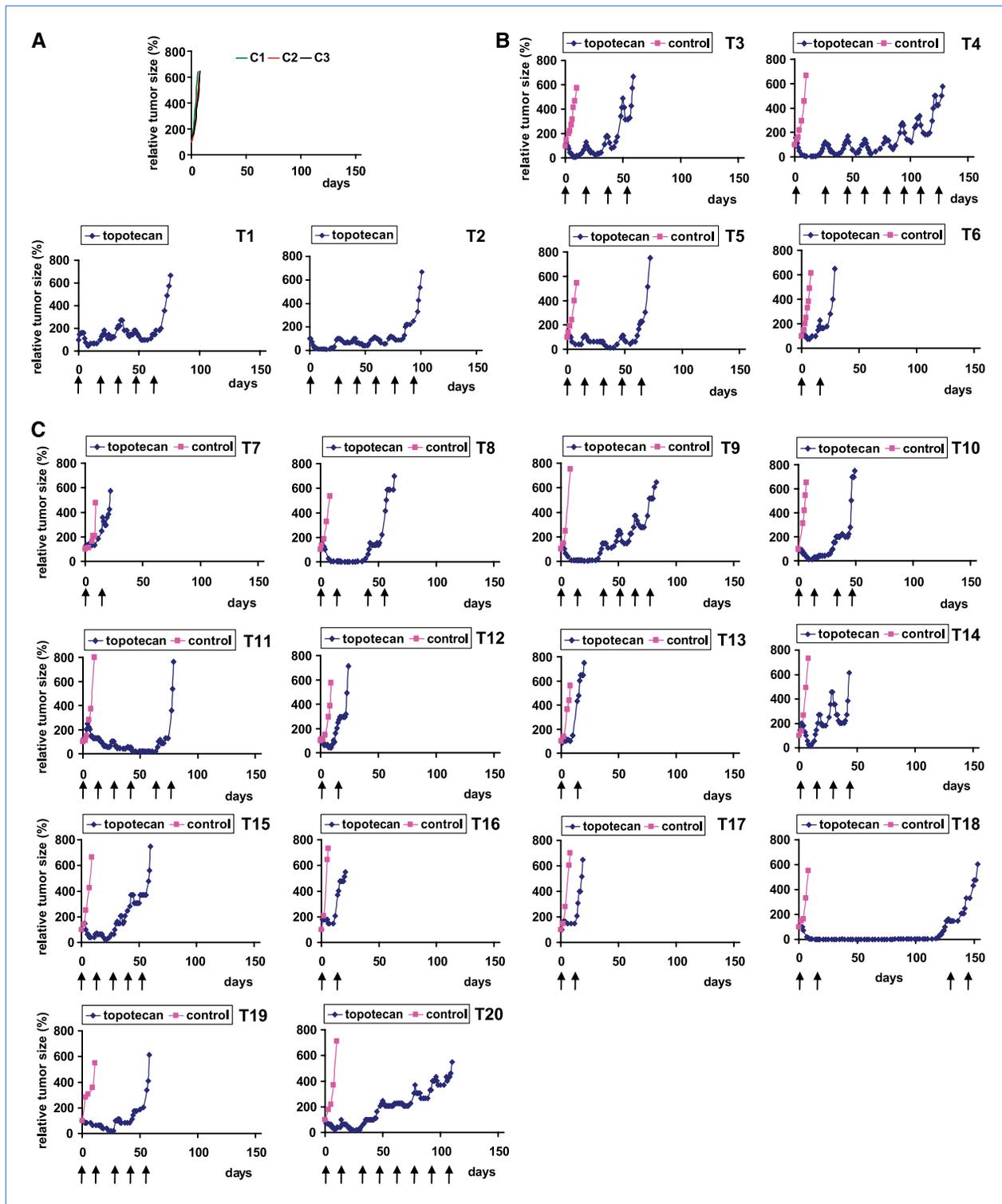


Figure 1. Response of *Brca1;p53*-deficient tumors to the MTD of topotecan. **A**, two individual *K14cre,Brca1^{F/F},p53^{F/F}* animals carrying a spontaneous mammary tumor of ~ 200 mm³ (T1 and T2) were treated with 4 mg/kg topotecan i.p. on days 0 to 3. For comparison, the growth of untreated spontaneous tumors in three individual *K14cre,Brca1^{F/F},p53^{F/F}* animals (C1–C3) is also shown. **B**, animals with four individual orthotopically transplanted *Brca1;p53*-deficient mammary tumors (volume, ~ 200 mm³) were left untreated (pink line) or treated as in **A**, but on days 0 to 4. **C**, animals bearing 14 individual orthotopically transplanted *Brca1;p53*-deficient mammary tumors (volume, ~ 200 mm³) were left untreated (pink line) or treated as in **A**, but on days 0 to 4 and 14 to 18 (dose-intensified treatment). When tumors in **A** to **C** relapsed or showed progressive growth (tumor size, $\geq 50\%$) after a recovery time of 14 d after the day 0 injection, treatment was resumed as indicated by the arrows.

Eventually, both tumors acquired resistance to the MTD of topotecan. In an attempt to eradicate the tumors by intensifying the topotecan therapy, we used wild-type animals with orthotopically transplanted *Brca1*;*p53*-deficient tumors, because wild-type animals could take an additional dose of topotecan (4 mg/kg drug i.p. on 5 consecutive days; Supplementary Fig. S1B). The orthotopically transplanted tumors responded to topotecan like the primary tumors, but also the intensified topotecan therapy was unable to eradicate the tumors (Fig. 1B, T3–T6). We therefore added a second MTD schedule after a 14-day recovery period after the first topotecan injection and tested this treatment in 14 individual *Brca1*^{-/-};*p53*^{-/-} tumors (4 mg/kg drug i.p. on days 0–4 and 14–18; Fig. 1C, T7–T20). Although some tumors became nonpalpable after this dose-dense treatment, eventually all tumors relapsed and acquired topotecan resistance. Interestingly, the time until resistance developed varied substantially between individual tumors, showing the response heterogeneity of these tumors.

In drug-sensitive tumors we identified a strong increase in nuclear DNA damage foci 24 hours after a 5-day topotecan schedule using γ -H2AX as marker (Supplementary Fig. S2). γ -H2AX foci are associated with DSBs (18); hence, our finding supports the notion that topotecan-induced SSBs are converted into DSBs in the tumors. Moreover, we observed a significant decrease in the proliferation marker Ki-67 and an increase in senescence-associated β -galactosidase activity, as well as increased nuclear p19^{ARF} staining (Supplementary Fig. S3) after topotecan treatment. On the basis of published findings in cell lines (19), we expected to find many apoptotic cells. However, only a modest increase in apoptosis-related cell death [cleaved caspase-3 and terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) positive] was detected in shrinking tumors (Supplementary Fig. S3A).

***Abcg2* is frequently upregulated in topotecan-resistant tumors.** We looked for alterations in gene expression using a two-class paired significance of microarray analysis (SAM) comparing 39K oligo expression arrays of resistant to matched untreated control tumor samples. We found elevated expression of 150 genes at a δ of 1.058 (false discovery rate of 2.21%; Fig. 2A and Supplementary Table S2). Of these 150 genes only *Abcg2* has previously been linked to topotecan resistance. *Abcg2* encodes the ATP-binding cassette (ABC) drug efflux transporter ABCG2/BCRP, which is known to cause topotecan resistance in cultured cells (20, 21). Quantification of *Abcg2* transcript levels confirmed that the level of *Abcg2* was at least 2-fold elevated in 9 of 20 tumors (Fig. 2B, T1, T2, T4, T6, T9, T11, T13, T15, and T16). Moreover, ABCG2-positive tumor cells could be detected *in situ* (Fig. 2C) with the ABCG2-specific antibody Bxp53 (16), and immunoreactivity correlated with *Abcg2* transcript levels (Supplementary Table S3). The increased *Abcg2* gene expression was not due to DNA amplification: we quantified the DNA content in whole tumor lysates by MLPA and found no copy number alterations of *Abcg2* (Supplementary Fig. S4).

ABC transporters other than ABCG2 that have been linked to topotecan resistance by others (13, 22–27) include ABCB1A (MDR1A), ABCB1B (MDR1B), ABCC1 (MRP1), and

ABCC4 (MRP4). Of these, only the expression of *Abcb1a* and/or *Abcb1b* was elevated in a number of tumors: 9 of 20 tumors showed at least a 2-fold increase in *Abcb1a* or *Abcb1b* transcript levels (Fig. 2B).

Doxorubicin-resistant and Abcb1a/b-overexpressing tumors respond to topotecan after orthotopic transplantation. To investigate whether the mouse P-glycoproteins ABCB1A and ABCB1B can cause topotecan resistance in our tumor model, three individual doxorubicin-resistant and docetaxel cross-resistant *Brca1*^{-/-};*p53*^{-/-} tumors were orthotopically transplanted into syngeneic wild-type hosts and treated with the MTD of several drugs (Fig. 3A). The tumors maintained their elevated *Abcb1a* and *Abcb1b* RNA levels (Fig. 3B) and accordingly their resistant phenotype after transplantation but were completely sensitive to topotecan. This shows that upregulation of the mouse P-glycoproteins by itself does not lead to topotecan resistance in this tumor model.

Introduction of Abcg2 null alleles into the mouse model.

To study ABCG2-independent topotecan resistance mechanisms, *Abcg2* null alleles were introduced into the *K14cre*, *Brca1*^{F/F};*p53*^{F/F} mouse model (Supplementary Fig. S5). *Abcg2*^{-/-};*K14cre*,*Brca1*^{F/F};*p53*^{F/F} animals with spontaneous mammary tumors cannot be treated with the topotecan MTD established in *Abcg2*^{+/+} mice, because the ablation of ABCG2 alters topotecan pharmacokinetics (28, 29). This problem was solved by orthotopic transplantation of tumors into syngeneic wild-type animals (Fig. 4A). In comparison with *Abcg2*-proficient tumors, we found an increased topotecan sensitivity in *Abcg2*-deficient tumors resulting in an augmented overall survival ($P < 0.001$; Fig. 4B). This confirms that ABCG2 is contributing to topotecan resistance *in vivo*. Nevertheless, ablation of *Abcg2* did not result in tumor eradication. Eventually, most of the *Abcg2*^{-/-};*Brca1*^{-/-};*p53*^{-/-} tumors developed topotecan resistance after a median latency of 137 days ($n = 11$) after start of treatment.

ABCG2-independent mechanisms of topotecan resistance. Alterations of the drug target Top1 result in camptothecin resistance in cultured cells (12, 13, 25). Several point mutations, which disable camptothecin (analogue)-mediated stabilization of Top1 cleavage complexes, have been detected in the core and catalytic domain of *Top1* (12, 13, 25, 30). We therefore sequenced nucleotides 1051 to 2257 (starting from ATG) of *Top1* cDNA, which include the previously described mutations. However, using *Top1* cDNA derived from five individual topotecan-resistant *Abcg2*^{-/-};*Brca1*^{-/-};*p53*^{-/-} tumors (TB1, TB2, TB4, TB5, and TB8), we did not find a single-point mutation analyzing eight independent clones per tumor (not shown). Another alteration reported to cause topotecan resistance is a reduction in the level of the drug target (12, 31, 32). Yet, in both *Abcg2*-proficient and -deficient tumors, we did not observe a significant decline of *Top1* transcripts (Fig. 2B; Supplementary Fig. S6). Only T10 showed a reduction of *Top1* mRNA to 48% in the resistant tumor compared with the control. At the protein level, however, immunoblotting revealed substantially decreased Top1 levels in five of nine topotecan-resistant *Abcg2*^{-/-};*Brca1*^{-/-};*p53*^{-/-} tumors compared with their matched untreated controls (Fig. 5A, TB1, TB2, TB8, TB9, and TB10). In addition, five

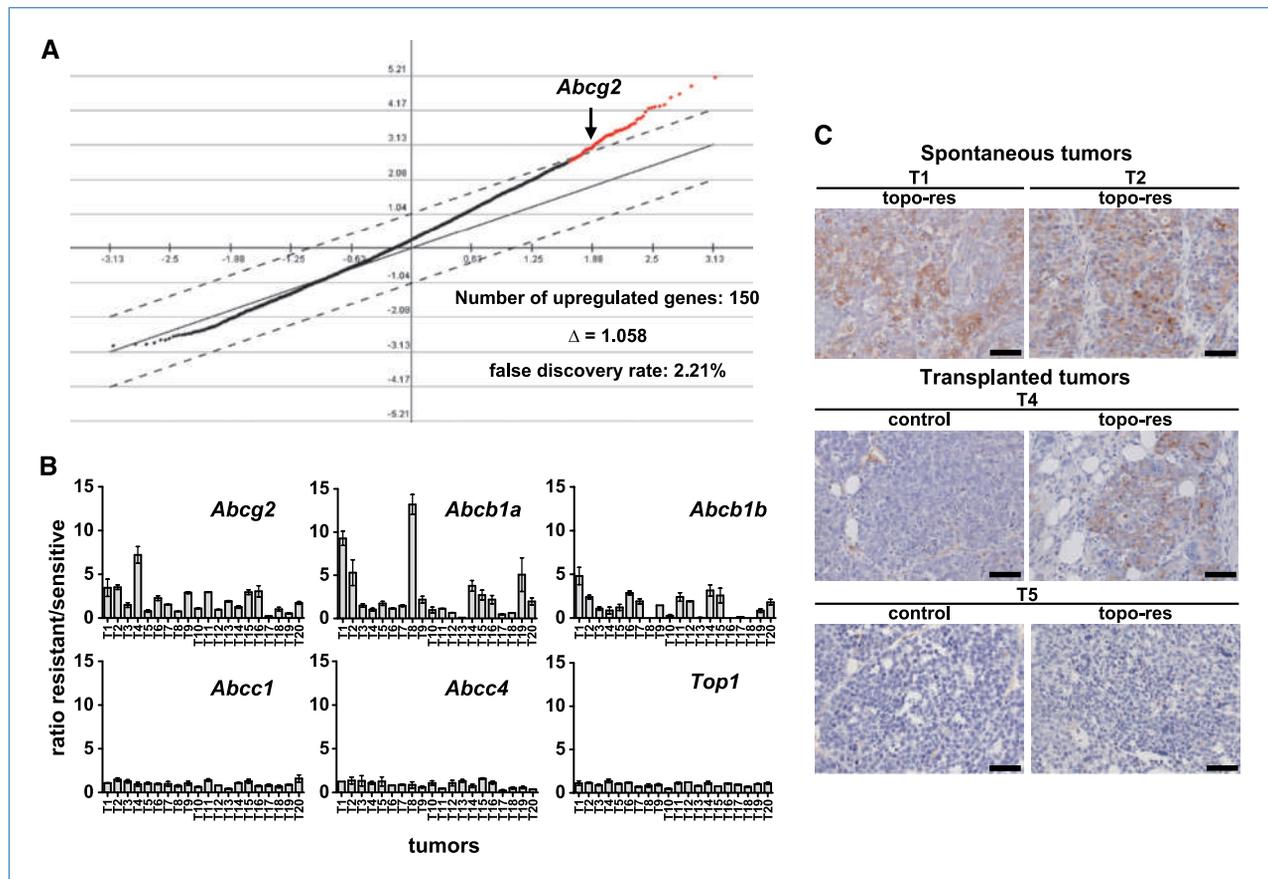


Figure 2. Alterations in gene expression in topotecan-resistant *Brca1*^{-/-}, *p53*^{-/-} tumors. A, two-class paired SAM of 20 tumors. Gene expression profiles were determined by hybridizing both topotecan-resistant and matched untreated control tumors to a pooled *p53*^{-/-} tumor reference (8). Significantly upregulated genes in the resistant tumors ($\Delta = 1.058$, false discovery rate, 2.21%) are indicated in red. The observed score of *Abcg2* was 2.81. The complete list of the 150 upregulated genes is shown in Supplementary Table S2. B, transcript levels of *Abcg2*, *Abcb1a*, *Abcb1b*, *Abcc1*, *Abcc4*, and *Top1* quantified by RT-MLPA. Columns, mean resistant/sensitive ratios of three independent reactions, using *Actβ* as internal reference. Error bars, SDs. C, examples of ABCG2 immunoreactivity in spontaneous (T1 and T2) and transplanted (T4 and T5) topotecan-resistant *Brca1*^{-/-}, *p53*^{-/-} tumors using the rat monoclonal Bxp-53 antibody (16). Scale bars, 100 μ m.

of eight *Brca1*^{-/-}, *p53*^{-/-} tumors, lacking *Abcg2* upregulation, also showed marked decreases in *Top1* protein levels (Fig. 5B, T5, T10, T18–T20). Downregulation of *Top1* is therefore another major biochemical mechanism of topotecan resistance in this model.

Topotecan-olaparib combination therapy does not eradicate tumors either. *Brca1*^{-/-}, *p53*^{-/-} tumors are sensitive to the PARP inhibitor olaparib, and its combination with cisplatin or carboplatin increased the overall survival of tumor-bearing mice (10). Topotecan might even be more effective in combination with olaparib, because topotecan increases SSBs, which require PARP-mediated repair. In the absence of functional PARP, topotecan should drastically increase SSBs and thus increase synthetic lethality with BRCA1 deficiency. When we tested 4 mg/kg topotecan i.p. on days 0 to 4 in combination with 50 mg/kg olaparib i.p. daily, the mice had to be killed due to diarrhea with accompanying weight loss that could not be compensated by additional fluid supplementation (Fig. 6A). This is not surprising,

as PARP deficiency is known to sensitize mammalian cells to *Top1* poisons (33). The combination with olaparib was tolerated only when topotecan was lowered to 0.5 mg/kg i.p. on 5 consecutive days (Fig. 6A). We then treated *Abcg2*-deficient *Brca1*^{-/-}, *p53*^{-/-} tumors to avoid ABCG2-mediated topotecan resistance and to maximize the chance of tumor eradication. Nevertheless, the topotecan-olaparib combination did not increase the relapse-free survival of four individual tumors compared with olaparib as single agent (Fig. 6B, TB1, TB5, TB7, and TB11). Even the addition of the P-glycoprotein inhibitor tariquidar to the regimen, to prevent *Abcb1a/b*-mediated olaparib resistance (10), did not result in the eradication of the tumors (Fig. 6C).

Discussion

We show here that spontaneous and transplanted *Brca1*^{-/-}; *p53*^{-/-} mammary tumors are sensitive to topotecan, albeit with considerable variability in drug response. The median

overall survival of mice with transplanted tumors under the dose-dense topotecan therapy (T7-T20) was 53.5 days (± 39 SD) compared with 8 days (± 1 SD) of their matched untreated controls (Fig. 1C). Such intertumoral differences, which did not correlate with a specific histomorphology, were also observed with other drugs in this model (9, 10) and seem to mimic the response heterogeneity seen in patients. We know that tumors differ in additional mutations acquired after the initial *Brca1* and *p53* deletion (8), and these must be responsible for response variability.

Despite their initial sensitivity, tumors eventually acquire resistance to the MTD of topotecan. Research with cell lines has identified several mechanisms of camptothecin/topotecan resistance. These include overexpression of the drug efflux transporters ABCG2, ABCC4, ABCB1, ABCC2, reduced expression of Top1, Top1 mutations, Top1 mislocalization, elevated DNA repair, and resistance to apoptosis (reviewed in refs. 1, 12, 13). Which of these mechanisms contribute to topotecan resistance in real tumors has remained unclear, however (34, 35). We show here that increased levels of ABCG2 are a major mechanism of topotecan resistance *in vivo*, as in cultured cells (21, 36, 37). The importance of this mechanism is illustrated by the *Abcg2*^{-/-} tumors in which the development of topotecan resistance was substantially delayed (Fig. 4B). *Abcg2*^{-/-};*Brca1*^{-/-};*p53*^{-/-} tumors can still de-

velop full topotecan resistance, however, and a substantial decrease in the level of Top1 can explain resistance in several of these tumors. Remarkably, this profound decrease in Top1 protein was not accompanied by a corresponding decrease in *Top1* mRNA in most tumors, in sharp contrast with the observations on camptothecin-resistant tumor cell lines (12, 32, 38). If downregulation of Top1 would also be posttranscriptional in human tumors, its detection by gene expression profiling would be impossible. Top1 degradation is mediated by ubiquitin- or SUMO1-associated posttranscriptional modification (39–42), and which mechanism applies in our mouse tumors is under investigation.

It is notable that some mechanisms of topotecan resistance identified in cell lines, such as upregulation of ABCC4 or mutations in *Top1*, have not yet turned up in our tumor model. ABCG2-mediated drug efflux or downregulation of Top1 can explain topotecan resistance in 14 of 20 individual *Brca1*^{-/-};*p53*^{-/-} tumors, and the study of topotecan resistance in the remaining tumors is ongoing.

P-glycoprotein is not such an effective transporter of topotecan as ABCG2 (23, 43, 44), but how effective it is cannot be deduced from the literature. Relative resistance levels up to 20-fold have been reported (25), although most recent papers find much lower P-glycoprotein-mediated resistance or transport (45–47). Our results show that even a 53-fold

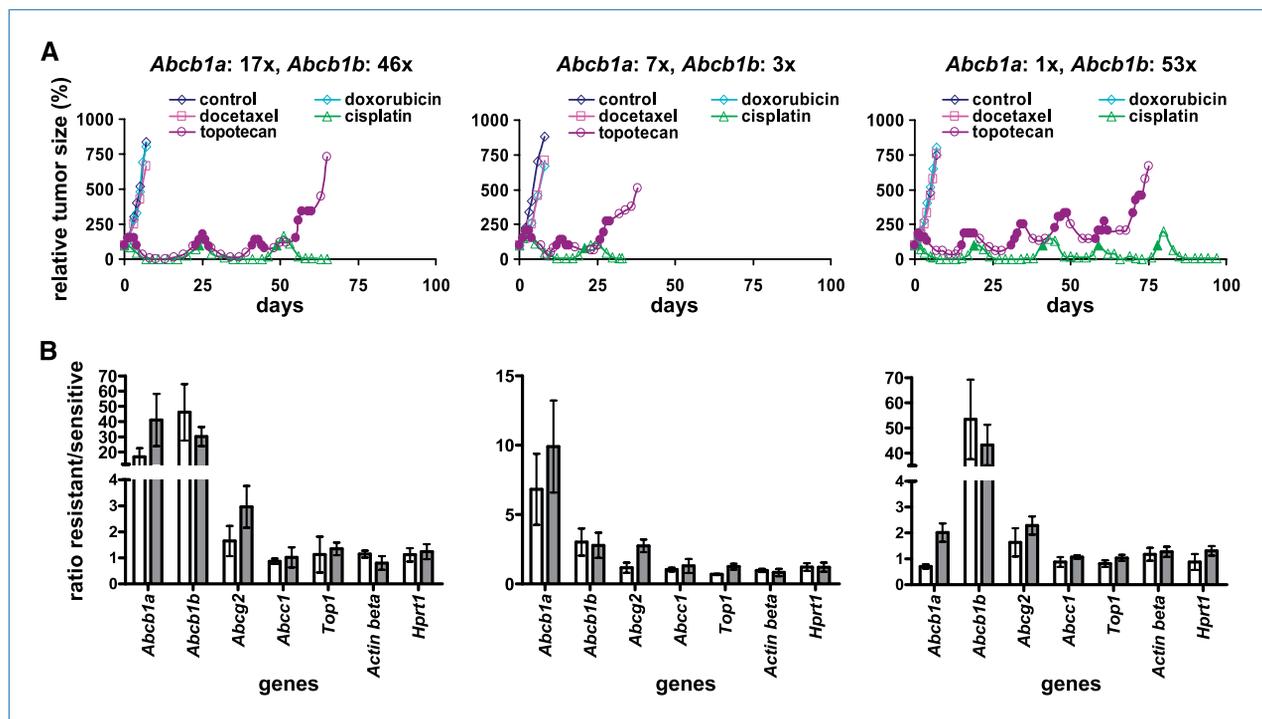
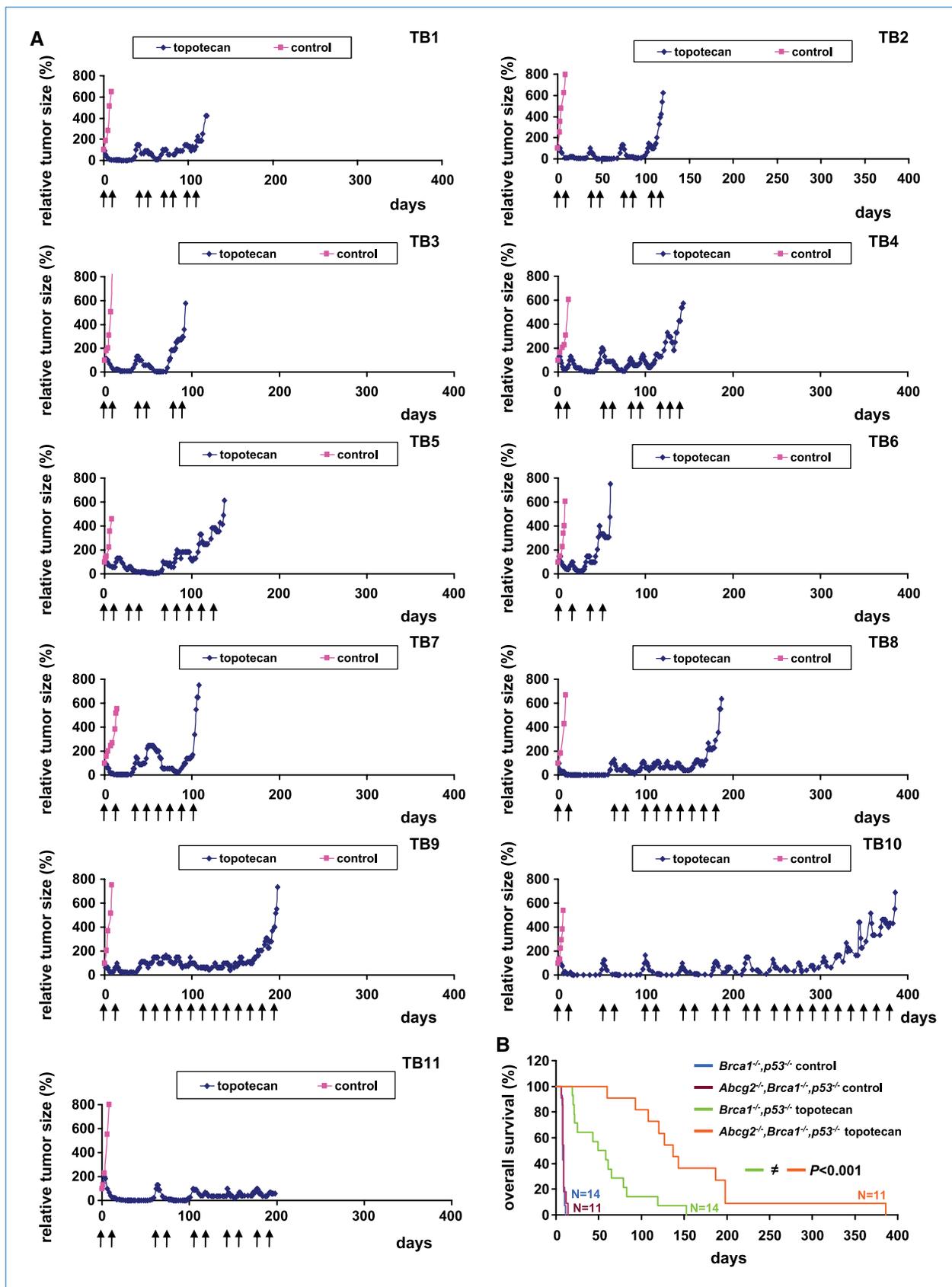


Figure 3. Sensitivity of *Abcb1a/1b*-overexpressing doxorubicin-resistant *Brca1*^{-/-};*p53*^{-/-} tumors to topotecan. A, response of three doxorubicin-resistant tumors (T²³ dox2-res, T²³ dox2-res, T²³ dox4-res; see ref. 10) to doxorubicin, docetaxel, topotecan, and cisplatin MTD treatments after orthotopic transplantation into syngeneic mice. Levels of *Abcb1a* and *Abcb1b* overexpression, as measured by RT-MLPA before transplantation, are indicated at the top. Filled symbols in the responses of topotecan- and cisplatin-treated tumors indicate MTD treatments. B, transcript levels of *Abcb1a*, *Abcb1b*, *Abcg2*, *Abcc1*, *Top1*, *Actin beta*, and *Hprt1* before (open columns) and after (gray columns) transplantation. Columns, mean resistant/sensitive ratios of three independent reactions, using the sum of the values for *Actin beta* and *Hprt1* as internal reference. *Hprt1* was compared with *Actin beta* and vice versa. Error bars, SDs.



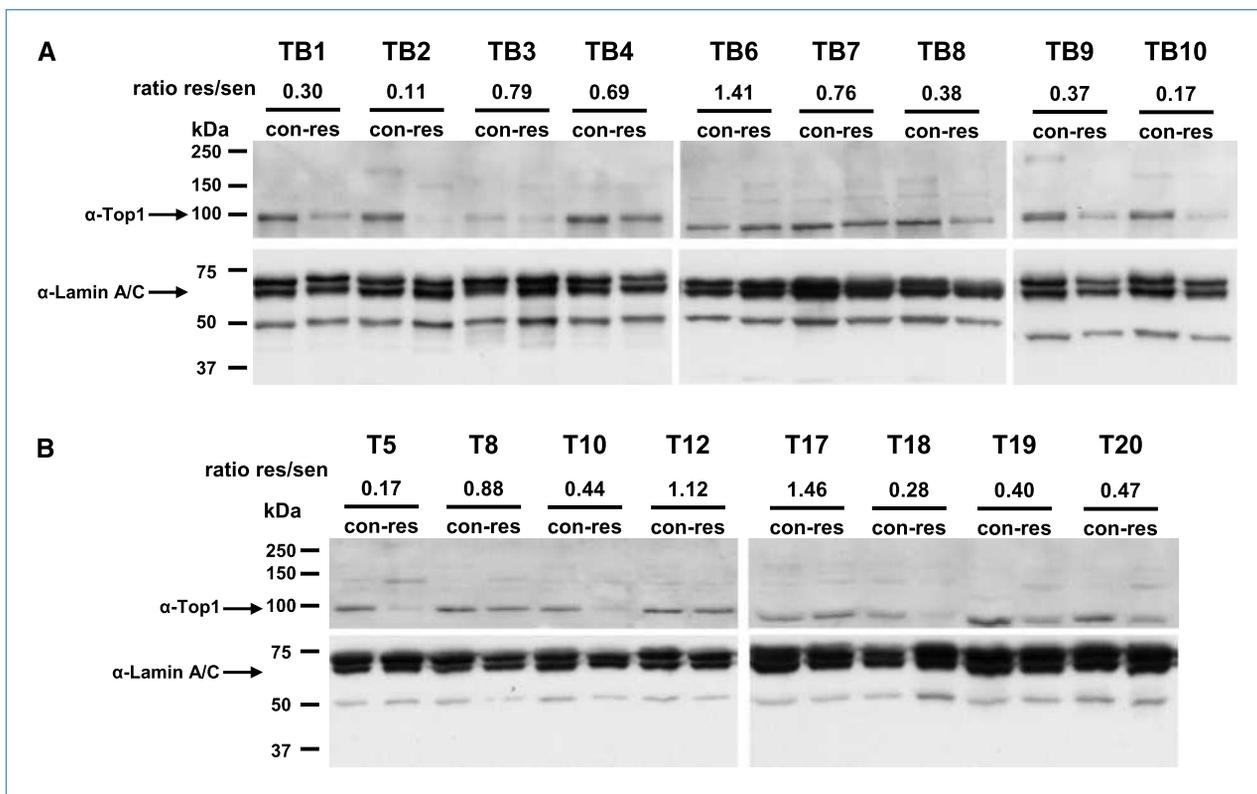


Figure 5. Western blot analysis of Top1 levels in topotecan-resistant tumors. Lysates of nine resistant *Abcg2*^{-/-}, *Brca1*^{-/-}, *p53*^{-/-} tumors (TB1–TB4 and TB6–TB10; A) and lysates of eight resistant *Brca1*^{-/-}, *p53*^{-/-} tumors without increased *Abcg2* gene expression (T5, T8, T10, T12, and T17–T20; B) were probed with a polyclonal rabbit anti-human Top1 antibody (Topogen) and compared with matched topotecan-sensitive controls (Top1 MW = 100 kDa). As loading control we used Lamin A/C, detected with a polyclonal rabbit anti-human antibody (Santa Cruz; Lamin A MW = 69 kDa, Lamin C MW = 62 kDa). Resistant/control ratios of relative Top1 band intensities are indicated for each individual tumor.

upregulation of P-glycoprotein, which makes the tumor completely resistant to doxorubicin, does not result in clear-cut resistance to topotecan. In real tumors, P-glycoprotein is therefore unable by itself to cause topotecan resistance. Nevertheless, we found in many tumors a significant (2- to 13-fold) upregulation of the *Abcb1* genes (Fig. 2B). Although this could be incidental, we think that P-glycoprotein, in conjunction with other weak resistance mechanisms, could allow the tumor cell sufficient time to develop more robust resistance. Effective cooperation between ABCG2 and P-glycoprotein in the defense against topotecan penetration into the brain has been documented by de Vries and colleagues (48). Whereas only minor increases (up to 2-fold) of brain topotecan were found in *Abcg2*^{-/-} or *Abcb1a/1b*^{-/-} mice,

the compound knockout mice showed 12-fold increased brain penetration relative to wild-type mice. This shows that P-glycoprotein can significantly contribute to the prevention of cellular topotecan accumulation, if it gets help.

It is remarkable that deletion of *Abcg2* did not result in tumor eradication by topotecan. ABCG2 has been proposed as a critical factor in the chemotherapy resistance of tumor-initiating cells of various tumor types (14, 15, 22), but our results argue against this proposal. We cannot exclude that topotecan does not reach remnant tumor cells but we consider this unlikely, as there are no obvious histologic sanctuaries in the remnants. Another possibility is that the tumor-initiating cells are protected by another drug efflux transporter, but there is no evidence for this either. Our

Figure 4. Topotecan response of *Abcg2*;*Brca1*;*p53*-deficient tumors and differential survival of *Abcg2*^{-/-} versus *Abcg2*^{+/+} tumor-bearing animals under topotecan therapy. A, response of *Abcg2*;*Brca1*;*p53*-deficient tumors to the MTD of topotecan. Animals with orthotopically transplanted *Abcg2*;*Brca1*;*p53*-deficient mammary tumors (volume, ~200 mm³) were left untreated (pink line) or treated with 4 mg/kg topotecan i.p. on days 0 to 4 and 14 to 18. When tumors relapsed or showed progressive growth (tumor size, ≥50%) after a recovery period of 14 d after the day 0 injection, treatment was resumed as indicated by the arrows. B, overall survival (%) over time (d) of wild-type animals carrying orthotopically transplanted *Abcg2*-proficient (T7–T20) or -deficient (TB1–TB11) *Brca1*^{-/-}, *p53*^{-/-} mammary tumors. Topotecan was administered as in A and Fig. 1C. All animals carrying *Abcg2*-proficient tumors and 10 of 11 animals carrying *Abcg2*-deficient tumors had to be killed, because the resistant tumor reached a volume of ~1,500 mm³. One animal (TB11) had to be sacrificed due to a rectum prolapse on day 198, before having acquired full topotecan resistance. The *P* value was calculated using the Wilcoxon rank sum test.

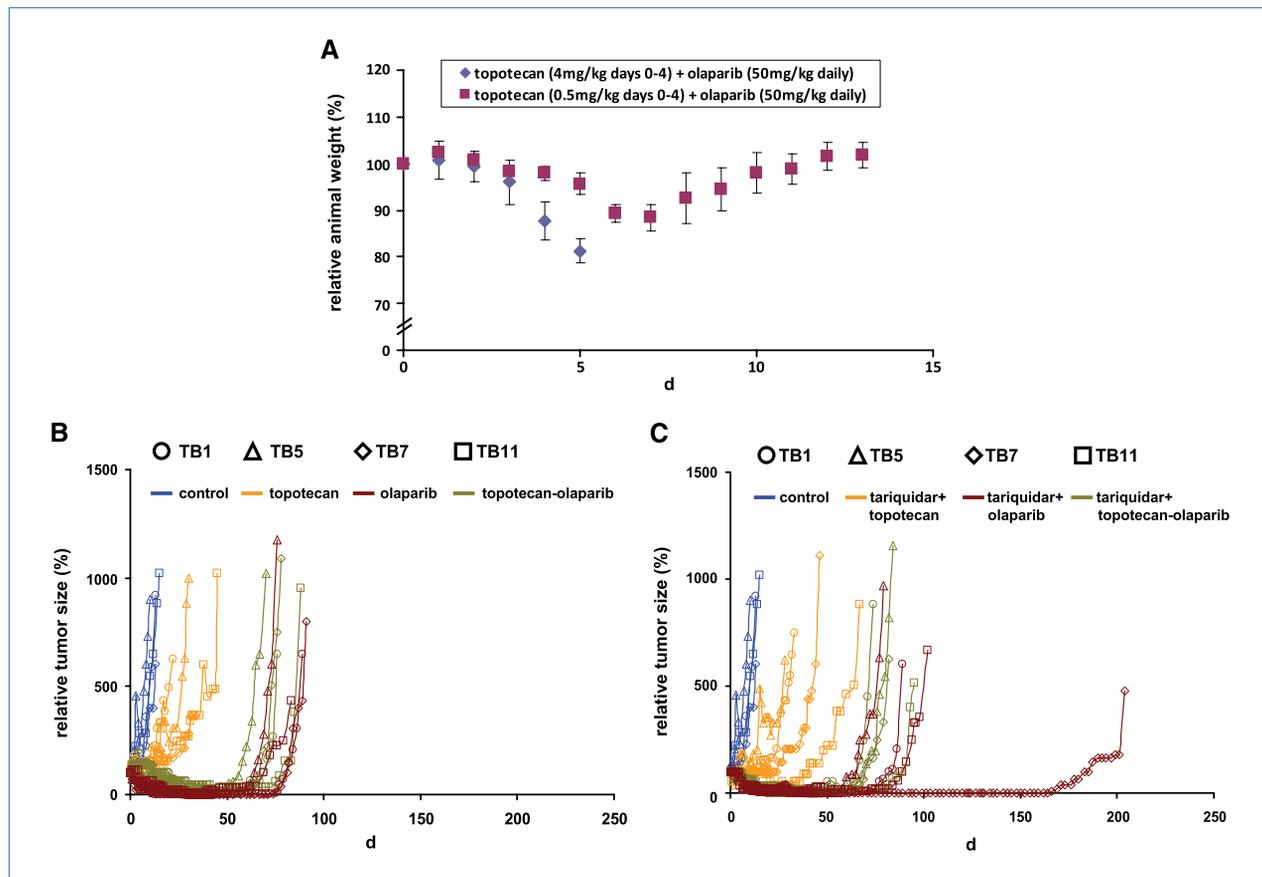


Figure 6. Response of transplanted *Abcg2*^{-/-}, *Brca1*^{-/-}, *p53*^{-/-} tumors to topotecan-olaparib combination therapy. A, relative animal weights (%) in response to topotecan-olaparib combination treatment. Female wild-type (FVB/N × 129/Ola)F1 animals were treated with 4 mg/kg topotecan i.p. on days 0, 1, 2, 3, and 4 in combination with 50 mg/kg olaparib i.p. daily (blue triangles). On day 5 the animals had to be killed due to severe weight loss caused by diarrhea. Weight monitoring in response to the 8-fold lower dose of 0.5 mg/kg is shown as red squares. The average of five animals is presented. Error bars, SD. B, response of four *Abcg2*^{-/-}, *Brca1*^{-/-}, *p53*^{-/-} tumors (TB1, TB5, TB7, and TB11) to topotecan (orange) and olaparib (brown) monotherapy or topotecan-olaparib (green) combination therapy (untreated controls in blue). Animals received 0.5 mg/kg topotecan i.p. on days 0 to 4, 14 to 18, and 28 to 32; 50 mg/kg olaparib i.p. daily for 42 d or both antitumor agents in these dosages combined. After a 42-d dosing schedule animals were left untreated and screened for tumor relapse. C, response of the same *Abcg2*^{-/-}, *Brca1*^{-/-}, *p53*^{-/-} tumors as in B to tariquidar-topotecan and tariquidar-olaparib treatment and tariquidar-topotecan-olaparib combination therapy. In addition to the same olaparib and topotecan dosages as in B, animals received 2 mg/kg tariquidar i.p. every second day during 42 d of drug administration to prevent ABCB1A/B-mediated olaparib resistance. After a 42-d dosing schedule animals were left untreated and screened for tumor relapse.

current hypothesis is that remnant tumor cells arrest in the cell cycle and thereby escape anticancer drug-mediated cell death (49). This hypothesis is supported by the observation that cycling cells (Ki-67 positive) are drastically reduced after treatment. Because we found many senescent-like cells in the topotecan-sensitive tumors after treatment, it is possible that therapy-induced senescence allows escape from topotecan-induced cell death.

How cells in topotecan-sensitive tumors are killed is unclear. We found only a slight increase in apoptosis-related cleaved caspase-3 and TUNEL immunoreactivity, as was also observed in response to olaparib (10). Necrosis or mitotic catastrophe (50) may be more important mechanisms of cell death in this tumor, but we cannot exclude that cleaved caspase-3-mediated cell cycle-dependent apoptosis is only detectable for a short period of time and therefore underestimated in

tumor sections. *In vitro* studies with *Brca1*^{-/-}; *p53*^{-/-} cells are in progress to settle this issue. In any case, our data show that *in vivo* imaging of apoptosis is not a suitable readout for therapy response in this tumor model.

To explore new therapeutic alternatives for breast cancers associated with BRCA1 defects, we tested the combination of topotecan with the PARP inhibitor olaparib. This combination did not result in tumor eradication, even if we prevented efflux of topotecan by using *Abcg2*^{-/-} tumors and P-glycoprotein-mediated olaparib extrusion by inhibiting this transporter with tariquidar. We also observed this lack of complete cell kill when olaparib was combined with platinum drugs (10). It is clinically relevant that the topotecan-olaparib combination proved so toxic in mice that we had to reduce the topotecan concentration 8-fold. We have not seen this increased toxicity in the combination of olaparib

with cisplatin or carboplatin (10). We do not think that topotecan toxicity is increased because olaparib inhibits ABCG2, because methotrexate transport by ABCG2-overexpressing cells was not altered by olaparib (data not shown). It seems more likely that the level of endogenous DNA damage requiring PARP-mediated repair is already close to the maximum that normal cells can deal with. A further increase in SSBs induced by topotecan would overtax the DNA repair capacity remaining in the absence of functional PARP.

Our study shows that GEMMs of human cancer are not only useful for studying the response of real tumors to drugs but also for the identification of resistance mechanisms actually occurring *in vivo*. Eventually, novel strategies to prevent or reverse topotecan resistance can be tested in this model as well.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

References

- Pommier Y. Topoisomerase I inhibitors: camptothecins and beyond. *Nat Rev Cancer* 2006;6:789–802.
- Fedier A, Steiner RA, Schwarz VA, et al. The effect of loss of *Brca1* on the sensitivity to anticancer agents in *p53*-deficient cells. *Int J Oncol* 2003;22:1169–73.
- Foulkes WD, Stefansson IM, Chappuis PO, et al. Germline *BRCA1* mutations and a basal epithelial phenotype in breast cancer. *J Natl Cancer Inst* 2003;95:1482–5.
- Turner N, Tutt A, Ashworth A. Hallmarks of 'BRCAness' in sporadic cancers. *Nat Rev Cancer* 2004;4:814–9.
- Turner NC, Reis-Filho JS, Russell AM, et al. *BRCA1* dysfunction in sporadic basal-like breast cancer. *Oncogene* 2007;26:2126–32.
- Cleator S, Heller W, Coombes RC. Triple-negative breast cancer: therapeutic options. *Lancet Oncol* 2007;8:235–44.
- Jaspers JE, Rottenberg S, Jonkers J. Therapeutic options for triple-negative breast cancers with defective homologous recombination. *Biochim Biophys Acta* 2009;1796:266–80.
- Liu X, Holstege H, van der Gulden H, et al. Somatic loss of *BRCA1* and *p53* in mice induces mammary tumors with features of human *BRCA1*-mutated basal-like breast cancer. *Proc Natl Acad Sci U S A* 2007;104:12111–6.
- Rottenberg S, Nygren AO, Pajic M, et al. Selective induction of chemotherapy resistance of mammary tumors in a conditional mouse model for hereditary breast cancer. *Proc Natl Acad Sci U S A* 2007;104:12117–22.
- Rottenberg S, Jaspers JE, Kersbergen A, et al. High sensitivity of *BRCA1*-deficient mammary tumors to the PARP inhibitor AZD2281 alone and in combination with platinum drugs. *Proc Natl Acad Sci U S A* 2008;105:17079–84.
- Fong PC, Boss DS, Yap TA, et al. Inhibition of poly(ADP-ribose) polymerase in tumors from *BRCA* mutation carriers. *N Engl J Med* 2009;361:123–34.
- Pommier Y, Pourquier P, Urasaki Y, Wu J, Laco GS. Topoisomerase I inhibitors: selectivity and cellular resistance. *Drug Resist Updat* 1999;2:307–18.
- Rasheed ZA, Rubin EH. Mechanisms of resistance to topoisomerase I-targeting drugs. *Oncogene* 2003;22:7296–304.
- Bleau AM, Hambarzumyan D, Ozawa T, et al. *PTEN/PI3K/Akt* pathway regulates the side population phenotype and ABCG2 activity in glioma tumor stem-like cells. *Cell Stem Cell* 2009;4:226–35.
- Dean M, Fojo T, Bates S. Tumour stem cells and drug resistance. *Nat Rev Cancer* 2005;5:275–84.
- Jonker JW, Buitelaar M, Wagenaar E, et al. The breast cancer resistance protein protects against a major chlorophyll-derived dietary phototoxin and protoporphyria. *Proc Natl Acad Sci U S A* 2002;99:15649–54.
- Pajic M, Iyer JK, Kersbergen A, et al. Moderate increase in *Mdr1a/1b* expression causes *in vivo* resistance to doxorubicin in a mouse model for hereditary breast cancer. *Cancer Res* 2009;69:6396–404.
- Bonner WM, Redon CE, Dickey JS, et al. γ H2AX and cancer. *Nat Rev Cancer* 2008;8:957–67.
- Sordet O, Khan QA, Kohn KW, Pommier Y. Apoptosis induced by topoisomerase inhibitors. *Curr Med Chem Anticancer Agents* 2003;3:271–90.
- Allen JD, Brinkhuis RF, Wijnholds J, Schinkel AH. The mouse *Bcrp1/Mxr/Abcp* gene: amplification and overexpression in cell lines selected for resistance to topotecan, mitoxantrone, or doxorubicin. *Cancer Res* 1999;59:4237–41.
- Ishikawa T, Ikegami Y, Sano K, Nakagawa H, Sawada S. Transport mechanism-based drug molecular design: novel camptothecin analogues to circumvent ABCG2-associated drug resistance of human tumor cells. *Curr Pharm Des* 2006;12:313–25.
- Chen AY, Yu C, Potmesil M, et al. Camptothecin overcomes MDR1-mediated resistance in human KB carcinoma cells. *Cancer Res* 1991;51:6039–44.
- Chen ZS, Furukawa T, Sumizawa T, et al. ATP-Dependent efflux of CPT-11 and SN-38 by the multidrug resistance protein (MRP) and its inhibition by PAK-104P. *Mol Pharmacol* 1999;55:921–8.
- Chu XY, Suzuki H, Ueda K, et al. Active efflux of CPT-11 and its metabolites in human KB-derived cell lines. *J Pharmacol Exp Ther* 1999;288:735–41.
- Gounder MK, Nazar AS, Saleem A, et al. Effects of drug efflux proteins and topoisomerase I mutations on the camptothecin analogue gimatecan. *Invest New Drugs* 2008;26:205–13.
- Tian Q, Zhang J, Tan TM, et al. Human multidrug resistance associated protein 4 confers resistance to camptothecins. *Pharm Res* 2005;22:1837–53.
- Tian Q, Zhang J, Chan SY, et al. Topotecan is a substrate for multidrug resistance associated protein 4. *Curr Drug Metab* 2006;7:105–18.
- Jonker JW, Smit JW, Brinkhuis RF, et al. Role of breast cancer resistance protein in the bioavailability and fetal penetration of topotecan. *J Natl Cancer Inst* 2000;92:1651–6.
- Kruijtzter CM, Beijnen JH, Rosing H, et al. Increased oral bioavailability of topotecan in combination with the breast cancer resistance

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- protein and P-glycoprotein inhibitor GF120918. *J Clin Oncol* 2002; 20:2943–50.
30. Chrencik JE, Staker BL, Burgin AB, et al. Mechanisms of camptothecin resistance by human topoisomerase I mutations. *J Mol Biol* 2004; 339:773–84.
 31. Burgess DJ, Doles J, Zander L, et al. Topoisomerase levels determine chemotherapy response *in vitro* and *in vivo*. *Proc Natl Acad Sci U S A* 2008;105:9053–8.
 32. Liao Z, Robey RW, Guirouilh-Barbat J, et al. Reduced expression of DNA topoisomerase I in SF295 human glioblastoma cells selected for resistance to homocamptothecin and diflomotecan. *Mol Pharmacol* 2008;73:490–7.
 33. Pommier Y, Barcelo JM, Rao VA, et al. Repair of topoisomerase I-mediated DNA damage. *Prog Nucleic Acid Res Mol Biol* 2006;81: 179–229.
 34. Robey RW, Polgar O, Deeken J, To KW, Bates SE. ABCG2: determining its relevance in clinical drug resistance. *Cancer Metastasis Rev* 2007;26:39–57.
 35. Robey RW, To KK, Polgar O, et al. ABCG2: a perspective. *Adv Drug Deliv Rev* 2009;61:3–13.
 36. Rabindran SK, Ross DD, Doyle LA, Yang W, Greenberger LM. Fumitremorgin C reverses multidrug resistance in cells transfected with the breast cancer resistance protein. *Cancer Res* 2000;60:47–50.
 37. Yang CH, Schneider E, Kuo ML, et al. BCRP/MXR/ABCP expression in topotecan-resistant human breast carcinoma cells. *Biochem Pharmacol* 2000;60:831–7.
 38. Pfister TD, Reinhold WC, Agama K, et al. Topoisomerase I levels in the NCI-60 cancer cell line panel determined by validated ELISA and microarray analysis and correlation with indenoisoquinoline sensitivity. *Mol Cancer Ther* 2009;8:1878–84.
 39. Desai SD, Liu LF, Vazquez-Abad D, D'Arpa P. Ubiquitin-dependent destruction of topoisomerase I is stimulated by the antitumor drug camptothecin. *J Biol Chem* 1997;272:24159–64.
 40. Desai SD, Li TK, Rodriguez-Bauman A, Rubin EH, Liu LF. Ubiquitin/26S proteasome-mediated degradation of topoisomerase I as a resistance mechanism to camptothecin in tumor cells. *Cancer Res* 2001;61:5926–32.
 41. Lin CP, Ban Y, Lyu YL, Desai SD, Liu LF. A ubiquitin-proteasome pathway for the repair of topoisomerase I-DNA covalent complexes. *J Biol Chem* 2008;283:21074–83.
 42. Mao Y, Sun M, Desai SD, Liu LF. SUMO-1 conjugation to topoisomerase I: a possible repair response to topoisomerase-mediated DNA damage. *Proc Natl Acad Sci U S A* 2000;97:4046–51.
 43. Hendricks CB, Rowinsky EK, Grochow LB, Donehower RC, Kaufmann SH. Effect of P-glycoprotein expression on the accumulation and cytotoxicity of topotecan (SK&F 104864), a new camptothecin analogue. *Cancer Res* 1992;52:2268–78.
 44. Hoki Y, Fujimori A, Pommier Y. Differential cytotoxicity of clinically important camptothecin derivatives in P-glycoprotein-overexpressing cell lines. *Cancer Chemother Pharmacol* 1997;40:433–8.
 45. Crouthamel MH, Wu D, Yang Z, Ho RJ. A novel MDR1 G1199T variant alters drug resistance and efflux transport activity of P-glycoprotein in recombinant Hek cells. *J Pharm Sci* 2006;95:2767–77.
 46. Li H, Jin HE, Kim W, et al. Involvement of P-glycoprotein, multidrug resistance protein 2 and breast cancer resistance protein in the transport of belotecan and topotecan in Caco-2 and MDCKII cells. *Pharm Res* 2008;25:2601–12.
 47. Marchetti S, Oostendorp RL, Pluim D, et al. *In vitro* transport of gimatecan (7-t-butoxyiminomethylcamptothecin) by breast cancer resistance protein, P-glycoprotein, and multidrug resistance protein 2. *Mol Cancer Ther* 2007;6:3307–13.
 48. de Vries NA, Zhao J, Kroon E, et al. P-glycoprotein and breast cancer resistance protein: two dominant transporters working together in limiting the brain penetration of topotecan. *Clin Cancer Res* 2007; 13:6440–9.
 49. Borst P, Rottenberg S, Jonkers J. How do real tumors become resistant to cisplatin? *Cell Cycle* 2008;7:1353–9.
 50. Roninson IB, Broude EV, Chang BD. If not apoptosis, then what? Treatment-induced senescence and mitotic catastrophe in tumor cells. *Drug Resist Updat* 2001;4:303–13.

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