General introduction
1. BRCA1-deficient breast cancer

Nearly 20 years ago it was found that germ-line mutations in the human breast cancer susceptibility gene *BRCA1* confer a lifetime risk of 60 to 85 per cent of breast cancer and 15 to 40 per cent of ovarian cancer (1). Hereditary breast cancers comprise about 5% of all breast cancers (2), and mutations in the *BRCA1* and the other major breast cancer susceptibility gene *BRCA2* underlie this disease (3, 4). BRCA1 loss of function is usually the result of loss of heterozygosity (LOH), and a classical example of the Knudson two-hit paradigm for loss of tumor suppressor gene function (5). Tumors arising in *BRCA1* germline mutation carriers show morphological and gene expression features of the basal-like breast cancer subtype (6). Typically, they are invasive ductal carcinomas of no special type (IDC-NST) with a high histological grade, and characterized by high mitotic indices, central necrotic or fibrotic zones, pushing borders and a lymphocytic infiltrate. The majority of basal-like breast cancers lack hormone receptor expression and lack HER2 protein overexpression or gene amplification, which is referred to as “triple negative” (TN, (7)). Moreover, they express genes usually found in basal or myoepithelial cells of the normal breast, including the cytokeratins (CK) 5/6, 14 and 17, P-cadherin, caveolins 1 and 2, nestin, αB crystallin, CD109 and EGFR (6). Most, if not all, BRCA1-deficient breast cancers have in addition *TP53* gene mutations (8). Alterations of the pRB and p16 G1/S cell-cycle checkpoints are also common in these cancers.

Of the sporadic breast cancers, about 25% have a molecular fingerprint that is reminiscent of the TN basal-like tumors that develop in germline *BRCA1* mutation carriers. *BRCA1* mutations in sporadic cancers are much rarer than in hereditary breast cancers. Nevertheless, lack of BRCA1 expression occurs in 11 to 14% of these cases (9) due to promoter hypermethylation (10, 11). Hence, BRCA1-deficient breast cancers are not restricted to patients with a hereditary predisposition, but are also found among patients with sporadic tumors.

Since its discovery as a tumor suppressor gene, several functions of BRCA1 have been identified (12). The most salient of these is its involvement in the homologous recombination (HR) repair pathway, essential for the error-free repair of double-stranded DNA breaks (DSB) (13, 14). As a result of BRCA1 dysfunction and the DNA repair defect that goes along with it, tumor cells become genomically unstable, which is revealed by a characteristic copy number aberration (CNA) profile and an associated “BRCAness” phenotype (9). Array comparative genomic hybridization (aCGH) has been found to be a useful tool to identify patients that may carry HR-deficient breast tumors, based on BRCA1-specific aCGH classifiers (15-18).
Importantly, the HR-deficiency of BRCA1-deficient breast cancers creates an Achilles heel that can be directly targeted with high-dose DSB-inducing chemotherapy (as reviewed in (19)). The topoisomerase inhibitors are an important class of DSB-inducing agents.

2. Physiology of DNA topoisomerases and inhibitors of these enzymes as anticancer agents

DNA topoisomerases are essential enzymes for maintaining DNA topology during transcription, replication and chromatin remodelling (20). These enzymes evolved to resolve helical supercoiling, knotting and catenation of DNA and to avoid DNA damage as a result of these topology problems. Topoisomerases are classified into two categories: type I enzymes cleave one DNA strand at a time creating single-stranded DNA breaks (SSB), while type II enzymes introduce DSB. Topoisomerases cleave the DNA phosphodiester backbone by nucleophilic attack from a catalytic tyrosine residue, which becomes covalently linked to the phosphate end of the DNA break (21). This reaction is highly reversible under physiological conditions and leaves the DNA sequence unchanged following topoisomerization. Human DNA topoisomerase I (TOP1, 100 kDa) is a type IB topoisomerase, which functions as a monomer and becomes transiently linked to the 3'-phosphoryl end after nicking the DNA (22). This TOP1-DNA cleavable complex is the drug target of the clinically used TOP1 inhibitors topotecan and irinotecan. These camptothecin derivatives prevent TOP1 from religating introduced SSB by stabilizing the TOP1-DNA cleavable complexes (TOP1cc). Toxic DSB are generated from SSB when replication forks collide with these complexes. When transcription complexes collide with the TOP1cc, RNA-DNA flaps are generated.

Human cells have two DNA topoisomerase II (TOP2) isoforms (23): α (170 kDa) and β (180 kDa). TOP2α and β are type IIA topoisomerases which function as dimers and become transiently linked to the 5'-phosphoryl ends after introduction of DSB. The catalytic cycle of these enzymes involves furthermore the unique capability of passing one DNA duplex (the T or transported helix) through a second duplex (the G or gated helix) by several conformational changes which require ATP hydrolysis (24). The unknotting and decatenation activities of TOP2 cannot be taken over by TOP1, which operates entirely without ATP hydrolysis. There is, however, partial redundancy in function, because TOP2 is also able to resolve positively and negatively supercoiled DNA, just like TOP1. TOP2 is targeted by several anticancer agents like the anthracycline doxorubicin and the epipodophyllotoxin etoposide (25). These
drugs are also called TOP2 poisons, because they corrupt the physiological function of TOP2 by stabilizing the TOP2-DNA cleavable complex (TOP2cc). As these TOP2cc persist, DSB are accumulated.

Hence, both TOP1 and TOP2 inhibitors increase the frequency of DSB of replicating cells. Such lesions are especially toxic to BRCA1-deficient breast cancers, which lack error-free DNA repair by HR.

3. Other therapeutic options to treat BRCA1-deficient breast cancers

The DNA damage hypersensitivity of HR-deficient breast cancers has been targeted by other classical cytotoxic agents like bifunctional alkylators and platinum agents, which induce DNA intra- and interstrand cross-links (25, 26). However, a major disadvantage of these agents is their considerable toxicity to non-tumorous tissues.

PARP inhibition (PARPi) is a novel and less toxic therapeutic approach that has recently been introduced to target specifically the HR repair defect of BRCA1- (and BRCA2)-deficient breast and ovarian cancer cells (26, 27). This therapeutic strategy is one of the first successful examples of a molecularly targeted therapy, in which the concept of a synthetic lethal interaction is exploited (28, 29). In this case, the interaction stems from the fact that PARPi is only toxic to the BRCA-deficient cancer cells, but not to BRCA-proficient cells in all other tissues. Recently published evidence has shed new light on our current understanding of the mechanism for PARP and BRCA synthetic lethality (as reviewed in (30)). In contrast to previous reports, in which PARP1 was hypothesized to be a critical component of the base excision repair pathway (BER), Ström and colleagues found that PARP1 is not directly required for efficient BER completion (31). In this alternative model, it is proposed that trapped PARP1 on SSB impairs efficient religation through the BER pathway and that collapsed replication forks at these persisting SSB generate toxic DSB. This mechanism of PARPi action is very reminiscent of that of TOP1 inhibitors, in which tumor cells also indirectly accumulate DSB through persisting SSB. But even this mechanistic basis of PARPi efficacy has recently been questioned by others, who found that deregulation of the error-prone non-homologous end-joining (NHEJ) pathway is responsible for generating increased levels of genomic instability and therefore cytotoxicity in HR-deficient tumor cells (32). Regardless of what the exact mechanism of action will turn out to be, PARP inhibitors as targeted monotherapy for BRCA1-deficient breast cancers have already shown great promise in the clinic (33, 34) and will undoubtedly become an important part of the clinical management of these cancer patients in the coming years (35).
4. Synergism between TOP1 and PARP inhibition

It follows from the previous discussion about mechanisms of action of PARPi monotherapy that the cytotoxicity induced by TOP1 inhibitors may be enhanced when these two therapeutic agents are combined. This has indeed been found in vitro, but there is still some debate about how this synergism exactly plays out. PARP inhibitors increase DNA breaks in response to TOP1cc, but without a concomitant increase in TOP1cc formation (36). PARPi has therefore no effect on TOP1 activity as indicated by DNA relaxation assays or the level and persistence of TOP1cc. Interestingly, sensitization towards camptothecin as indicated by γ-H2AX induction was observed at concentrations of veliparib (ABT-888) that on their own had no detectable effects (37, 38). It may be that PARP inhibitors enhance the persistence of SSB already generated by stabilized TOP1cc through additional trapping of PARP1 on these SSB, preventing BER from religating these SSB (30). Alternatively, Zhang et al. (38) reported evidence for the involvement of the nucleotide excision repair (NER)-associated XPF-ERCC1 endonuclease as an important back-up repair pathway in the absence of the PARP-TDP1 pathway that usually repairs TOP1cc. Transfection experiments in Parp1−/− mouse embryonic fibroblasts (MEFs) with catalytically inactive PARP1 or PARP1 DNA binding domains provides further evidence for a model in which PARP inhibitors act as PARP1 poisons, where DNA-bound but inactive PARP1 prevents proper DNA repair (37). Irrespective of the exact mechanism of synergy, TOP1 - PARP inhibitor combination therapy has already been tested in phase I trials of solid tumor patients with synergistic tumor cell killing, but also considerably increased haematological toxicity (39, 40). This side effect made dose lowering inescapable. Nevertheless, if there is still synergy at low-dosed PARP inhibitor and TOP1 inhibitor combination therapy in patients, this therapeutic approach may considerably increase recurrence-free survival of BRCA1-deficient breast cancer patients, who have to face a rather poor prognosis.

5. Mechanisms of resistance to topoisomerase inhibitors

Resistance to chemotherapy is a major problem in the cancer clinic, because most patients succumb to tumors that no longer respond to the available anticancer drugs. Successful systemic treatment of patients (and model animals) implies first of all that the drug has to reach its intended pharmacological target at a sufficient concentration that kills tumor cells, but not the cancer patient. In a genetically engineered mouse model of pancreatic ductal adenocarcinoma, for instance, it was
recently shown that inhibition of sonic hedgehog signaling considerably improves gemcitabine delivery and efficacy due to depletion of the tumor-associated stroma, which impairs tumor perfusion (41). Once a drug has been administered, it has to be absorbed and distributed to the intended target tissue without being inactivated by metabolism along its way. If tumors are located in tissue sanctuary sites like the brain, they may be shielded from drugs by the blood-brain-barrier (42). In addition, tumors often develop leaky fenestrated vasculature without proper lymphatics. The increased interstitial pressure that accompanies this pathological angiogenesis may also compromise adequate drug delivery (43). But even if these barriers can be breached, tumors can still mount an effective cell-intrinsic defense by broadly three classes of resistance mechanisms:

- downregulation of uptake transporters or upregulation of efflux transporters, lowering intracellular drug availability
- drug target-related mechanisms
- drug target-independent mechanisms

5.1. ABC efflux transporters

ATP-binding cassette (ABC) transporters are well-known for their role in mediating drug resistance by effluxing substrate drugs across the cell membrane in an ATP-dependent manner. There are seven distinct subfamilies of ABC transporters (ABCA-ABCG, (44)) of which P-glycoprotein (P-gp;MDR1;ABCB1), BCRP (ABCG2) and MRP1 (ABCC1) have been most extensively studied and shown to transport a wide variety of anticancer drugs, including the topoisomerase inhibitors (45). ABC transporters are also expressed in the epithelia of the gut, liver and kidney or the endothelium of the blood-brain-barrier, where they play a critical role in drug excretion or prevention of uptake. P-gp was the first discovered ABC transporter and shown to efflux doxorubicin in Chinese hamster ovarian cells (46). The topotecan transporter BCRP was much later identified as an ABC half transporter that has to homodimerize before becoming fully functional (47-49). Studies with P-gp and BCRP knockout mice showed that these transporters co-operate in limiting topotecan penetration into the mouse brain (42).

5.2. Drug target-related resistance mechanisms

Topoisomerase inhibitors interact with very well-defined cellular target enzymes that induce DNA lesions. If tumor cells acquire topoisomerase alterations that negatively affect this interaction, they can avoid these lesions and therefore
become resistant. Downregulation of TOP1, for instance, will result in less SSB and replication-associated DSB when TOP1cc are stabilized by the TOP1 inhibitors (50-57). Similarly, downregulation of TOP2 will also result in less DSB, providing an escape route to TOP2 inhibitors (58-60). In addition, numerous topoisomerase point mutations have been reported, which disable TOP1 or 2 cleavable complex stabilization (61-64). Finally, relocalization of topoisomerases away from DNA has been shown to be another drug target-related resistance mechanism (65-67). Importantly, most of these topoisomerase-related resistance mechanisms were identified in cultured cancer cells and the clinical validation of these findings has proven to be difficult. Limited sample availability and methodological differences between studies were frequently put forward as reasons for contradictory reports (68). Using genetically engineered mouse models as surrogate patients to study in vivo chemoresistance may fill this caveat and improve the translation from bench to bed side.

5.3. Drug target-independent resistance mechanisms

One obvious way to overcome DNA damage is to increase repair capacity. Recently, secondary BRCA1 (and BRCA2) mutations have been implicated in restoration of HR (69-71). This genetic reversion caused resistance to platinum agents in ovarian carcinomas, but may also be relevant for resistance against topoisomerase inhibitors. Alternatively, there is evidence that TDP1 and other endonucleases (72) enhance the removal of 3’-phosphotyrosyl-DNA complexes that remain after partial proteasomal degradation of TOP1 (73). Similarly, TDP2 has recently been linked to the removal of the 5’-phosphotyrosyl-DNA complexes that remain after TOP2 degradation (74). Interestingly, a novel pathway has recently been identified in BRCA1-deficient ES cells that leads to partial restoration of HR by loss of 53BP1 expression (75, 76).

In addition to DNA repair, tumors may inactivate a drug by converting it into inactive metabolites. An important shortcoming of the currently used camptothecin analogues topotecan and irinotecan is the rapid conversion of the active lactone form into the inactive carboxylate form, which is tissue pH-dependent. The pro-drug irinotecan requires in addition a prior carboxyl esterase-mediated cleavage step to generate the active SN-38. This compound is glucuronidated by the UGT1A conjugating enzymes in the liver to improve water solubility and subsequent excretion. This metabolic pathway has also been shown to be responsible for resistance in the anthracycline-cross-resistant MCF-7 AdVp 3000 breast cancer cells (77).
6. Therapeutic strategies to prevent or overcome resistance to topoisomerase inhibitors

It has been a long-standing goal in the ABC transporter field to develop effective inhibitors that may be combined with anticancer agents to improve tumor drug delivery into the tumor, thereby overcoming clinical chemoresistance. However, in a retrospective analysis of drug resistance reversal studies in breast and lung cancer, a recent review concluded that after 35 years the role of P-gp in clinical oncology is still not established (78). For P-gp, there are highly potent and non-toxic third generation inhibitors available and we showed in our mouse model that addition of tariquidar overcomes P-gp-mediated doxorubicin and olaparib resistance (79, 80). Specific inhibitors for ABCG2 are in a much less advanced stage of clinical development (81) and we tested whether Ko143 could reverse ABCG2-mediated topotecan resistance after showing that tumor-specific ablation of this ABC transporter considerably improved overall survival of tumor-bearing animals under topotecan monotherapy (82). Indeed, addition of Ko143 also improved overall survival of the topotecan-treated animals.

Another therapeutic approach to increase the intra-tumoral concentration of topoisomerase inhibitors may be the use of polyethylene glycol (PEG)-conjugated ('pegylated') compounds as an alternative strategy to target ABCG2-expressing tumor cells. Using xenograft models, Sapra and coworkers (83) reported that administration of the pegylated topoisomerase I inhibitor SN-38 (ENZ-2208) results in higher and longer-lasting tumor exposure to SN-38 compared with CPT-11, its pro-drug. In addition to substantially improving water solubility of the SN-38, pegylation has been suggested to increase tumor-specific accumulation of polymeric compounds due to aberrant and leaky tumor-associated vasculature (enhanced permeability and retention effect or EPR). This strategy to improve tumor drug availability by optimizing its pharmacological formulation may be a useful approach that avoids the pharmacokinetic interactions that are associated with ABC transporter inhibitors.

As described above, drug target downregulation is an important resistance mechanism to topoisomerase inhibitors. Interestingly, it has been observed in vitro that tumor cells, which downregulate TOP1 in response to TOP1 inhibitor treatment, may upregulate TOP2α and become hypersensitive to TOP2 inhibitors (84, 85). This is not unexpected as upregulation of TOP2α may be helpful to increase the cell’s capacity to resolve DNA supercoiling and compensate for very low levels of TOP1 expression. No compensatory upregulation of TOP1 in TOP2 inhibitor-treated cells
has been reported, however, and this is probably due to the fact that TOP2 can in addition decatenate and unknot DNA, whereas TOP1 can only resolve supercoiling. Based on these considerations, it was investigated whether there may be a synergistic effect of combining TOP1 and 2 inhibitors. Unexpectedly, it was found that this combination was antagonistic rather than synergistic, because of inhibition of nucleic acid synthesis by the TOP1 inhibitor (86). Subsequently, others showed that sequential, instead of simultaneous, treatment with TOP1 and 2 inhibitors does enhance cytotoxicity (87, 88). In the clinic, however, even this strategy turned out to be problematic, because of excessive and dose-limiting bone marrow toxicity (89, 90). Also dual TOP1 and 2 inhibiting compounds were explored, but probably never gained significant momentum due to related clinical problems (91-93).

7. The \textit{K14cre;Brca1}^{F/F};\textit{p53}^{F/F} mouse model of BRCA1-deficient breast cancer as a tool to study resistance mechanisms to topoisomerase inhibitors

Compared with other fields in medicine, oncology is infamous for its high attrition rate in novel therapeutics development, making it a challenging arena for the pharma industry to deliver the new drugs that are so badly needed in the war against cancer (94). An important reason why it is so difficult to successfully develop new anticancer drugs is the lack of good preclinical models that predict compound behaviour in patients (95). Cultured cell systems may have been instrumental in the understanding of the fundamental aspects of cell biology, but were proven to be rather poor reflections of the cancers from which they were originally derived decades ago (96). Although xenografted mice may overcome some cell culture-related artefacts, modelling pharmacokinetics and the tumor microenvironment to some extent, the host animals are still immunocompromised, which is an important shortcoming when it comes to chemotherapy response. The newer generation genetically engineered mouse models (GEMMs), however, more faithfully mimic many aspects of human cancers and hold great promise as platforms to study chemotherapy response with unprecedented genetic detail (97).

The mammary tumors that spontaneously develop in our conditional \textit{K14cre;Brca1}^{F/F};\textit{p53}^{F/F} mouse model closely mimic many aspects of the pathomorphology and biology of human BRCA1-deficient breast cancers (98). Spontaneous mammary tumors that arise in this model can be orthotopically transplanted into wild-type syngeneic hosts, while retaining their individual morphologies and gene expression profiles (79). Intervention studies can be
carried out testing individual tumor responses to several drugs in parallel. Samples from matched resistant and untreated control tumors can be easily obtained and compared to identify molecular differences that underlie resistance development. If the GEM model cannot reflect a particular resistance mechanism like genetic reversion (69-71), additional genetic modifications can be introduced (99). These approaches are impossible to perform in human patients and make these models valuable tools that may accelerate the development and approval of new anticancer agents in the clinic (100).

8. Objectives and outline of this thesis

The main purpose of the research presented in this thesis was to investigate mechanisms of acquired resistance to topoisomerase inhibitors. These anticancer agents are part of many standard-of-care chemotherapy regimens in the clinic. We used the conditional $K14\text{cre};Brca1^{F/F};p53^{F/F}$ mouse model as a tool to investigate this problem. We found that ABCG2 and P-gp were frequently causing resistance to topotecan and doxorubicin, respectively (Chapter 1 and (79)). Subsequently, we tested whether adding the specific ABCG2 inhibitor Ko143 (Chapter 2) could overcome resistance to topotecan, for which we developed a validated RP-HPLC assay to measure Ko143 pharmacokinetics (Chapter 3). In addition, we also introduced Abcg2 and P-gp null alleles into the mouse model to identify ABC transporter-independent resistance mechanisms that may be relevant to the clinic (Chapters 1 and 4). While breeding the required mice for this purpose, we made the observation that loss of ABCG2 decreased latency of spontaneous tumor development in our mouse model. As a side project, we investigated whether the phytoestrogens resveratrol and genistein could affect this tumor latency phenotype (Chapter 6). We also tested the potential for synergism between TOP1 and PARP inhibitor combination therapy (Chapter 1). Chapter 5 discusses partial restoration of homologous recombination through loss of 53BP1 as a novel mechanism of acquired resistance to topotecan and the PARP inhibitor olaparib.
References


in combination with topotecan for the treatment of patients with advanced solid


87. Bertrand R, O’Connor PM, Kerrigan D, Pommier Y. Sequential administration of camptothecin and etoposide circumvents the antagonistic cytotoxicity of simultaneous


