General summary
Drug resistance remains a major and unsolved problem in clinical oncology as most patients die of tumors that no longer respond to any form of chemotherapy (1). The research presented in this thesis is focused on investigating mechanisms of acquired resistance to topoisomerase inhibitors. Many standard-of-care chemotherapy regimens, including those used to treat breast cancer, comprise these anticancer agents. We used the conditional \( K14\text{cre};Brca1^{+/+};p53^{+/+} \) mouse model of hereditary breast cancer (2) to investigate molecular mechanisms of acquired chemoresistance. Spontaneous tumors, arising in this model, were orthotopically transplanted into syngeneic mice and treated with the maximum tolerable dose to mimic the clinical situation as closely as possible. Identifying resistance mechanisms to topoisomerase inhibitors in a tractable mouse model and developing effective strategies to overcome resistance may help improve chemotherapy in BRCA1-deficient breast cancer patients.

**Chapter 1**

BRCA1 dysfunction results in defective homology-directed DNA repair and hypersensitivity towards double-stranded DNA breaks (DSB). This tumor-specific repair defect may serve as an Achilles heel and we evaluated this putative synthetic lethality in chapter 1, using the topoisomerase I (Top1) inhibitor topotecan as monotherapy or 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. Of the various mechanisms reported in cultured cells to cause resistance to Top1 inhibitors (3-5), we identified only two in our mouse model: an increased expression of the drug efflux transporter ABCG2 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 \textit{in vivo} relevance of ABCG2 for topotecan resistance in a novel approach. Despite the lack of ABCG2, a putative tumor-initiating cell marker \( (6, 7) \), 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 patients.
Chapter 2
In chapter 2, we tested the polyethylene glycol-conjugated ('pegylated') SN38 compound EZN-2208 as a novel approach to treat \textit{BRCA1}-mutated tumors that express ABCG2. In xenografts, EZN-2208 results in higher and longer-lasting exposure of tumors to the irinotecan metabolite SN38 compared with irinotecan itself (8-10). We found that EZN-2208 therapy resulted in more pronounced and durable responses of ABCG2-positive tumors than topotecan or irinotecan therapy. We also evaluated tumor-specific ABCG2 inhibition by Ko143 in \textit{A}bc\textit{g}2\textsuperscript{−/−} host animals that carried tumors with topotecan-induced ABCG2 expression. Addition of Ko143 moderately increased overall survival of these animals, but did not yield tumor responses like those seen after EZN-2208 therapy. Our results suggest that pegylation of Top1 inhibitors may be a useful strategy to circumvent efflux transporter-mediated resistance and to improve their efficacy in the clinic.

Chapter 3
The fumitremorgin C analogue Ko143 is a potent and selective inhibitor of the ATP-binding cassette transporter ABCG2. To support the \textit{in vivo} ABCG2 resistance studies of chapter 2, we developed a sensitive and selective method for Ko143 quantification in plasma and tumor samples, using the parent compound fumitremorgin C as internal standard. Sample pretreatment by liquid-liquid extraction in diethyl ether yielded a recovery of 50\% from human and mouse plasma. Pretreated samples were separated by reversed-phase high-performance liquid chromatography with fluorescence detection at 295 nm excitation and 350 nm emission wavelengths. Sharp chromatographic peaks were obtained with a 5 \textmu{}m GraceSmart C18 column. The mobile phase consisted of methanol : 10 mM ammonium acetate pH 5.0 (62:38 \textit{v/v}), delivered at a flow rate of 0.2 mL/min. Acceptable accuracy and precision of ±15\% were achieved within the linear dynamic range of the calibration curve (2-500 ng/mL) for human and mouse plasma samples. Mouse tumor tissue samples required the use of a calibration curve prepared in the same matrix due to the lower recovery of 40\% from this matrix. Then, accuracy and precision were within the generally accepted range of ±15\% for bioanalytical assays. Ko143 was stable in human plasma for up to 3 repeated freeze-thaw cycles and when stored at room temperature for up to 72 hours. However, when kept at room temperature in mouse plasma, Ko143 was rapidly degraded by esterase activity, which could be prevented by collection of
blood into sodium fluoride-containing tubes and maintaining samples on ice during pretreatment.

**Chapter 4**

P-glycoprotein (P-gp) is the most studied ATP-binding cassette transporter, known to cause multidrug resistance. The relevance of P-gp for clinical drug resistance remains controversial, however (11). We previously found that modestly elevated expression levels of the mouse P-gp genes *Mdr1a* (*Abcb1a*) and *Mdr1b* (*Abcb1b*) are sufficient to cause resistance to the topoisomerase II (TOP2) inhibitor doxorubicin (12). Since P-gp-mediated drug resistance may be rare in breast cancer patients, we studied in chapter 4 P-gp-independent doxorubicin resistance mechanisms. For this purpose, we crossed the *K14cre;Brca1*^{−/−};*p53*^{−/−} mouse model onto a P-gp-deficient background to generate tumors, in which the function of both mouse P-gps is completely abrogated (13). When transplanted into P-gp-proficient mice, these tumors were hypersensitive to the maximum tolerable doxorubicin dose and usually did not acquire drug resistance. Only when we lowered the dose to 50%, resistance eventually occurred. Tumors that were resistant to the 50% dose showed a stable resistance phenotype upon transplantation into new recipient animals and subsequently also acquired resistance to the full dose. As a known mechanism of resistance we identified low TOP2α transcript and protein levels in about half of the 50% dose-resistant tumors. Some tumors without TOP2α downregulation were nevertheless cross-resistant to the TOP2 inhibitor etoposide, but not to the TOP1 inhibitor topotecan. RNA sequencing analysis of these tumors did not identify *Top2* mutations, but instead revealed lower *Top2β* RNA levels as a possible alternative resistance mechanism for some of the tumors. Our results suggest that low expression and not mutation of the drug target TOP2 explains many cases of P-gp-independent doxorubicin resistance in our mouse model.

**Chapter 5**

Inhibition of poly(ADP-ribose) polymerase (PARP) is a promising therapeutic strategy for homologous recombination-deficient tumors, such as BRCA1-deficient breast cancers. We previously reported that BRCA1-deficient mouse mammary tumors may acquire resistance to the clinical PARP inhibitor (PARPi) olaparib through activation of the P-gp drug efflux transporter (14). In chapter 5, we set out to identify novel mechanisms of PARPi resistance that cannot be explained by P-gp-mediated drug efflux, residual BRCA1 activity or restoration of BRCA1 function (15).
For this purpose, we have used the K14cre;Brca1\textsuperscript{F/F};p53\textsuperscript{F/F} conditional mouse model, in which mammary tumors arise that contain a large, irreversible Brca1 mutation on a P-gp-deficient background. Tumor-specific genetic inactivation of P-gp increased the long-term response of BRCA1-deficient mouse mammary tumors to olaparib, but these tumors eventually developed PARPi resistance. In about 25\% of studied cases this is caused by partial restoration of homologous recombination due to somatic loss of 53BP1 (16, 17). Importantly, PARPi resistance was minimized by long-term treatment with the novel PARP inhibitor AZD2461, which is a poor P-glycoprotein substrate. Together, our data suggest that restoration of homologous recombination is an important mechanism for PARPi resistance in BRCA1-deficient mammary tumors and that the risk of relapse of BRCA1-deficient tumors can be effectively minimized by using AZD2461, a novel PARPi with lower affinity to P-gp.

Chapter 6

In addition to their role in drug resistance, the ATP-binding cassette (ABC) transporters ABCG2 and ABCB1 have been suggested to protect cells from a broad range of substances that may foster tumorigenesis. Phytoestrogens or their metabolites are substrates of these transporters and the influence of these compounds on breast cancer development is controversial. Estrogen-like properties might accelerate tumorigenesis on the one hand, whereas their proposed health-protective properties might antagonize tumorigenesis on the other. As a side-line project, we addressed this issue in chapter 6, using the newer generation K14cre;Brca1\textsuperscript{F/F};p53\textsuperscript{F/F} mouse model of BRCA1-mutated breast cancer. We also studied the ability of phytoestrogens to restore mammary tumorigenesis in ovariectomized animals. The exposure of premalignant mammary epithelial cells to genistein or resveratrol might be influenced by the ABC transporters ABCB1 or ABCG2. It has been shown that ABCG2-deficient animals have increased resveratrol or genistein plasma levels compared with wild-type mice (18-20). Hence, to increase the exposure of mammary epithelial cells to genistein or resveratrol, we also investigated mammary tumor formation in Abcb1a/b\textsuperscript{-/-};K14cre;Brca1\textsuperscript{F/F};p53\textsuperscript{F/F} and Abcg2\textsuperscript{-/-};K14cre;Brca1\textsuperscript{F/F};p53\textsuperscript{F/F} females. Compared with the ABC transporter proficient model, ABCG2-deficient animals showed a reduced median tumor latency of 17.5 days (P < 0.001), whereas no significant difference was observed for ABCB1-deficient animals. Neither genistein nor resveratrol altered this latency reduction in Abcg2\textsuperscript{-/-};K14cre;Brca1\textsuperscript{F/F};p53\textsuperscript{F/F} animals. Ovariectomy resulted in nearly complete loss of mammary tumor development, which was not restored by genistein or resveratrol. Our results show that ABCG2 contributes to the protection
of genetically instable epithelial cells against carcinogenesis. Diets containing high levels of genistein or resveratrol had no effect on mammary tumorigenesis, whether mice were lacking ABCG2 or not. Because genistein and resveratrol only delayed skin tumor development of ovariectomized animals, we conclude that these phytoestrogens are no effective modulators of mammary tumor development in our mouse model.

Collectively, the results presented in this thesis show that genetically engineered mouse models are useful tools to study drug resistance to both benchmarked agents like topoisomerase inhibitors and novel targeted chemotherapeutics like PARP inhibitors. As more and more molecular insights of breast cancers become available by novel approaches that characterize cancer (epi-)genomes and proteomes at a high resolution, the mouse models mimicking human cancer will undoubtedly continue to evolve. I am convinced that these models are helpful to achieve the goal of optimizing chemotherapy efficacy in the clinic (21).
References


