Summary, discussion and future perspectives
Summary

First-line treatment strategy for locally-advanced cervical cancer (LACC) patients consists of radiotherapy with concomitant platinum-based chemotherapy. However, 30-50% of the patients show therapy resistance or develop recurrent disease [1]. Therefore, novel therapeutic strategies are needed to further improve survival of cervical cancer patients, for instance by overcoming therapy resistance. Therapeutic targeting of components of the DNA damage response (DDR) might be an attractive option to achieve this goal, since this approach may increase the cellular sensitivity for chemo- and/or radiotherapy.

The DDR orchestrates the cellular response to DNA damage in normal cells but also in malignant cells. In cervical cancer cells, DDR function is partially impaired, since the DDR components p53 and Rb1 are inactivated due to persistent oncogenic human papilloma virus (HPV) E6 and E7 expression. Compared to normal cells, cervical cancer cells therefore have partially defective DDR signaling, and consequently depend stronger on their residual DDR signaling factors. Therefore, the inhibition of these residual DDR signaling axes may be exploited therapeutically to achieve potentiation of chemo- and/or radiotherapy. In this thesis, we investigated the DDR as therapeutic intervention target in cervical cancer and aimed to identify the potentiating effects of DDR targeting on chemo- and/or radiotherapy in preclinical in vitro and in vivo cervical cancer models.

The general introduction and subsequent chapters were outlined in Chapter 1.

Improving efficacy of genotoxic treatments, such as chemo- and radiotherapy, by therapeutic targeting of the DDR is not straightforward, especially because the DDR consists of multiple parallel signaling pathways, which display crosstalk at multiple levels. Therefore, it is currently unclear which DDR component is most suitable as a therapeutic target, and which chemo- and radio-therapeutic treatments this should preferably be combined with. To investigate the current status of DDR targeting in cervical cancer, a literature review on preclinical and clinical studies on DDR inhibition in cervical cancer is provided in Chapter 2. Drugs acting on kinases upstream within the DDR pathway were our focus of interest, as they have been investigated most extensively. We searched available literature on PubMed (including Medline), Embase and the Cochrane database. Additionally, information about completed and ongoing clinical trials were retrieved from www.clinicaltrials.gov. We specifically focused on current knowledge of the chemo/radio-potentiating effects of ATM, ATR, DNA-PK, Chk1, Chk2 and MK2 inhibitors in cervical cancer cells. Notably, we described a major gap in the current knowledge. The majority of current literature assessed the use of DDR inhibitors either alone or in combination with either radiation or a single chemotherapeutic agent. In clinical practice, however, most regimens combine radiotherapy with genotoxic chemotherapy in the advanced-stage disease setting. In addition, further improvement of the pharmacokinetics of DDR compounds is necessary to allow translation of in vitro chemo-/radiotherapy potentiating drugs to the in vivo and clinical setting.

In the context of LACC treatment, both cisplatin and radiotherapy cause cytotoxicity by inducing DNA double-strand breaks (DSBs). However, their cytotoxic effects are limited by the activity of DDR kinases, including the ataxia telangiectasia mutated (ATM) kinase, which is a central regulator in DSB signaling. We therefore investigated in Chapter 3 the therapeutic potential of targeting ATM or its substrate 53BP1 (p53-binding protein-1) in cervical cancer.
Firstly, we demonstrated that inactivation of either ATM or 53BP1 resulted in cell cycle defects in response to ionizing radiation. However, clonogenic survival analysis revealed that only ATM inhibition, and not 53BP1 inactivation, resulted in decreased survival in cervical cancer cell lines. Notably, a high activation status of ATM prior to ionizing radiation coincided with increased radioresistance. This notion was in line with immunohistochemical analysis of ATM and activated (phospho)-ATM in cervical cancer specimens, which revealed that high levels of active ATM prior to chemoradiation were related to increased radiotherapy resistance (HR 1.650; 95% CI 1.076-2.528; P=0.022). Therefore, both our \textit{in vitro} and \textit{in vivo} data suggested a beneficial effect of ATM inhibition along with chemoradiation to improve clinical outcome in cervical cancer.

In addition to ATM, multiple other DDR components are activated upon chemoradiation. Therefore, in Chapter 4 we investigated the effects of chemical inhibition of other major DDR kinases including ATR, DNA-PK, Chk1, Chk2 and MK2 in cervical cancer cell lines. Immunohistochemical analysis revealed that all these DDR kinases are expressed prior to therapy in LACC tumor samples. Of all analyzed DDR kinases, only MK2 and ATR appeared to be already active prior to treatment, as revealed by the phosphorylation status of respectively threonine-334 on MK2 and serine-33 on the ATR substrate RPA32 in treatment-naïve cancer tissue. Concerning the effects of chemical DDR kinase inhibition on cisplatin-induced cytotoxicity, only inhibition of Chk1/2 and ATR caused chemosensitization. Conversely, increased radiosensitization was observed upon inhibition of ATM, ATR and DNA-PK. However, as concomitant chemoradiation is the standard treatment in LACC patients, we studied the clinically more relevant setting of studying DDR inhibition in combination with combined cisplatin-based chemoradiation. This analysis revealed a strong chemoradiosensitizing effect of ATR inhibition. From these studies, we conclude that ATR inhibition is the most promising DDR target to potentiate chemoradiation in cervical cancer cells. Furthermore, exploratory immunohistochemical analysis suggested that a high activation of the ATR-Chk1 pathway, as assessed by high expression levels of activated Chk1, might be related to a worse disease-specific survival in LACC patients who were primarily treated with chemoradiation (HR 2.268; 95% CI: 1.058-4.860; P=0.035).

Cervical cancer is predominantly caused by persistent infection with the HPV types 16 and 18. Expression of the HPV oncoproteins E6 and E7 results in inactivation of p53 and Rb1 respectively. As a consequence, cervical cancers mainly rely on the G2/M cell cycle checkpoint for repairing DNA lesions. Wee1 is a key controller of the G2/M cell cycle checkpoint. Previous reports showed that chemical inhibition of Wee1, as expected, sensitize p53-mutant cervical cancer cell lines to chemo- and radiotherapy. Therefore, the potentiating effects of Wee1 inhibition in cervical cancer were investigated in Chapter 5. We found that 99.5% of the tumor specimens from LACC patients, who were primarily treated with chemoradiation (n=204), showed abundant Wee1 expression. \textit{In vitro}, Wee1 inhibition alone demonstrated cytotoxicity in HPV-positive cervical cancer cells and also sensitized cells for cisplatin. Surprisingly, not all HPV-positive cervical cancer cell lines showed similar levels of sensitivity for Wee1 inhibition. Importantly, we found that in these cell lines Rb1 depletion, but not p53 depletion, rescued Wee1 inhibitor sensitivity. In conclusion, the results in Chapter 5 implicate that LACC patients could potentially benefit of Wee1 inhibition and that sensitivity to Wee1 inhibition in cervical cancer cells is determined by both p53 and Rb1 inactivation.
In vitro findings can be further evaluated in 3D models, to test potential value prior to translation to the clinical situation. Unfortunately the availability of reliable models to translate in vitro findings to in vivo models is limited. Therefore, in Chapter 6 we established the chicken chorioallantoic membrane assay as platform for efficient testing of novel therapeutic interventions in combination with concomitant chemoradiation in cervical cancer. We confirmed that cervical in ovo tumors can be established from the HeLa cervical cancer cell line. Subsequently, we observed tumor growth reduction with either single therapy with cisplatin or ionizing radiation. We observed high levels of replication stress upon cisplatin treatment and high levels of γ-H2AX in response to ionizing radiation, indicating that these therapy modalities were successfully delivered to the tumor cells in ovo. Furthermore, also after concomitant treatment with cisplatin and radiation, we were able to observe tumor growth reduction. Therefore, we here provide a novel model, which may be useful to efficiently triage a variety of therapeutic compounds for use as candidates in further (pre)clinical development.

Discussion and future perspectives

Treatment of cervical cancer

Addition of platinum-based chemotherapy to primary radiotherapy as treatment strategy in LACC patients has resulted in a ~6% gain in overall survival [2-7]. However still 30-50% of the patients fail to respond to therapy or develop recurrent disease [1]. Therefore, novel treatment strategies are needed to improve therapy efficacy. Platinum-based chemotherapy and radiotherapy exert their anti-cancer effects by inducing high levels of DNA lesions, including stalled-replication forks and DNA DSBs. However, one of the mechanisms involved in the cytotoxicity of these DNA lesions -and consequently therapy efficacy- is the ability of cancer cells to halt cell proliferation and repair the DNA damage. The cellular mechanisms that coordinate the cellular response to genotoxic stress are collectively called the DDR. Multiple studies have demonstrated that chemo- and/or radiotherapy can be potentiated by targeting DDR pathways [8-13]. This potentiating effect can either be achieved by deregulation of cell cycle checkpoints or by decreasing the ability of cancer cells to repair treatment-induced DNA lesions. This approach may be especially relevant for cervical cancers, since they already have an impaired DDR, due to p53 and Rb1 inactivation by HPV oncoproteins. As a consequence, cervical cancer cells rely strongly on their residual DDR pathways to cope with DNA damage, while normal cells still have an intact DDR function.

Taken together, targeting the -already compromised- DDR in cervical cancer may result in potentiating chemo- and/or radiotherapy. Therefore, therapeutic targeting of residual DDR pathways in cervical cancer cells appears to be a promising strategy to improve efficacy of chemoradiation in cervical cancer.
Therapeutic targeting of the DNA damage response in cervical cancer

The majority of the reported small molecule chemical inhibitors that are used to potentiate the cytotoxic effects of genotoxic agents inhibit the enzymatic activity of DDR kinases. We showed that ATM inhibition results in increased cervical cancer cell death upon irradiation-induced DNA damage (Chapter 3). In addition, we showed that inhibition of ATR or Chk1 potentiates the cytotoxic effects of platinum-containing drugs in cervical cancer cells (Chapter 4). Depending on the type of DNA damage induced, the inactivation of specific DDR components is required to achieve potentiating effects of either genotoxic agent. Taking this into account, it is not unexpected that the majority of DDR inhibitors are tested as monotherapy or in combination with either ionizing radiation or a single genotoxic agent [14]. However, standard of care for many advanced-staged cancers consists of a multimodality approach, including cervical cancer. In chapter 4 we demonstrated the importance of the nature of the induced DNA damage type in relation with expected potentiating effects of DDR targeting. Therefore, it is essential for further in vivo studies to enable proper target selection by testing DDR compounds together with treatment modalities currently used in patients.

A second concern regarding the currently available DDR targeting agents is their specificity. In vitro studies using chemical DDR agents as anti-cancer therapy often confirm phenotypes obtained after knockdown of the specific DDR-gene. When results between pharmacological and genetic inhibition are overlapping, the observed (chemical) phenotype is very likely associated with the specific inhibition of the DDR-target in that particular experiment. However, other kinases may still be (partially) inhibited and thereby influence other cellular processes. For example, in a cell-based screen to identify novel ATR targeting compounds, NVP-BEZ235 was identified as a highly potent ATR inhibitor [15]. Especially in p53-deficient cancers in combination with replication-stress inducing agents, ATR inhibition is a promising anti-cancer therapeutic modality. However, in literature NVP-BEZ235 was already described as an inhibitor of phosphatidylinositol-3-kinase (PI3K) and mammalian target of rapamycin (mTOR) [16]. Inhibition of either PI3K or mTOR results in a strong G1-arrest [17,18]. Consequently, the anti-proliferation property of mTOR inhibition will limit the generation of stalled-replication forks, and thereby limit the cytotoxicity induced by ATR inhibition. Consequently, the off-target effects of NVP-BEZ235 towards mTOR (from the perspective as an ATR inhibitor) may nullify the anti-cancer effect of ATR inhibition [15]. In addition, the cross reactivity of certain DDR inhibitors is not unexpected as kinases from the same kinase-family are structurally related. For example, DNA-PK, a member of the PI3K-related kinase (PIKK) family, is structurally very similar to PI3K and many of the DNA-PK inhibitors also target PI3K p110α [19].

Another important issue to address is the long-term effect of DDR inhibition in patients. In normal cells, the DDR functions as an anti-cancer barrier thereby preventing carcinogenesis [20]. Exposure to DDR inhibiting agents might impair the protective DDR function in normal cells and may have tumorigenesis-promoting effects upon long-term treatment. This is underscored by the phenotypes of patients with, for example, the ataxia-telangiectasia (AT) disorder. AT is caused by mutations in the ATM gene and is characterized by extreme sensitivity to ionizing radiation, and a predisposition for malignancies [21-23]. Use of reversible DDR inhibitors might limit side effects [15,24]. For example, ETP-46464 has been described to transiently block ATR activity in
U2OS cells after ultra-violet induced DNA damage [25]. In the context of cervical cancer, ETP-46464 was shown to potentiate HeLa and SiHa cancer cells for cisplatin and ionizing radiation [26]. Ideally, proof-of-principle experiments in cervical cancer patient-derived xenograft (PDX) models could be used to study the anti-cancer effects of reversible DDR inhibitors and hopefully aid in bringing these drugs to the clinic.

The number of clinical trials in which DDR inhibitors are tested, specifically in cervical cancer, is limited and mostly concern phase I trials. Furthermore, several clinical trials with DDR targeting compounds were prematurely terminated. For example, the use of AZD7762, a Chk1 inhibitor, was halted due to unexpected cardiac cytotoxicity [27,28]. Currently, only one clinical trial (phase I) is ongoing which specifically evaluates a DDR inhibitor in cervical cancer patients, namely the Wee1 inhibitor AZD-1775 (NTC10958658). In chapter 5 we observed differences in sensitivity to Wee1 inhibition between various HPV-positive cell lines. We found that besides inactivation of p53, also Rb1 inactivation is an important determinant in the response to Wee1 inhibition. Therefore, it would be interesting to include analysis of expression and/or mutational status of p53 and Rb1 in the current ongoing clinical trial with AZD-1775. If the status of these proteins is indeed important for the cytotoxic responses to AZD-1775 in HPV-positive cervical cancer cells, this might provide a tool to better select cervical cancer patients that may benefit of Wee1 inhibition.

Future challenges for the use of DDR inhibitors in cervical cancer

Compared to normal cells, cancer cells have distinct biological characteristics, which are encapsulated in the hallmarks of cancer [29]. The aim of DDR inhibitors is to target some of these cancer-specific properties to ultimately kill cancer cells and spare normal tissue. Gradually, concepts from fundamental research are being translated into the clinical setting [14]. To understand challenges ahead, a closer understanding of the DDR in relation to the hallmarks of cancer is needed.

Uncontrolled cell proliferation triggers activation of the DDR to protect precancerous cells from progressing into cancer cells [20,30,31]. When the DDR barrier is impaired, for example by mutation of the tumor suppressor p53, tumorigenesis is thought to progress [31]. In cervical carcinogenesis this barrier is predominantly breached after persistent infection with HPV16 or 18, which results in p53 and Rb1 inactivation [32]. As a consequence of compromised DDR pathways, cancer cells depend on their residual DDR capacity to cope with DNA damage [33,34]. This dependency may constitute an 'Achilles heel' and a therapeutic opportunity to specifically kill cancer cells and spare normal cells. In this context, the first DDR inhibitor, the poly(ADP-ribose) polymerase (PARP) inhibitor Olaparib, has recently been approved in Europe and the USA for monotherapy in advanced ovarian cancer patients, who harbor a germline BRCA mutation following at least three lines of chemotherapy [35]. This combination of a cancer-specific mutation and cancer cell death after inhibition of a specific (enzymatic) protein is based on the concept called synthetic lethality [36]. Of note, only 13-15% of ovarian cancer patients harbor a BRCA1/2 mutation [37]. As a consequence, only a small proportion of ovarian cancer patients theoretically benefit from olaparib, and (mutation-based) patient selection is required to identify those patients that are expected to benefit from this treatment. The fact that BRCA1/2 mutant cancers are also more
sensitive to multiple genotoxic agents has raised the question whether PARP inhibitor treatment may be potentiated by addition of conventional DNA damaging therapies.

At this time, similar synthetic lethal combinations have not been identified in cervical cancers. Although a recent shRNA screen demonstrated a lethal combination in HeLa cervical cancer cells between loss of p53 and SGK2 (serum/glucocorticoid regulated kinase 2) or PAK3 (p21-activated kinase 3), further fundamental research is needed to explore the clinical relevance of these interactions [38]. Possibly due to its HPV-mediated carcinogenesis, only a limited amount of somatic mutations are found in cervical cancers, which therefore might require combination therapy [39].

So, how can we exploit the DDR to improve the susceptibility of cervical cancer cell for chemoradiation? In order to cope with DNA damage, normal and cancer cells depend on cell cycle checkpoints and DNA repair machineries [36]. We propose that DDR inhibition as a therapeutic strategy in cervical cancer should aim for a maximization of the chemoradiotherapy-induced replication stress and DNA DSBs by either 1) impairment of DNA repair machineries and/or 2) by impairment of the G2/M checkpoint.

In this thesis we showed that, out of several key DDR kinases, inhibition of ATR has the greatest potential to chemoradiosensitize cervical cancer cells. One rational behind the increased potentiation is probably related with an increase in DNA damage. When -in case of DNA damage- ATR activity is inhibited, both the mitotic cell cycle checkpoint is impaired and replication forks collapse, which eventually lead to increased DNA damage [15]. In addition, we and others demonstrated involvement of ATR in HR repair [40-42]. How and to which extent the impairment of DNA repair by ATR contributes to the chemoradiosensitization needs further investigation. Although several specific ATR inhibitors are available, none of these is specifically tested in clinical trials in the context of advanced cervical cancer [43]. After further validation of proof-of-principle in in vivo models, therapeutic targeting of ATR with small molecule inhibitors is of interest to be tested in clinical studies for cervical cancer.

In cervical cancer, the consequence of HPV-mediated p53 inactivation is the loss of the G1/S cell cycle checkpoint, which is required for proper DNA repair [32,44]. Therefore, cervical cancer cells rely on residual cell cycle checkpoints, like the G2/M, in case of DNA damage [45]. This provides a rational to target kinases involved in the G2/M checkpoint. An important downstream regulator involved in this cell cycle checkpoint is the Wee1 kinase [46]. When Wee1 is inhibited, CDK1 is activated which forces replicating and damaged cells to enter mitosis [47]. These events ultimately lead to mitotic catastrophe and apoptosis [47,48]. Recent observation from our laboratory as well as others, is that Wee1 is also involved in S-phase progression, and that Wee1 inhibition leads to excessive replication fork collapse and a defect in HR repair [49,50]. So again –in analogy to ATR inhibitors- Wee1 inhibition induced both an increase of DNA damage, and inactivation of cell cycle checkpoints.

Our results and reports in literature demonstrated chemo- or radiosensitizing effects in p53-deficient tumors cells, including cervical cancer cells [51-53]. Currently, one phase I study (NCT01958658) is underway addressing safety and tolerability issues of a specific Wee1 inhibitor, MK-1775, when combined with chemoradiation in advanced cervical cancer patients. Results of this study are eagerly awaited, but will not yet answer the therapeutic index of Wee1 inhibition
in advanced cervical cancer patients. In p53-mutant ovarian cancer, Wee1 (Mk-1775) combined with carboplatin demonstrated interesting anti-cancer effects and patient tolerability [54].

Clinical application of DDR agents is still in the early phase of development. More understanding how to combine DDR agents with conventional cancer therapies, dosing schedules, patient toxicities, is needed. Most studies focus on the use of DDR inhibitors as monotherapy, while in most cancer therapies chemoradiation is standard care [43]. Understanding of molecular responses will contribute to our understanding on how to most efficiently exploit DDR targeting. Another aspect that obtained more awareness is the genetic background of cancers in patients. In the near future more tumors will be genetically profiled prior to start of therapy to personalize the therapeutic strategy.

Also modulation of other signaling pathways, like the immune pathway, might be interesting to improve therapy outcome in cervical cancer patients. One of the mechanisms for tumors to avoid immune responses is by blocking immune checkpoint receptors [55]. The recent encouraging clinical results of monoclonal antibodies targeting receptors like CTLA-4 (cytotoxic T-lymphocyte antigen-4) and PD-1 (programmed death-1), might also be of interest for cervical cancer [55-57]. Specifically, flow cytometric analysis revealed increased expression of PD-1 and PD-L1 (programmed death ligand-1) on cervical T cells in high-risk HPV-related cervical intraepithelial neoplasia (CIN) [58]. In addition, immunohistochemical analysis in stage IB and II cervical cancer patients demonstrated only in 19% of the cases PD-L1 positivity, while more than half of the cytotoxic T cells were positive for PD-1 [59]. These studies suggest that targeting of PD-1 might be an interesting target in cervical cancer. Although multiple clinical trials with immune checkpoint antibodies are ongoing, only three studies were specifically orientated on cervical cancer (NCT02635360, NCT02257528, NCT01693783). Two phase II clinical trials are investigating an anti-PD-1 antibody (NCT02635360, NCT02257528), while one phase II study is investigating intervention with an anti-CTLA-4 antibody (NCT01693783). In the context of targeting the DDR, an interesting thought is that blocking DNA repair, inactivation of cell cycle checkpoint or promoting replication fork collapse will likely increase the mutational load of cancer cells. If these additional mutations lead to the expression of neo-epitopes, this will likely further boost the activity of immune checkpoint inhibitors. Unfortunately, both in vitro and in vivo results backing this hypothesis are lacking.

**Conclusion**

In this thesis preclinical studies demonstrated the ability of specific DDR targeting agents to potentiate chemo-/radiotherapy in cervical cancer. Since DNA damage inducing therapies remain the main anti-cancer strategy, a better understanding of the differential functions of DDR components and development of improved DDR targeting therapeutics are needed. Together this will help to optimally exploit and implement DDR inhibition as novel strategy in cervical cancer therapy to further improve patient outcome.
References


