Breaking the DNA damage response
to improve cervical cancer treatment

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Abstract
Every year, cervical cancer affects ~500,000 women worldwide, and ~275,000 patients die of this disease. The addition of platin-based chemotherapy to primary radiotherapy has increased 5-year survival of advanced-stage cervical cancer patients, which is, however, still only 66%. One of the factors thought to contribute to treatment failure is the ability of tumor cells to repair chemoradiotherapy-induced DNA damage. Therefore, sensitization of tumor cells for chemoradiotherapy via inhibition of the DNA damage response (DDR) as a novel strategy to improve therapy effect, is currently studied pre-clinically as well as in the clinic. Almost invariably, cervical carcinogenesis involves infection with the human papillomavirus (HPV), which inactivates part of the DNA damage response. This HPV-mediated partial inactivation of the DDR presents therapeutic targeting of the residual DDR as an interesting approach to achieve chemoradio-sensitization for cervical cancer. How the DDR can be most efficiently targeted, however, remains unclear. The fact that cisplatin and radiotherapy activate multiple signaling axes within the DDR further complicates a rational choice of therapeutic targets within the DDR. In this review, we provide an overview of the current preclinical and clinical knowledge about targeting the DDR in cervical cancer.
2. Breaking the DNA damage response to improve cervical cancer treatment

Introduction
Cervical cancer is a major malignant disease among women worldwide and is estimated to account for 8% of the total cancer-related mortality [1]. Survival rates vary dramatically between cervical cancer patients, with 5-year survival rates of 66%, 40%, 42% and 22% for stages IIB, IIIA, IIIB and IVA respectively [2]. These numbers indicate that current treatment modalities, which for advanced cervical cancer consist primarily of radiotherapy combined with platinum-based chemotherapy, leave ample room for improvement.

Radiotherapy induces high levels of DNA damage in tumor cells with the aim to promote apoptosis. Addition of cisplatin to radiotherapy increases anti-tumor effects, and has been shown to improve therapy outcome in cervical cancer [3]. Tumor cells respond to DNA damage by activation of the DNA damage response (DDR). This complex signaling network coordinates a cell cycle arrest with the repair of DNA lesions [4]. As can be expected, DDR-proficient tumor cells repair therapy-induced DNA damage and are more resistant to therapy when compared to tumor cells in which DDR components are inactivated [5–11]. As combined chemoradiotherapy is standard of care for advanced cervical cancer, and DNA repair capacity apparently limits treatment responses, therapeutic targeting of the DDR is a potentially attractive strategy to improve cervical cancer treatment efficacy.

A second reason why targeting the DDR may be relevant for cervical cancer relates to its carcinogenesis, which involves persistent infection with the human papillomavirus (HPV) [12]. Oncogenic transformation by HPV is mediated by the viral oncogenes HPV-E6 and HPV-E7 [13]. The E7 oncoprotein exerts its effect by inactivation of the Retinoblastoma 1 (Rb1) tumor suppressor gene, while E6 promotes ubiquitin-mediated degradation of the p53 tumor suppressor [14,15]. Since p53 constitutes an integral component of the DDR, HPV-positive cervical cancer cells display a significantly impaired ability to activate cell cycle checkpoints and to induce apoptosis upon DNA damage [16]. Additionally, expression of HPV oncoproteins was reported to negatively impact on various DNA repair pathways [17–23]. For example, radiosensitization of head-and-neck squamous cancer cell lines was only observed in HPV-positive cells, as a result of impaired DNA repair [17]. Also, one of the preferred integration sites of HPV is in RAD51B, a well-known DNA repair gene [24,25]. Thus, HPV-positive cancer cells have impaired control of the cell cycle in the context of DNA damage, and show decreased capacity to repair DNA lesions. Targeting residual cell cycle checkpoint components or DNA repair factors may therefore be especially useful for cervical cancers, of which 99.7% is associated with HPV [12]. Conversely, studies in cervical cancer may be relevant to many other cancer types, in which p53 and/or Rb1 are inactivated through somatic mutations and are also treated with genotoxic therapies.

We here provide a literature overview of pre-clinical and clinical studies of DDR kinase inhibitors in the context of potentiating the response to chemoradiotherapy in cervical cancer.

Search strategy
Literature was searched (until October 2015), with the following search criteria in PubMed (including Medline), Embase and the Cochrane database: [cervical cancer] combined with [DDR OR DNA damage response] or [Caffeine], [Wortmannin], [KU55933], [KU60019], [CP466722], [CGK733], [Schisandrin B], [Compound 45], [NVP-BEZ235], [VE-821], [VE-822], [VX-970], [NU6027],
AZD6738, [AZ20], [ETP-46464], [LY294002], [NU7441], [KU0060648], [NU7062], [NVP-BKM120], [AZD7762], [UCN-01], [SCH900776], [LY2603618], [SAR-020106], [MK2 inhibitor] and variations thereof. Only studies published in the English language were included. The clinical trial register (www.clinicaltrials.gov) was used to evaluate completed and ongoing clinical trials with DDR kinases inhibitors.

Impact of HPV infection and somatic mutations on DNA damage signaling in cervical cancer

HPV

Fifteen carcinogenic HPV subtypes have been identified, which together account for almost all cervical cancer cases [26]. Of these subtypes, HPV16 and 18 are most carcinogenic and are associated with 70% of the cervical cancers [27]. Both HPV16 and HPV18, importantly, are classified as ‘high-risk HPV subtypes’. The differences between the various HPV subtypes in the context of cervical carcinogenesis are not fully understood at the molecular level. Although a comprehensive overview is lacking which compares the HPV oncogenes from the various HPV subtypes, it appears that sequences of ‘high-risk HPV subtypes’ share similarities, including in the C-terminal PDZ recognition domain within the E6 oncoprotein [28]. Phenotypically, however, squamous cell carcinomas are more associated with HPV16, while HPV18 is more prevalent in adenocarcinomas [29]. HPV viruses share that their integration results in over expression of the viral E6 and E7 oncoproteins [29,30].

The oncogenic effects of E6 and E7 have been elucidated in great detail [16]. The E7 oncoprotein binds the Rb1 protein [14]. Under physiological conditions Rb1 binds and thereby prevents the transactivation of proliferation-stimulating genes by the E2F transcription factors [31]. Upon phosphorylation of Rb1 by the cyclin-dependent kinases Cdk4 and Cdk6, the E2F transcription factors are released from Rb1 [32]. Since Cdk4/6 activity is under control of mitogens, Rb1 prevents uncontrolled cell proliferation in the absence of growth-promoting signals [33]. As a consequence of HPV infection, E7 expression by passes the need for extracellular proliferation signals and consequently promotes uncontrolled growth [33].

In response to aberrant proliferation, such as caused by Rb1 inactivation, the DNA damage response (DDR) is activated [34,35]. Activation of the DDR leads to sustained p53 activation and promotes apoptosis and cell cycle arrest [36]. In many solid cancers, the ensuing genetic pressure on TP53 leads to its loss or mutational inactivation [35,37]. HPV-mediated oncogenesis, however, is fundamentally different. In a one-step process, both the proliferation-regulatory machinery as well as the p53-pathways are deregulated, since p53 is being destabilized by the HPV-E6 oncoprotein [15]. As a consequence of p53 inactivation, either through mutation or persistent HPV infection, cells can no longer install a solid G1/S cell cycle checkpoint in response to DNA damage, as this checkpoint in large part depends on p53 [37]. Cells require such checkpoints to provide time for DNA repair. Consequently, p53-defective cells rely more on their remaining checkpoint capacity for survival after DNA damage [30]. In addition, expression of HPV oncogenes induces aberrant initiation of replication, which leads to imbalanced nucleotide pools and ensuing replication fork stalling [38]. Again, this characteristic of HPV-positive cancer cells will very likely result in increased dependence on pathways that respond to DNA lesions for cellular survival.
Somatic mutations

The combined inactivation of DNA damage checkpoints and proliferation control upon HPV-E6 and HPV-E7 expression result in enhanced genomic instability and facilitates the further loss and gain of tumor suppressors and oncogenes [39]. According to the COSMIC database (www.cancer.sanger.as.uk), recurrent somatic mutations in the cancer-related genes PIK3CA (9%), CDKN2A (7%), STK11 (5%) and HRAS (4%) have been observed in squamous cervical cancer [40], confirming earlier genetic analyses of cervical cancers [41–43]. Compared to other solid cancers, and in line with its HPV-mediated oncogenesis, mutations in TP53 (5%) are relatively infrequent in cervical cancers [40]. When mutations in 139 cancer-related genes were studied in a broader genotyping screen of cervical cancer patients, mutations in PIK3CA (31.3%) and PTEN loss (7.8%) appeared to occur in dependently of histological subtype [44]. Conversely, oncogenic KRAS mutations (17.5%) were only detected in adenocarcinomas, while EGFR mutations (7.5%) were solely detected in squamous cell carcinomas [44]. Recent whole exome sequencing of both cervical cancer and corresponding normal tissues of 115 patients revealed additional recurrent mutations in EP300 (16%), FBXW7 (15%), HLA-B (9%), MAPK1 (8%) and NFE2L2 (4%) in squamous cell carcinoma and mutations in ELF3 (13%) and CBFB (8%) in adenocarcinoma [24]. Regarding genetic alterations in DDR pathway components, none current aberrations were identified, besides the relatively low frequency mutation of TP53 [24,40].

Cell cycle checkpoints and DNA damage signaling in cervical cancer

The observation that p53-deficient cervical cancers depend strongly on their residual checkpoint signaling components [37,45], and the fact that these tumors do not display obvious mutations in other DNA damage checkpoint genes, underscore the potential of therapeutic targeting of DDR pathways in cervical cancer. Below, a brief overview of canonical DDR signaling pathways is provided, to illustrate key components and to highlight the interconnected nature of the multiple signaling axes in the DDR.

DNA damage signaling through the ATM and ATR pathways

DNA double strand breaks (DSBs) are considered highly cytotoxic, and are induced in large quantities by radiotherapy [46]. Upon DSB induction, the Mre11-Rad50-Nbs1 (MRN) complex recognizes and directly binds the DNA ends of the DSB [47]. Subsequently, ATM is activated via autophosphorylation at Ser-1981, enabling phosphorylation of many ATM substrates (Fig. 1) [48,49]. This results in the recruitment of DNA repair factors, cell cycle arrest and, when DNA damage is sustained, apoptosis induction [49]. Concerning the DSB-induced cell cycle arrest, Chk2 is of particular importance. Chk2 is activated through phosphorylation on Thr-68 by ATM [50]. In turn, Chk2 inactivates the Cdc25C phosphatase, which normally promotes cell cycle progression by activating cyclin-dependent kinases (CDKs) [51].

In parallel to the above-mentioned kinase-driven cellcycle arrest, ATM also phosphorylates p53, to promote it transcriptional activity, which drives expression of targets genes including the CDK inhibitor p21 [52,53]. Although this transcriptional response requires hours to be installed, the combined kinase- and transcription-driven in activation of CDKs provides a robust cell cycle arrest, allowing time for DNA breaks repair.
In addition to radiotherapy, patients with advanced cervical cancer receive concomitant platin-based chemotherapy. Cisplatin and other platinum-based agents create inter- and intra-strand DNA crosslinks, which interfere with DNA unwinding and consequently obstruct DNA replication, leading to cytotoxicity predominantly in S-phase. The single stranded DNA (ssDNA)
that becomes exposed at stalled replication forks, recruits the ssDNA-binding complex RPA and ATRIP, which in turn efficiently trigger activation of the ATR signaling axis within the DDR [54]. Subsequently, ATR activates numerous proteins involved in stabilizing and restarting stalled replication forks [54]. ATR also regulates cell cycle progression through its downstream effect or kinase Chk1, which inactivates the Cdc25 phosphatases, while activating p53, and thereby links replication stress detection to establishing a cell cycle arrest [55].

Although ATR is not directly activated in response to DNA DSBs, it also plays a critical role in repairing DNA DSBs. When DSBs are repaired through homologous recombination (HR) repair, DNA-ends require 5’-to-3’ resection to facilitate pairing with a homologous DNA template. This process creates long stretches of ssDNA, which activates ATR and actually facilitates HR repair by activating, among others, the BRCA1 repair factor [59].

Also in the absence of exogenous DNA damage, HPV-positive cells may increasingly depend on the ATR kinase for their survival. Expression of E6 and E7 was shown to deregulate the DNA replication machinery, which leads to imbalanced nucleotide pools and consequent stalling of replication forks and genome instability [38]. Although it has not been shown for cervical cancer cells specifically, increased replication stress results in increased signaling of the ATR and Chk1 kinases [56,57]. Importantly, tumor cells with increased levels of replication stress also depend for their survival on ATR and Chk1 kinases [58], which again underscores why these kinases may have value as therapeutic targets in HPV-positive cancers, including cervical cancers.

Concluding, the ATM/Chk2 axis is crucially involved in the cellular response to DNA DSBs, while the ATR/Chk1 axis appears a key regulator of the cellular response to replication stress. Since combined chemo-radiotherapy induces both these types of DNA lesions and activates multiple DDR pathways, a challenge is posed in selecting optimal DDR targets for improving chemoradiotherapy in cervical cancer.

p38-MAPK/MK2, a DDR pathway parallel to ATM/Chk2 and ATR/Chk1

The recent description of a third DDR signaling pathway further complicates the rational choice for DDR targets. In this specific pathway, p38-MAPK activates its downstream target MK2, and functions in parallel to the ATM and ATR pathways [60]. Under physiological conditions, the p38/MK2 axis controls general stress responses, under scored by its activation upon inflammation, oxidative stress and ultraviolet light [61]. MK2 heterodimerizes with p38α and resides in the nucleus as an un-phosphorylated complex [62,63]. In response to genotoxic stress, including ionizing radiation (IR) and cisplatin, p38α is phosphorylated by the MAP kinase-kinase-kinase (MAPKKK) TAO in an ATM-dependent manner, resulting in export of the p38/MK2 complex to the cytoplasm, where it phosphorylates the Cdc25 phosphatases to prevent S-phase progression and mitotic entry [62,64,65]. In parallel, p38 and MK2 control various mRNA stability factors to stabilize Gadd45α, leading to cell cycle arrest maintenance [66]. p38/MK2 appears to be especially important for the survival of p53-deficient cancer cells, since genetic or chemical interference with p38 MK2 selectively blocked the ability of p53-deficient cancer cells to arrest during S-phase and at the G2/M transition, and potentiated the effects of genotoxic chemotherapeutics in vitro and in vivo [60]. These observations provide a rational to target either p38 or MK2 in p53-defective cells, like cervical cancer cells, to increase the effects of genotoxic therapy.
HPV, cervical cancer and DNA repair pathways

Cancer cells that can efficiently repair treatment-induced DNA damage appear to be more resistant to treatment. This has been convincingly shown for various cancers, including glioblastomas, in which epigenetic silencing of the DNA repair gene *MGMT* leads to temozolomide sensitivity [5]. Analogously, inactivating mutations in the DNA repair genes *BRCA1* and *BRCA2* are accompanied with increased sensitivity to cisplatin and poly-(ADP-ribose) polymerase (PARP) inhibitors in breast and ovarian cancer [7,8].

Also studies in cervical cancer provided a link between DNA repair capacity and therapy sensitivity, both in vitro and in vivo [10,11]. For example, in advanced cervical cancer patients treated with chemoradiotherapy, high expression levels of the nucleotide excision repair (NER) protein ERCC1 were associated with a decreased progression-free survival and worse overall survival [11]. Conversely, impaired non-homologous end-joining (NHEJ) repair, due to low Ku70 levels, was related to increased survival of cervical cancer patients treated primarily with radiotherapy [10]. These observations suggest that therapeutic inhibition of DNA repair may be useful in potentiating chemoradiotherapy.

**DNA repair pathways associated with cervical cancer therapy**

DNA DSBs, as induced by radiotherapy, can be repaired by two fundamentally distinct pathways: non-homologous end-joining (NHEJ) and homologous recombination (HR). During NHEJ, the two DNA ends are ligated in a sequence-independent manner, in a process depending fully on DNA-dependent protein kinase (DNA-PK), in conjunction with its subunits Ku70/Ku80 [67]. Since the free ends of a DNA break may require processing of damaged nucleotides, NHEJ can lead to loss of genetic information and is therefore potentially mutagenic [68]. In contrast, HR is error-free as it uses for repair a homologous template, which is typically the sister chromatid. However, since sister chromatids only become available in S/G2 phase, HR is restricted to the subset of proliferating cells. To allow pairing with homologous DNA templates, DNA ends require extensive resection, leading to long stretches of ssDNA, which are ultimately used by the Rad51 recombinase to facilitate the process of homology search and recombination [69].

Repair of cisplatin-induced DNA damage is more complex when compared to DNA DSB repair, and is largely context-dependent. When DNA is quiescent (i.e. not being replicated or transcribed), DNA cross-links are primarily repaired by NER. When DNA is actively transcribed, intra-strand crosslinks interfere with ongoing transcription, which activates transcription-coupled repair (TCR). The repertoire of DNA repair mechanisms involved in crosslink repair changes drastically when DNA is replicated. When replication forks stall at cross-linked DNA, the Fanconi Anemia (FA) pathway is activated by local ubiquitination [70]. Subsequently, homologous recombination components are recruited together with translesion synthesis (TLS) polymerases to continue replication [71].

**HPV and DNA repair**

HPV infection has been linked to altered DNA repair through various mechanisms. Early on, expression of the E6 and E7 from HPV16 oncoproteins were shown to independently induce numerical and structural chromosomal aberrations, anaphase bridges as well as elevated levels of γ-H2AX, all pointing at defective DNA repair [72–74]. In line with these observations, expression
of HPV16-E6 resulted in sustained Chk1 phosphorylation upon carcinogen-induced DNA damage [75], and HPV-infected cells were shown to have an impaired capacity to repair UV-induced thymidine dimers [76].

Several reports have provided mechanistic evidence that HPV E6/E7 directly affect DNA repair. Hufbauer et al. showed that the sensing of DNA damage was impaired upon expression of HPV8-E6, as judged by decreased phosphorylation of ATM, ATR and Chk1 [19], which was in accordance to data from Wallace and co-workers [77]. Also, expression of E6 from HPV5 and HPV8 subtypes was shown to negatively impact on the expression of the BRCA1 and BRCA2 genes, and functionally impaired repair of DNA breaks through homologous recombination [21]. Also the repair of DNA breaks through non-homologous end-joining is influenced by HPV. Although HPV16-E6 was shown to impair NHEJ fidelity through p53-dependent as well as independent fashions, the direct NHEJ component that is affected remains elusive [23].

HPV E6 was also shown to block NER [78]. Specifically, E6 of HPV1, 8 and 16 was shown to directly bind and thereby impair the function of XRCC1 [22]. In parallel, the up-regulation of the NER repair protein DDB2 in response to UV was shown to be blocked by expression of HPV E6, likely reflecting the role of p53 in repair of DNA damage [79]. Also the MGMT (O\(^\text{6}\)-methylguanine DNA methyltransferase) DNA repair protein, which is involved in the protection of cells against alkylating agents, was shown to be regulated by HPV. Specifically, E6 in conjunction with the E6-associated ubiquitin ligase E6-AP was reported to directly bind MGMT, which leads to the proteasomal degradation of MGMT [80], which will likely affect repair of DNA lesions.

In conclusion, radiotherapy and cisplatin-based chemotherapy induce different types of DNA damage. While DNA DSBs are repaired using NHEJ or HR, repair of cisplatin-induced DNA lesions depends on the NER pathway, the FA pathway, TLS polymerases, as well as HR [81,82]. Importantly, expression of HPV oncogenes appears to negatively impact the ability to repair DNA, pointing at an increased sensitivity of HPV-positive cells, such as most cervical cancer cells, for genotoxic agents.

Therapeutic inactivation of DNA repair components may therefore be effective in cervical cancer cells for two reasons: firstly, as cervical cancer is commonly treated with DNA damaging agents, inactivation of DNA repair may increase the efficacy of such treatments. In this context, multiple DNA repair pathways may have to be targeted to efficiently sensitize cervical cancers for combined chemoradiotherapy as multiple different DNA lesions are inflicted, and hence multiple different DNA repair pathways are involved. Secondly, as HPV oncogenes negatively impact on DNA repair, HPV-positive cells may be more dependent on their remaining DNA repair capacity. Therapeutic targeting of these repair components may therefore be especially beneficial in cervical cancer, perhaps most effectively in combination with chemo-radiation.

Therapeutic targeting of the DDR in cervical cancer

The DDR in HPV-mediated cervical cancer is partly compromised, which may result in a stronger dependency of cancer cells on their residual DDR signaling axes. For this reason, DDR components, especially DDR kinases, have been coined as therapeutic targets in oncology. In this section, the reported results on targeting DDR kinases in cervical cancer are discussed for the most-studied components in this signaling network.
The notion that ATM inhibition could improve efficacy of genotoxic agents originates from observations in A–T patients, who harbor homozygous ATM mutations [83]. This autosomal-recessive hereditary disorder is characterized by a strong cancer predisposition [84]. Importantly, A–T patients also display extreme radiosensitivity, caused by partially defective DSB repair [85]. Further, this observation implied a potential benefit of therapeutic ATM targeting, when combined with radiotherapy.

Genome sequencing efforts, including those by The Cancer Genome Atlas (TCGA), identified frequent somatic mutation of ATM in several cancers, including lung and breast cancer [86]. Squamous cervical cancers, however, do not appear to harbor recurrent somatic ATM mutations (5.6%) according to the TCGA database [87,88]. Rather, ATM is abundantly expressed in cervical cancers, as was observed in 375 treatment-naive tumors using immunohistochemistry [89]. In addition, 52.4% of these tumors showed high levels of phosphorylated ATM [89], which correlated with a worse response to chemoradiotherapy and a shorter disease-specific survival [89]. So, targeting the ATM kinase in advanced stage cervical cancer might be an interesting therapeutic strategy increase sensitivity to chemoradiotherapy.

Even prior to the identification of the ATM gene, caffeine was found to have radio-sensitizing effects in vitro, including in cervical cancer cells and other p53-inactivated cells [90,91]. Much later, caffeine was found to inhibit ATM, although it also inhibited other kinases, including ATR (Table 1) [92]. Due to its non-specific effects and since the required caffeine concentration to inhibit ATM can not be achieved clinically, caffeine never exceeded beyond an in vitro laboratory tool. Also Wortmannin was shown to block ATM activity and induce radiosensitization [93]. However, like caffeine, Wortmannin is non-specific and targets a large variety of phosphoinositide 3-kinases [94].

The first selective ATM inhibitor, KU55933, was described in 2004 and allowed more specific investigation of the sensitizing effects of ATM inhibition [95]. Also, KU55933 treatment lead to in vitro sensitization to radiation and other DSB-generating drugs, including etoposide and doxorubicin, confirming the role for ATM in controlling cell cycle arrest with DNA repair [89,95]. KU55933 analogs with improved characteristics, KU60019 and KU59403, showed chemo- and radio-sensitizing effects both in vitro and in vivo [96,97]. When compared to KU55933, KU60019 showed ~10-fold higher potency, displayed similar target selectivity, was more hydrophilic, and did not induce major adverse effects in an orthotopic glioblastoma mouse xenograft model [96,98]. KU59403 showed even better pharmacokinetics, was even more potent and showed >1000-fold selectivity for ATM over other kinases [97]. In vivo experiments in mice, xenografted with colon or breast cancer cell lines, demonstrated clear sensitizing effects of intraperitoneal-administered KU59403 upon etoposide and irinotecan treatment [97]. Currently, however, no studies are available on the use of KU60019 or KU59403 in cervical cancer models. Although preclinical evidence points at ATM as a promising target for radio/chemo-sensitization, none of the ATM inhibitors have entered clinical studies. This might be due to unfavorable pharmacokinetic properties or anticipated adverse effects of ATM inhibition, with the clinical features of cerebellar dysfunction, immuno-incompetence and malignancies of A–T patients in mind [86]. For this reason, reversible ATM inhibitors would be preferable to allow transient ATM inactivation. The observation that the ATM inhibitor CP466722 is reversible, while it still potently radiosensitized cervical cancer cells [99], may be an important step toward further clinical investigation.
CDK2, cyclin-dependent kinase 2; IL-6, interleukin-6; PI3K, phosphoinositide 3-kinases; PIKK, phosphoinositide 3-kinase-related kinase; RSK1, ribosomal protein S6 kinase polypeptide 1; TNFa, tumor necrosis factor alpha; VEGFR2, vascular endothelial growth factor receptor 2.

ATR
ATR has significant sequence homology with ATM, and both kinases share the phosphorylation consensus motif Ser/Thr-Gln ([S/T]-Q) [49]. Unlike ATM however, ATR is required for embryonic development and cell proliferation [100]. This essential function of ATR for a long time disqualified ATR as a therapeutic target. However, several ATR inhibitors have recently been developed, some of which have entered clinical trials.

The ATR inhibitor Schisandrin B increased cytotoxicity in fibroblasts and lung cancer cells induced by ultraviolet (UV) radiation, but not ionizing radiation [101]. The 'Vertex compound 4508760-L-bw-Wieringa' is the first irreversible ATR inhibitor, and reduces p38β phosphorylation, inhibits MK5, and inhibits MDR1 [102]. Table 1 lists the compounds targeting key DDR kinases in preclinical and clinical studies.

### Table 1: Overview of chemotherapeutic compounds targeting key DDR kinases in preclinical and clinical studies

<table>
<thead>
<tr>
<th>Drug target</th>
<th>Compound</th>
<th>IC50</th>
<th>Target specificity</th>
<th>Drug development phase</th>
<th>ClinicalTrials.gov identifier (NCT number)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATM</td>
<td>Caffeine</td>
<td>200 nM</td>
<td>Inhibits multiple PI3K members</td>
<td>preclinical phase I/II</td>
<td>n/a</td>
</tr>
<tr>
<td></td>
<td>Wortmannin</td>
<td>150 nM</td>
<td>Inhibits multiple PI3K members</td>
<td>preclinical phase I/II</td>
<td>n/a</td>
</tr>
<tr>
<td></td>
<td>KU55933</td>
<td>12.9 nM</td>
<td>First selective ATM inhibitor, 100-fold selective for ATM compared to other PIKK enzymes</td>
<td>preclinical phase I/II</td>
<td>n/a</td>
</tr>
<tr>
<td></td>
<td>KU60019</td>
<td>6.3 nM</td>
<td>Analogue of KU55933</td>
<td>preclinical phase I/II</td>
<td>n/a</td>
</tr>
<tr>
<td></td>
<td>KU55933</td>
<td>3 nM</td>
<td>Analogue of KU55933</td>
<td>preclinical phase I/II</td>
<td>n/a</td>
</tr>
<tr>
<td></td>
<td>CFP468722</td>
<td>6.3 nM</td>
<td>Selective and reversible ATM inhibitor</td>
<td>preclinical phase I/II</td>
<td>n/a</td>
</tr>
<tr>
<td>ATR</td>
<td>Schisandrin B</td>
<td>7.25 μM</td>
<td>Moderately selective ATR inhibitor</td>
<td>preclinical phase I/II</td>
<td>n/a</td>
</tr>
<tr>
<td></td>
<td>Vertex compound 4508760-L-bw-Wieringa</td>
<td>0.42 μM</td>
<td>Inhibits multiple PI3K members</td>
<td>preclinical phase I/II</td>
<td>01656101, 01717898, 00620094, 01453559, 01343498, 01482156, 01634061, 01285466, 01471847, 01756118, 01337765, 01290406, 01028913, 01495247, 01538104, 01693971, 01288902, 01166376, 01248494, 01658436, 01300962, 02430063</td>
</tr>
<tr>
<td>DNA-PK</td>
<td>ETP-46464</td>
<td>25 nM</td>
<td>First reversible ATR inhibitor</td>
<td>preclinical phase I/II</td>
<td>02264479, 02222393, 01955668</td>
</tr>
<tr>
<td></td>
<td>NU6027</td>
<td>6.7 μM</td>
<td>Inhibits also CDK2</td>
<td>preclinical phase I/II</td>
<td>n/a</td>
</tr>
<tr>
<td></td>
<td>AZD7738</td>
<td>1 nM</td>
<td>Phase I</td>
<td>preclinical phase I/II</td>
<td>00477736, 00437203, 0039403, 00212194, 00772189, 0045747, 0034550, 00347242, 0042861, 0001444, 00072287, 00116838, 0003329, 0003888, 00040095, 00043464, 0045175, 0040513, 00082017, 00085955</td>
</tr>
<tr>
<td></td>
<td>AZD2225</td>
<td>5 μM</td>
<td>First selective ATR inhibitor</td>
<td>preclinical phase I/II</td>
<td>n/a</td>
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<tr>
<td></td>
<td>VE322/VE970</td>
<td>19 nM</td>
<td>Analogue of VE281</td>
<td>preclinical phase I/II</td>
<td>n/a</td>
</tr>
<tr>
<td>Chk1</td>
<td>NU-7441</td>
<td>14 nM</td>
<td>Inhibits MDR1 also</td>
<td>preclinical phase I/II</td>
<td>01363625, 00038777, 00031681, 00301038, 00084263, 00038403, 00012194, 00772189, 0045747, 0034550, 00347242, 0042861, 0001444, 00072287, 00116838, 0003329, 0003888, 00040095, 00043464, 0045175, 0040513, 00082017, 00085955</td>
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<tr>
<td></td>
<td>NU-7026</td>
<td>230 nM</td>
<td>Phase I</td>
<td>preclinical phase I/II</td>
<td>n/a</td>
</tr>
<tr>
<td></td>
<td>CC-115</td>
<td>13 nM</td>
<td>Inhibits mTOR</td>
<td>preclinical phase I/II</td>
<td>00477736, 00437203, 0039403, 00212194, 00772189, 0045747, 0034550, 00347242, 0042861, 0001444, 00072287, 00116838, 0003329, 0003888, 00040095, 00043464, 0045175, 0040513, 00082017, 00085955</td>
</tr>
<tr>
<td></td>
<td>KL-6648</td>
<td>19 nM</td>
<td>Inhibits multiple PI3K members</td>
<td>preclinical phase I/II</td>
<td>n/a</td>
</tr>
<tr>
<td></td>
<td>UCN-01</td>
<td>11 nM</td>
<td>Non-selective Chk1 inhibitor</td>
<td>preclinical phase I/II</td>
<td>n/a</td>
</tr>
<tr>
<td></td>
<td>AZD7762</td>
<td>5 nM</td>
<td>Inhibits Chk2</td>
<td>phase I</td>
<td>0037670, 00413688, 00473616</td>
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<tr>
<td></td>
<td>PF-0047736</td>
<td>0.49 μM</td>
<td>Inhibits Chk2</td>
<td>phase I</td>
<td>0047203</td>
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<td></td>
<td>XL-844</td>
<td>2.2 μM</td>
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<td>00475817, 00234481</td>
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<td></td>
<td>SCH900776</td>
<td>3 nM</td>
<td>Inhibits Chk1</td>
<td>phase I</td>
<td>01670596, 0097517, 00779584, 01521299</td>
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<tr>
<td></td>
<td>MK-8776</td>
<td>7 nM</td>
<td>Phase I</td>
<td>preclinical phase I/II</td>
<td>n/a</td>
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<tr>
<td></td>
<td>LY2006388</td>
<td>&lt;1 nM</td>
<td>Inhibits Chk2 and RSK1</td>
<td>phase I</td>
<td>01139775, 01266588, 00839332, 00415636, 01341497, 00888588</td>
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<td>GDC-0425</td>
<td>n/a</td>
<td>Phase I</td>
<td>preclinical phase I/II</td>
<td>n/a</td>
</tr>
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<td>n/a</td>
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<td>7 nM</td>
<td>Phase I</td>
<td>preclinical phase I/II</td>
<td>n/a</td>
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<tr>
<td>p38/MK2</td>
<td>PF-3644022</td>
<td>5.2 nM</td>
<td>Inhibits TNFα and IL-6 production, inhibits MK5</td>
<td>preclinical phase I/II</td>
<td>02322383, 02364206, 01663857, 01393900</td>
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<tr>
<td>MK2 inhibitor III</td>
<td>8.5 nM</td>
<td>Inhibits MK5</td>
<td>preclinical phase I/II</td>
<td>n/a</td>
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45' showed more potency and appears selective for ATR [102]. Lung cancer cells treated with compound 45 demonstrated radiosensitivity, and displayed a 7-fold higher sensitivity for cisplatin [102]. As expected, the chemosensitizing effects of compound 45 were much more pronounced than its radiosensitizing effects [102]. Since ATR primarily functions in resolving replication stress, most studies investigated ATR inhibition in combination with agents that perturb DNA replication. However, the fact that ATR also functions in other DNA repair processes, including DSB repair, may explain why transient ATR blocking by ETP-46464 showed increased radiosensitizing effects in breast cancer cells when compared to ATM inhibition [103]. The reversibility of ETP-46464 combined with its radio- and chemo-sensitizing effects makes this compound a promising candidate for clinical testing. Besides ETP-46464, also NVP-BEZ235 was identified as an ATR-targeting compound [104], although NVP-BEZ235 is known to also inhibit phosphatidylinositol-3-kinase and the mammalian target of rapamycin (mTOR) [105]. Also NU6027 non-specifically inhibits ATR, as it also potently blocks cyclin-dependent kinase 2 (Cdk2) [106,107]. Nevertheless, NU6027 potentiated ionizing radiation and various chemotherapeutic compounds in breast and ovarian cancer cell lines in vitro, including cisplatin [107].

To further assess the in vivo potential of ATR inhibition as chemo- and/or radiosensitizer, more hydrophilic ATR compounds were required. AZ20 was the first ATR inhibit or to show beneficial anti-cancer effects in vivo [108]. Already without DNA damage-inducing agents, daily oral administration of AZ20 to mice bearing a colorectal adenocarcinoma xenograft showed 77% tumor growth reduction on day 21 of treatment [108]. Also the ATR inhibitor VE-822 (also called VX-970) is suitable for in vivo use. Whereas in vitro work with its analog VE-821 enhanced radiosensitivity in cervical cancer cells [109], in vivo studies with VE-822 showed increased sensitivity to ionizing radiation and gemcitabine in mice xenografts with TP53-mutant pancreatic cancer cells [110]. Interestingly for cervical cancer, the addition of VE-822 to combined chemoradiotherapy resulted in a 2.5 to 3-fold delay before xenograft tumors reached 400 mm³ [110]. VE-822 is currently tested in phase I and II (Table 1). Other ongoing clinical studies analyze the effects of ATR inhibitor AZD6738 (NCT02264678, NCT02223923, NCT01955668): one phase I trial has completed, and two studies are ongoing, in which ATR inhibition is combined with chemotherapy or with radiotherapy. To date, results of these studies are not publically available.

Concluding, in vitro approaches using ATR inhibition resulted in both radio- and chemosensitizing effects. This is of clear importance in the context of cervical cancer, and studies with recently developed ATR inhibitors, combined with platin-based chemoradiotherapy are eagerly awaited.

**DNA-PK**

In contrast to ATM and ATR, which play key roles in controlling the cell cycle in response to DNA damage, DNA-PK is mainly involved in repair of DNA. The majority of cellular DSBs is repaired via NHEJ, and the catalytic subunit of DNA-PK (DNA-PKcs) is the key regulator of NHEJ repair [111]. As radiotherapy is still the backbone in advanced-stage cervical cancer treatment and induces high numbers of DSBs, DNA-PKcs inhibition could be an attractive target to potentiate radiotherapy. Indeed, genetic DNA-PK in activation was associated with increased sensitivity for DSBs-inducing treatments, including ionizing radiation [112]. Conversely, preclinical and clinical studies showed
that increased radioresistance in cervical cancer cells was associated with increased DNA-PKcs activity or up-regulation of other NHEJ components [113]. The first identified DNA-PK inhibitors, including Wortmannin and LY294002, are neither specific nor suitable for clinical use, due to severe toxicity [114]. Two derivatives of LY294002, NU7441 and NU7026, more selectively inhibit DNA-PKcs [115,116], and enhanced tumor cell killing upon ionizing radiation and DSB-inducing chemotherapeutic agents [116–118]. In HeLa cells, DNA-PKcs inactivation via siRNA or NU7026 also resulted in radiosensitizing effects [118–120]. Using NU7441, a twofold etoposide-induced tumor reduction was observed in mice, harboring colon cancer xenografts [117]. Nonetheless, clinical studies of either NU7441 or NU7026 are currently lacking. KU-0060648, an NU7441 analogue with improved water-solubility and increased potency enhanced the cytotoxicity of DNA DSBs-inducing treatments both in vitro and in vivo, but also for these compound clinical trials are currently lacking [121]. Although not solely targeting DNA-PK, the dual DNA-PK and mTOR inhibitor CC-115 is in phase I of clinical testing in advanced solid and hematologic malignancies (NCT01353625).

Whereas targeting ATM/ATR affects both cell cycle control and DNA repair, DNA-PK inhibition acts primarily on NHEJ DNA repair capacity. Not surprisingly, blocking DNA-PKcs is primarily cytotoxic when combined with DNA DSB-inducing agents. Sensitivity of cervical cancer cells for NHEJ repair inhibition may be explained by the impairment of HR repair by the HPV E6 and E7 oncoproteins [21,22]. Specifically, E6 and E7 directly antagonize the critical HR regulator BRCA1 in cervical cancer cells [22]. Additionally, expression of E6 produced by HPV5 and HPV8 results in decreased levels of BRCA1 [21] and BRCA2 [21], which was accompanied by increased levels of DNA DSBs and impaired HR repair [21]. Another way by which DSB repair through HR is decreased in cervical cancer is though hypoxia [123]. Hypoxic tumor areas are as are observed in most locally-advanced cervical cancers (‘60%) [124]. A major therapeutic problem of cancer cells in hypoxic areas is their increased therapy resistance [125]. Interestingly, hypoxic cancer cells, including those in cervical cancer, feature decreased gene expression of RAD51 which is an important HR regulator [123]. This hypoxia-induced inactivation of RAD51 is maintained for prolonged time periods, even when normoxia is restored [123].

Combined, the reduced HR repair capacity, as caused by HPV oncoproteins or hypoxia, may create a window of treatment opportunity for cervical cancer cells in which NHEJ repair can be inhibited using DNA-PK targeting. The fact that p53-deficient cells appear increasingly sensitive to DNA-PK inhibition, again argues that this approach maybe useful for cervical cancer [117]. Whether DNA-PK targeting is valuable for cervical cancer remains unclear, since it has not been tested in the context of combined chemoradiotherapy in cervical cancer models.

Chk1/2

The Chk2 and Chk1 kinases are downstream of ATM and ATR respectively (Fig. 1). DSBs lead to direct phosphorylation at Thr-68 of Chk2 by ATM [126], whereas ATR phosphorylates Chk1 on Ser-345 and Ser-317 in response to replication stress [127]. Chk2 and Chk1 activation promotes cell cycle arrest, which is mainly accomplished by inhibition of the Cdc25 phosphatases. Both Chk1 and Chk2 can phosphorylate and thereby catalytically inhibit Cdc25C [51,128], making them control transition in the G2/M cell cycle checkpoint. The reliance on residual S and G2/M
checkpoint capacity in p53-deficient cancers provided a rational to investigate therapy-sensitizing effects of Chk1 and Chk2 inhibitors in p53-down regulated cancers [37]. In addition to its role in cell cycle regulation in situations of DNA damage, Chk1 was recently also shown to directly control the repair of DNA breaks [129]. Specifically, Chk1 was shown to directly interact with the Rad51 recombinase and to mediate phosphorylation of Rad51 [129]. In the context of cervical cancer, elevated replication stress due to E6/E7 expression may render cervical cancer cells increasingly dependent on homologous recombination pathways, and hence, Chk1 activity.

Concerning Chk1, in vitro data suggested that not all p53-deficient cancers might benefit from combined Chk1 inhibition with DNA damaging agents [130]. For cervical cancer, several papers demonstrated differential effects of combining Chk1 depletion with chemo- and/or radiotherapy [131–133]. Surprisingly, in HeLa cells Chk1 depletion potentiated only ionizing radiation, not cisplatin [131,132,134]. Others have shown additional growth delay in HeLa spheroids when chemoradiotherapy was combined with Chk1 targeting [133]. Most of these data are based on observations with the Chk1 inhibitor UCN-01 in HeLa cells. To reliably evaluate these results, however, these studies need to be confirmed using broader panels of cervical cancer cell lines, optimized treatment protocols, and more potent and selective Chk1-inhibitors. UCN-01 was the first available Chk1-inhibitor and sensitized p53-deficient cells in vitro for various genotoxic agents [135]. However, many other kinases are also affected by UCN-01, including MK2 and Chk2. Despite its non-selectivity, disruption of S and G₂ checkpoints appeared Chk1-dependent in p53-deficient cells [136]. Currently, phase I and II studies investigate the potential of UCN-01 either as single agent or combined with genotoxic chemotherapeutics in various cancer types [137]. Information for 4 out of 7 completed phase II studies is publicly available [138–141] and demonstrated a lack of significant anti-tumor effects of UCN-01 as single agent in metastatic melanoma (NCT00072189) or renal cell carcinoma (NCT00030888) [140,141]. Combination with topoisomerase I inhibitors, in respectively metastatic triple negative breast cancer and advanced ovarian cancer patients (NCT00072267), also did not show additional tumor-reducing effects [138,139]. Based on immunohistochemical stainings and assessment of UCN-01 binding to the plasma protein α1-acid glycoprotein (AAG), inefficient target engagement due to unfavorable pharmacokinetics of UCN-01 was thought to underlie these negative results [138,142]. Meanwhile, improved Chk1-inhibitors have been tested in phase I studies, including AZD7762. Although not studied in cervical cancer, AZD7762 mimics UCN-01 in its radiosensitization of p53-deficient tumor cells in vitro and in vivo [143]. These effects were explained by an impaired G₂/M checkpoint and HR inhibition [144]. In 2011, AZD7762 was discontinued from phase I trials (NCT00937664, NCT00413686, NCT00413686, NCT00473616), when two papers reported serious cardiac toxicity in patients [145,146]. Two other Chk1-inhibitors, PF-00477736 and XL-844, were also discontinued in phase I. The LY2603618 compound is currently the most promising Chk1-inhibitor as it efficiently mimics a Chk1-depletion phenotype in vitro [147]. In combination with to poiserase II inhibition, cervical cancer cells treated with LY2603618 showed an abrogated G₂/M checkpoint and impaired mitotic spindle formation[147]. In vivo studies with this compound are therefore warranted.

Taken together, the majority of studies with Chk1 inhibitors examined Chk1-inhibitors as a single agent or in combination with DNA damaging agents in vitro or in vivo. Strikingly, most studies in cervical cancer cells point at increased cytotoxicity only when Chk1 inhibition is combined
with ionizing irradiation, but not cisplatin. However, as these studies were mostly conducted with non-selective Chk1 compounds, more preclinical work with potent Chk1-inhibitors (LY2603618, MK-8776, LY2606368, or SAR-020106) is necessary. Such studies are needed to address whether Chk1 inhibition indeed has chemo- and/or radiosensitizing effects in cervical cancer.

**MK2**

Besides the Chk1/2 kinases, the parallel p38-MAPK/MK2 signaling pathway controls cell cycle progression in p53-deficient cells upon DNA damage [60]. Similar to the Chk1/2 kinases, MK2 inactivates Cdc25B/C via phosphorylation on Ser-323 and Ser-216 to halt cell cycle progression [66]. In vitro, MK2 depletion in HeLa cells resulted in cytotoxicity when combined with different genotoxic agents, which suggest MK2 as a possible target for radio/chemosensitization in cervical cancer [60,148]. However, the development of potent and specific ATP-competitive MK2 inhibitors is complicated. A low biochemical efficiency index limits inhibitor potency, while a small ATP pocket of MK2 restricts conformational changes to compounds to increase inhibitor selectivity [149]. Potent and selective ATP-competitive MK2 inhibitors are awaited to elucidate clinical relevance of targeting MK2 in cervical cancer.

**Conclusions**

In recent years the development of more selective inhibitors contributed to our understanding of the DDR wiring, and has revealed the therapeutic potential of DDR targeting. Many preclinical studies have shown improved cytotoxicity in different cancer types through chemical or genetic deregulation of the DDR. With the notion that the DDR serves as an anti-cancer barrier, it is not surprising that parts of the DDR are frequently compromised in cancers to facilitate tumor cell proliferation and survival [34,150]. In the case of cervical cancer, the HPV E6 oncoprotein mediated p53 down-regulation. With the DDR being partially inactivated, tumor cells are increasingly dependent on residual pathways in order to cope with certain types of DNA damage. In parallel, the expression of multiple oncogenes, including HPV E6/ E7, leads to increased levels of replication stress, and ensuing dependence on replication checkpoint kinases for cellular survival. These pathways are attractive to exploit therapeutically, although investigation of the DDR status in individual tumors is required to provide a rational for selecting eligible patient groups.

Based on *in vitro* and *in vivo* pre-clinical evidence, the response to chemo/radiotherapy can be increased using ATM, ATR, DNA-PK, Chk1 or MK2 inhibitors, although not for all these kinases suitable inhibitors are available and clinical data to support these claims, especially in the context of cervical cancer are currently lacking. An important point in this respect is that preclinical studies with chemical DDR inhibitors invariably assess these drugs either alone or combined with either ionizing radiation or a single chemotherapeutic agent. In clinical practice, however, most regimens combine multiple genotoxic agents in the advanced-stage disease setting. This also accounts for cervical cancer, in which patients receive combined platinum-based chemoradiotherapy.

In addition, results of phase I and II studies with DDR compounds (Table 1) emphasize major challenges ahead for clinical application of DDR inhibitors. Not only issues concerning bioavailability of drugs were reported (e.g. UCN-01), also patient safety is a concern. Cardiac
toxicity of AZD7762 was observed in the only completed phase I study [146]. In addition, two other phase I trials with AZD7762 (NCT00937664 and NCT00473616) were terminated prematurely after the risk–benefit profile was reviewed [145]. As a result, further clinical development of AZD7762 was discontinued. Other adverse effects of DDR inhibition remain largely unaddressed, such as the potential induction of secondary tumors.

Improving DDR targeting compounds is necessary to reduce off-target effects and to allow further translation of in vitro findings. In addition, more preclinical data in cervical cancer models are required to determine the most promising DDR target. Specifically, studies using panels of multiple primary cervical cancer cell lines in which DDR agents are combined with chemo-/radiotherapy are needed to select most promising targets. Moreover, advanced ex vivo or in vivo cervical cancer models, like patient-derived xenograft (PDX) mouse models, could be used to identify effective dosing schedules and combinations of treatment modalities, prior to clinical phase trials. Only when such experimental set-ups will be applied, full potential of DDR inhibition as treatment intervention in cervical cancer can be achieved.

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