Chapter 1

Sensitivity and acquired resistance of BRCA1;\(\text{p53}\)-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 (PARP) 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 Top 1 (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 11 Abcg2−/−;Brca1−/−;p53−/− tumors was 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.
Introduction

Topotecan is used to treat ovarian, cervical and small cell lung cancer patients. Topotecan inhibits topoisomerase I (Top1)-mediated religation of single-stranded DNA breaks (SSBs). When drug-stabilized covalent Top1-DNA complexes cause stalling of the replication machinery, SSBs are converted into double-stranded DNA breaks (DSBs). Such replication-mediated DSBs appear 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 (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”) (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;Brca1F/F;p53F/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). Since in our model olaparib treatment alone did not result in tumor eradication (10), we have tested whether increasing the induction of SSBs by a Top1 poison would enhance anti-tumoral efficacy of this PARP inhibitor.

Here we demonstrate 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 2 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<sup>−/−</sup>*;*Brca1<sup>−/−</sup>*;*p53<sup>−/−</sup>* 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;Brca1F/F;p53F/F mice to homozygocity (Abcg2+/−;K14cre;Brca1F/F;p53F/F) on a mixed 129/Ola and FVB/N genetic background. Abcg2 genotypes were confirmed by PCR with specific primers (forward: 5'-CTTCTCCATTCCATCGCTCG-3') for wildtype (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 µl/g of body weight by i.p. injection. When tumors reached a size of about 200 mm³, 4 mg topotecan per kg was given i.p. daily for 4 or 5 consecutive days or on days 0-4 and 14-18. Tumors that responded (volume <50% 14 days after the first topotecan injection of the first treatment course [days 0-4] or second course [days 0-4 and 14-18]) were left untreated until they relapsed to 100%. If tumors did not respond (volume remaining ≥50%), treatments were continued after a recovery time of 9 days following the last treatment. Olaparib and tariquidar were applied as reported (10, 17). In topotecan-olaparib combination-treated animals the topotecan dose was lowered 8-fold to 0.5 mg/kg and administered on days 0-4, 14-18 and 28-32, whereas 50 mg olaparib per kg was injected i.p. daily for 42 days and, when combined with topotecan, given 15 min after topotecan injection. 2 mg tariquidar per kg was administered i.p. every second day during this 42 day 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 (Central Microarray Facility, NKI Amsterdam) were used, containing 38,784 70-mer probes representing 23,527 genes and 35,172 gene transcripts (MEEBO, Illumina BV, Eindhoven, The Netherlands). The microarray data reported in this paper have been deposited in the ArrayExpress database of the European Bioinformatics Institute (Cambridge, UK, accession no. E-NCMF-28).

**Multiplex ligation-dependent probe amplification (MLPA) analysis**

(RT-)MLPA analyses on tumor DNA or RNA were carried out as before (9, 17).

**Immunohistological 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 topoisomerase I immunoblotting**

Snap-frozen tumor samples were ground into a fine powder by pestle in a liquid nitrogen cooled mortar and transferred into 1.5 ml Eppendorf vials containing 20 μl 1x Lämmli lysis buffer per mg tumor sample. Lysates were spun 5 min at 20,800xg 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 2x 15 min block with Blotto (5% w/v milkpowder in 1x TBS-0.1% Tween-20) both anti-topoisomerase 1 (rabbit polyclonal, 1:1000; Topogen, #2012-2) and anti-Lamin A/C (rabbit polyclonal, 1:1000; Santa Cruz, #sc-20681) antibodies were used as probes and visualized by enhanced chemoluminescence after incubation with horseradish peroxidase-conjugated swine anti-rabbit IgG (1:10000, 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<sup>−/−</sup>,p53<sup>−/−</sup> mammary tumors respond to the MTD of topotecan, but relapse and eventually acquire resistance*

We established the maximum tolerable dose (MTD) of topotecan in 12-14 week old female *K14cre,Brca1<sup>F/F</sup>,p53<sup>F/F</sup>* mice to maximize anti-tumoral drug efficacy and thereby mimicking a clinically relevant dose (Supplementary Figure 1, A). Four mg of topotecan per kg mouse i.p. on 4 consecutive days was tolerated and resulted in 10-20% weight loss. When two animals carrying “spontaneous” mammary tumors (~200 mm<sup>3</sup> in volume) were treated with this dose, tumors shrank (Figure 1, A; T1 and T2). Despite this good response, tumors were not eradicated and relapsed. Once the tumors grew back to a volume of about 200 mm<sup>3</sup> (100%) or showed progressive growth after a recovery period of 14 days following the first topotecan injection, treatments were resumed. 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, since wildtype animals could take an additional dose of topotecan (4 mg/kg drug i.p. on 5 consecutive days, Supplementary Figure 1, B). The orthotopically transplanted tumors responded to topotecan like the primary tumors, but also the intensified topotecan therapy was unable to eradicate the tumors (Figure 1, B; T3-T6). We therefore added a second MTD schedule following a 14 day recovery period after the first topotecan injection and tested this treatment in 14 individual *Brca1<sup>/−</sup>;p53<sup>/−</sup>* tumors (4 mg/kg drug i.p. on days 0-4 and days 14-18; Figure 1, C; T7-T20). Although some tumors became non-palpable following 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 Figure 2). 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<sup>ARF</sup> staining (Supplementary Figure 3) after topotecan treatment. On the basis of published findings in cell lines (19) we expected to find
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 mm$^3$ (T1 and T2) were treated with 4 mg topotecan per kg mouse i.p. on days 0-3. For comparison, the growth of untreated spontaneous tumors in three individual $K14cre,Brca1^{f/f},p53^{f/f}$ animals (C1-3) is also shown. B, animals with four individual orthotopically transplanted Brca1;p53-deficient mammary tumors (volume ~200 mm$^3$) were left untreated (pink line) or treated as in A, but on days 0-4. C, animals bearing 14 individual orthotopically transplanted Brca1;p53-deficient mammary tumors (volume ~200 mm$^3$) were left untreated (pink line) or treated as in A, but on days 0-4 and 14-18 (dose-intensified treatment). When tumors in A-C relapsed or showed progressive growth (tumor size ≥ 50%) after a recovery time of 14 days following the day 0 injection, treatment was resumed as indicated by the arrows.
many apoptotic cells. However, only a modest increase in apoptosis-related cell death (cleaved caspase 3 and TUNEL positive) was detected in shrinking tumors (Supplementary Figure 3, A).

**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 delta of 1.058 (false discovery rate of 2.21%, Figure 2, A and Supplementary Table 2). 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 (Figure 2, B; T1, T2, T4, T6, T9, T11, T13, T15 and T16). Moreover, ABCG2-positive tumor cells could be detected in situ (Figure 2, C) with the ABCG2-specific antibody Bxp53 (16), and immunoreactivity correlated with Abcg2 transcript levels (Supplementary Table 3). 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 Figure 4).

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 (Figure 2, B).

**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 wildtype hosts and treated with the MTD of several drugs (Figure 3, A). The tumors maintained their elevated Abcb1a and Abcb1b RNA levels (Figure 3, B) 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+/+,p53+/+ mouse model (Supplementary Figure 5). Abcg2-,K14cre,Brca1+/+,p53+/+ 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 (Figure 4, A). In comparison to Abcg2-proficient tumors we found an

Figure 2. Alterations in gene expression in topotecan-resistant Brca1-/-,p53-/- tumors. A, two-class paired Significance Analysis of Microarrays (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 (Δ = 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 2. B, transcript levels of Abcg2, Abcb1a, Abcb1b, Abcc1, Abcc4 and Top1 quantified by RT-MLPA. Bars indicate mean resistant/sensitive ratios of three independent reactions, using Actβ as internal reference. Error bars indicate standard deviations. C, examples of ABCG2 immunoreactivity in spontaneous (T1 and 2) and transplanted (T4 and 5) topotecan-resistant Brca1-/-,p53-/- tumors using the rat monoclonal Bxp-53 antibody (16). Scale bars indicate 100 μm.
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Increased topotecan sensitivity in Abcg2-deficient tumors resulting in an augmented overall survival ($P<0.001$, Figure 4, B). 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) following treatment start.

**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)-

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**Figure 3.** Sensitivity of Abcb1a/1b-overexpressing doxorubicin-resistant Brca1$^{-/-}$,p53$^{-/-}$ tumors to topotecan. A, response of three doxorubicin-resistant tumors (T*23 dox2-res, T*23 dox2-res, T*23 dox4-res; see reference (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, Actβ and Hprt1 before (open bars) and after (grey bars) transplantation. Bars show mean resistant/sensitive ratios of three independent reactions, using the sum of the values for Actinβ and Hprt1 as internal reference. Hprt1 was compared with Actinβ and vice versa. Error bars indicate standard deviations.
Figure 4. Topotecan response of Abcg2;Brca1;p53-deficient tumors and differential survival of Abcg2−/− versus Abcg2+/+ tumor-bearing animals under topotecan therapy.
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 1051-2257 (starting from ATG) of Top1 cDNA, which include the previously described mutations. However, using Top1 cDNA derived from 5 individual topotecan-resistant Abcg2⁻/⁻,Brca1⁻/⁻,p53⁻/⁻ tumors (TB1, 2, 4, 5 and 8), we did not find a single point mutation analyzing 8 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 (Figure 2, B and Supplementary Figure 6). Only T10 showed a reduction of Top1 mRNA to 48% in the resistant tumor in comparison to the control. At the protein level, however, immunoblotting revealed substantially decreased Top1 levels in 5 of 9 topotecan-resistant Abcg2⁻/⁻,Brca1⁻/⁻,p53⁻/⁻ tumors compared to their matched untreated controls (Figure 5, A; TB1, TB2, TB8, TB9 and TB10). In addition, 5 of 8 Brca1⁻/⁻,p53⁻/⁻ tumors, lacking Abcg2 upregulation, also showed marked decreases in Top1 protein levels (Figure 5, B; T5, T10, T18-20). 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

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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 topotecan per kg i.p. on days 0-4 and 14-18. When tumors relapsed or showed progressive growth (tumor size ≥ 50%) after a recovery period of 14 days following the day 0 injection, treatment was resumed as indicated by the arrows. B, Overall survival (%) over time (days) of wild-type animals carrying orthotopically transplanted Abcg2-proficient (T7-T20) or -deficient (TB1-TB11) Brca1⁻/⁻, p53⁻/⁻ mammary tumors. Topotecan was given as in A and Figure 1, C. All animals carrying Abcg2-proficient tumors and 10 out of 11 animals carrying Abcg2-deficient tumors had to be killed because the resistant tumor reached a volume of ~1500 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.
of functional PARP, topotecan should drastically increase SSBs and thus increase synthetic lethality with BRCA1-deficiency. When we tested 4 mg topotecan per kg i.p. on days 0-4 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 (Figure 6, A). This is not surprising as PARP deficiency is known to sensitize mammalian cells to Top1 poisons (33). Only when we lowered topotecan to 0.5 mg/kg i.p. on 5 consecutive days, the combination with olaparib was tolerated (Figure 6, A). We then treated Abcg2-deficient Brca1−/−, p53−/− tumors to avoid ABCG2-mediated topotecan resistance and to maximize the

**Figure 5.** Western Blot analysis of Top1 levels in topotecan-resistant tumors. A, lysates of 9 resistant Abcg2−/−, Brca1−/−, p53−/− tumors (TB1-4 and TB6-10) and B, lysates of 8 resistant Brca1−/−, p53−/− tumors without increased Abcg2 gene expression (T5, T8, T10, T12 and T17-20) 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.
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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 wildtype (FVB/N × 129/Ola)F1 animals were treated with 4 mg topotecan per kg i.p. on days 0, 1, 2, 3, 4 in combination with 50 mg olaparib per kg 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 5 animals is presented and the error bars indicate standard deviation. 

B, response of 4 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 topotecan per kg i.p. on days 0-4, 14-18 and 28-32; 50 mg olaparib per kg i.p. daily for 42 days or both anti-tumor agents in these dosages combined. After a 42 day 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, 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 tariquidar per kg i.p. every second day during 42 days of drug administration to prevent ABCB1A/B-mediated olaparib resistance. After a 42 day dosing schedule animals were left untreated and screened for tumor relapse.
chance of tumor eradication. Nevertheless, the topotecan-olaparib combination did not increase the relapse-free survival of 4 individual tumors, compared to olaparib as single agent (Figure 6, B; TB1, TB5, TB7 and TB11). Even addition of the P-glycoprotein inhibitor tariquidar to the regimen, to prevent Abcb1a/b-mediated PARPi-resistance (10), did not result in eradication of the tumors (Figure 6, C).
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-densest topotecan therapy (T7-T20) was 53.5 days (± 39 SD) compared to 8 days (± 1 SD) of their matched untreated controls (Figure 1, C). 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 mis-localization, elevated DNA repair and resistance to apoptosis (reviewed in (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 (Figure 4, B). Abcg2−/−;Brca1−/−;p53−/− tumors can still develop 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 post-transcriptional in human tumors, its detection by gene expression profiling would be impossible. Top1 degradation is known to be 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 out of 20 individual Brca1−/−;p53−/− tumors, and the study of topotecan resistance in the remaining tumors is ongoing.
P-glycoprotein (P-gp) 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-gp-mediated resistance or transport (45-47). Our results show that even a 53-fold upregulation of P-gp, which makes the tumor completely resistant to doxorubicin, does not result in clear-cut resistance to topotecan. In real tumors, P-gp 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-gp 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-gp 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 knock-out mice showed 12-fold increased brain penetration relative to wild-type mice. This shows that P-gp 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 histological 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 current hypothesis is that remnant tumor cells arrest in the cell cycle and thereby escape anti-cancer drug-mediated cell death (49). This hypothesis is supported by the observation that cycling cells (Ki-67 positive) are drastically reduced after treatment. Since 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, since 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 single-strand DNA breaks 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.
Acknowledgments

We thank Sjoerd Rodenhuis, Hein te Riele, Koen van de Wetering and Ron Kerkhoven for critical reading of the manuscript and Susan Bates from the NIH in Bethesda (USA) for providing tariquidar. Jorma de Ronde, Arno Velds and Daoud Sie were very helpful in processing the gene expression profiling data. We also thank members of the animal pathology facility of the NKI for technical assistance. Olaparib is a kind gift of KuDOS Pharmaceuticals, Cambridge, England and we are grateful to GlaxoSmithKline (England) for providing topotecan.

Grant support

References


Supplementary data

Supplementary Figure 1. Relative animal weights (%) in response to topotecan treatment. A, 12-14-week old female K14cre, Brca1<sup>F/F</sup>, p53<sup>F/F</sup> animals or B, wildtype (FVB/N × 129/Ola) F1 animals were treated with 200 ml saline i.p. (red squares) or 4 mg topotecan per kg mouse i.p. (blue rhombi) on days 0-3 in A or days 0-4 in B. The average of 5 animals is shown and the error bars indicate the standard deviation.
Supplementary Figure 2. γ-H2AX-immunoreactivity in a topotecan-treated Brca1−/−,p53−/− mammary tumor (T8). Tumor-bearing mice were treated with the topotecan MTD (4 mg/kg i.p.) on 5 consecutive days or left untreated. The left panels show micrographs taken from tissues collected at day 5 after the start of topotecan injections, whereas the right panels show micrographs taken from tissues collected at day 8. Upper panels show untreated control tissues and lower panels the matched topotecan-treated tissues. Scale bars indicate 50 μm.
Supplementary Figure 3. Immunohistochemical investigation of cell death mechanisms in a topotecan-treated Brca1<sup>-/-</sup>,p53<sup>-/-</sup> mammary tumor (T8). Orthotopically transplanted animals were either treated with 4 mg topotecan per kg mouse at days 0-4 or left untreated and sacrificed at day 8. A, mean relative cell counts (%) for Ki-67 (black bars), cleaved caspase 3 (green bars), TUNEL (orange bars) and p19<sup>ARF</sup> (blue bars) in 10 independent views (155x155 μm) per tumor sample are shown with error bars indicating standard deviations. B, topotecan induced senescence-associated β-galactosidase activity (SA-βgal) and nuclear p19<sup>ARF</sup> expression in the same Brca1<sup>-/-</sup>,p53<sup>-/-</sup> tumor as in A. HE stained sections of the same tumor tissues serve as morphological reference. All micrographs, except the control SA-βgal, are 400x magnification with scale bars indicating 50 μm. The untreated SA-βgal control is 200x magnification with a 100 μm scale bar.
Supplementary Figure 4. Relative gene copy numbers of \textit{Abcg2}, \textit{Abcb1a}, \textit{Abcb1b}, \textit{Abcc1}, \textit{Top1} and \textit{Actβ} in 20 Brca1^{−/−},p53^{−/−} mammary tumors resistant to topotecan. The DNA concentrations were determined by a DNA-specific MLPA and the signal intensities normalized to 6 independent internal controls (Pajic et al. Cancer Res 69(16):6396-404, 2009). Bars indicate mean resistant/sensitive ratios of three independent MLPA reactions. Error bars indicate standard deviations.
**Supplementary Figure 5.** ABCG2 immunoreactivity (brown) in liver sections from *K14cre,Brca1^{F/F},p53^{F/F}* or *K14cre,Abcg2^{-/-},Brca1^{F/F},p53^{F/F}* animals developing “spontaneous” *Brca1^{-/-},p53^{-/-}* or *Abcg2^{-/-},Brca1^{-/-},p53^{-/-}* mammary tumors, respectively. Scale bars indicate 100 μm.
Supplementary Figure 6. Transcript levels of *Abcb1a, Abcb1b, Abcc1, Abcc4* and *Top1* in 8
topotecan-resistant *Abcg2*/*, Brca1/*, *p53*/* mammary tumors quantified by RT-MLPA. Bars
indicate mean resistant/sensitive ratios of three independent reactions, using *Actβ* as an
internal reference. Error bars indicate standard deviations.
**Supplementary Table 1.** Primary and secondary antibodies used for immunohistochemistry.

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**Supplementary Table 2.** List of 150 genes found to be up-regulated in topotecan resistant Brca1<sup>-/-</sup>,p53<sup>-/-</sup> tumors by SAM (Δ=1.058, 2.21% median false positive). Genes are listed according to the descending observed score (see Figure 2, A for corresponding SAM graph).

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**Supplementary Table 3.** Morphology and immunoreactivity (ABCG2, Ki-67, F4/80) of 20 individual topotecan-sensitive (con) and -resistant (topo res) Brca1;\textit{p53}-deficient mammary tumors. n/a = not available; - = 0%; +/- = 1-10%; + = 11-50%; ++ = >50%.

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<td>++</td>
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</table>

* All of the biopsy taken from this tumor before topotecan treatment was used to isolate RNA/DNA.
**Supplementary Table 4.** Morphology and immunoreactivity (ABCG2, Ki-67, F4/80) of 11 individual topotecan-sensitive (con) and -resistant (topo res) Abcg2;Brca1;p53-deficient mammary tumors. n/a = not available; - = 0%; +/- = 1-10%; + = 11-50%; ++ = >50%.

<table>
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<td>n/a</td>
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<td>+/</td>
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<td>++</td>
<td>+/</td>
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<td>+/</td>
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* animal sacrificed during treatment due to rectum prolapse
Supplementary Materials and Methods

Histology

Tissues were fixed in 4% formaldehyde overnight, embedded in paraffin, and cut in 4 μm sections. After deparaffinization and rehydration, sections were stained with hematoxylin and eosin according to standard procedures.

Immunohistochemistry

After tissue deparaffinization and rehydration, endogenous peroxidase activity was blocked by incubating tissues in 3% (v/v) H₂O₂ in methanol for 10 min. Before staining, paraffin sections were pretreated by heat-induced epitope retrieval in citrate buffer pH 6.0 (γ-H2AX, cleaved caspase 3 and p19) or citraconic anhydride (for Ki-67). Slides were preincubated with 5% normal goat serum in PBS (PBSA) for 30 min followed by overnight incubation with the appropriate primary antigen-specific antibodies diluted in 1% PBSA at 4 °C (Supplementary Table 1). Immunoreactivity was detected with the streptavidin-biotin immunoperoxidase (sABC) method by using biotinylated secondary antibodies diluted in 1% PBSA at room temperature (Supplementary Table 1) and dianinobenzidine substrate for visualization (Dakocytomation, # K037711). After counterstaining with hematoxylin, slides were dehydrated and mounted. For negative controls primary antibodies were omitted. Positively labeled cells were counted in the tumor sections in 10 standardized microscopic fields (155x155 μm). These fields were defined by using an ocular morphometric grid and 400x magnifications.

Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL)

After deparaffinization, rehydration and quenching of endogenous peroxidase activity tissues were pretreated with Proteinase K, according to the manufacturer’s instructions (ApopTag® Peroxidase ISOL Kit, Millipore, #S7200). Following T4 DNA polymerase-mediated specific ligation of biotinylated blunt ended oligos to complementary blunt DNA ends in specimen nuclei, dianinobenzidine substrate was used for visualization by incubation with a horseradish peroxidase-conjugated streptavidin complex. After counterstaining with hematoxylin, slides were dehydrated and mounted for microscopic evaluation.

Senescence-associated (SA)-betagalactosidase activity

A senescence-associated β-galactosidase (SA-βgal) staining kit (#9860, Cell Signaling Technology) was optimized for use on 10 μm tumor cryosections. Following 1 hour drying at room temperature, sections were fixed in 1x fixative solution (2% formaldehyde, 0.2% glutaraldehyde in 1x PBS) for 15 min. After washing 2x in PBS for 5 min, slides were
incubated overnight at 37 °C in staining solution (0.1% w/v 5-bromo-4-chloro-3-indolyl-βD-galactopyranoside (x-gal), 5% v/v N-N-dimethylformamide (DMF), 372 mM citric acid/sodium phosphate (pH 6.0), 1.395 M NaCl, 18.6 mM MgCl₂, 5 mM potassium ferrocyanide and 5 mM potassium ferricyanide) according to the manufacturer’s instructions. After washing 2x in PBS for 5 min slides were mounted with aquatex (Merck). As morphological reference to the SA-βgal slides, tissue sections were also H&E stained.