Modulation of the TRAIL apoptotic pathway to optimize chemoradiation in preclinical models of cervical cancer
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ABSTRACT

Standard treatment of locally advanced cervical cancer currently consists of concurrent chemoradiation, leading to a 5-year disease-free survival of 66-79%, indicating that there is still ample room for improvement. Characteristic of cervical cancer is the presence of high risk (HR) human papillomavirus (HPV) DNA in more than 99% of these tumors. When the HR HPV genome integrates in the host genome, oncogenic E6 and E7 proteins become constitutively expressed. These oncogenes are also active earlier in the infection cycle and hence are available as therapeutic targets at the preneoplastic stages as well. E7 plays an important role in the early stage of carcinogenesis by stimulating proliferation. HR HPV E6-induced proteasomal degradation of p53 hampers p53 functionality in cell cycle arrest and apoptosis. As p53 plays a key role in the intrinsic apoptotic pathway, current chemoradiation cannot optimally activate this pathway. In this review, we focus on targeted anticancer drugs to eliminate the consequences of HR HPV E6 and E7 activity. Strategies for direct and indirect targeting of HR HPV E6 and E7, including RNA interference, small molecules, proteasome inhibitors, and histone deacetylase inhibitors, are described. In addition, the extrinsic apoptotic pathway as possible alternative therapeutic target for apoptosis induction is reviewed. The rational for implementing recombinant human TRAIL and death receptor agonists and the latest developments on combining these drugs with standard treatment in preclinical settings as well as clinical trials are discussed.
INTRODUCTION

Cervical cancer ranks third in cancer mortality in women worldwide, with the highest mortality rates in developing countries (1). Human papillomavirus (HPV) is the etiological factor in cervical carcinogenesis (2-4). Characteristic of cervical cancer is the presence of HPV DNA that is detected in more than 99% of these tumors and in up to 94% of women with high grade cervical intraepithelial neoplasias (CIN) (5-8). HPV 16 and 18 are the most frequent high risk (HR) HPV types and responsible for about 70% (HPV 16 54% and 18 17%) of the invasive cervical cancers (2-4).

Standard treatment of locally advanced disease consists of radiotherapy with cisplatin-based chemotherapy. Five randomized clinical trials showed the superiority of chemoradiation above radiation alone in Fédération Internationale de Gynécologie et d’Obstétrique (FIGO) stages IB to IVA. Since then chemoradiation is regarded as the standard of care (9). A recent randomized trial indicated that addition of gemcitabine and adjuvant cisplatin to cisplatin-based chemoradiation may lead to further improvement of current therapy efficacy (10). Overall survival following standard chemoradiation of 66-79% at 5 years shows, however, that there is still ample room for improvement (9).

Further enhancement of chemoradiation efficacy by either increasing radiotherapy or chemotherapy doses may be possible. Yet, this will be limited given the small therapeutic window for radiotherapy and chemotherapy. Alternatives by introducing new targeted drugs with toxicity profiles that only partly overlap standard treatment modalities might offer new opportunities to increase antitumor efficacy. This review focuses on a rational combination of irradiation and cisplatin with new drugs that target molecular pathways specifically dysregulated in cervical cancer. First, we will briefly summarize HPV-mediated changes in molecular pathways in cervical cancer, the effects of irradiation and cisplatin on molecular pathways in cervical cancer in general, and the activation of apoptotic pathways by chemoradiation. Thereafter we will focus on clinically potential modifiers of HPV-positive cervical cancers with an emphasis on drugs affecting the Tumor Necrosis Factor (TNF)-related apoptosis inducing ligand (TRAIL) apoptotic pathway.

HPV AND P53 INACTIVATION IN CERVICAL CANCER

HPVs are double-stranded DNA viruses that initially infect undifferentiated, basal keratinocytes. HPVs reprogram keratinocytes into a DNA replication competent state in which the HPV encoded E6 and E7 proteins play an important role. Higher expression levels of E6 and E7 are related to disease progression (11, 12). The increase is the consequence of a switch of the episomal HR HPVs into the integrated form in the genome of the host cells (13). Integration of HPV16 and 18 occurs early in cervical oncogenesis with 41% to 83% DNA integration rates found in low grade lesions (14, 15). However, integration does not automatically lead to high levels of the viral E6 and E7 proteins (14, 16). It facilitates though the next step for E6/E7 to escape from
immune response. Episomal E2 protein normally inhibits translation of integrated E6 and E7. When E2 is disrupted by host antiviral response, E6 and E7 overexpression occurs, leading to genomic instability and cancer development (15, 17, 18), yet more intracellular changes are still needed before HR HPV infection of cervical cells leads to malignancy (see Fig. 1A).

HR HPV encoded E7 protein forms complexes with hypophosphorylated retinoblastoma protein (pRb), which inhibits pRb function and induces E2F in infected cervical cells (19). In addition to its best known target pRb, E7 proteins directly target other cell cycle regulators, such as cyclin/cyclin-dependent kinase 2 (Cdk2) complexes and their natural inhibitors p21 and p27 (20, 21). Especially Cdk2 deregulation by E7 is crucial in preparing a fertile ground for cervical carcinogenesis since aberrant Cdk2 expression promotes chromosomal instability, such as centrosome multiplication, abnormal mitoses and aneuploidy (22). In summary E7 proteins hold the cell cycle in tight control to keep the infected cells in a constitutive replication state. Hence new therapeutic strategies to eliminate E7-mediated cell cycle dysregulation may be achieved by direct targeting E7 or via its downstream targets.

In addition, E2F induces p14ARF, an inhibitor of murine double minute 2 (Mdm2)-mediated p53 degradation (23, 24). Stabilization of p53 can trigger apoptosis. Therefore, the HR HPV encoded E6 protein is required to counteract this undesirable effect to permit proliferation of HPV infected cervical cells (2). E6 leads to degradation of a large number of proteins, including p53, B-cell lymphoma-2 (Bcl-2) homologous antagonist killer (Bak), c-Myc, p300/CREB binding protein (CBP), interferon regulatory factor 3 (IRF-3) and PDZ-proteins. P53 is the first E6 target identified and probably still the most important one. In normal cells, p53 is degraded by the ubiquitin proteasome pathway, mediated by Mdm2. However in case of HR HPV infection, the Mdm2 pathway in these infected cells is inactivated by E7 and, therefore, p53 turnover entirely depends on E6.

HPV16 and 18 E6 proteins consist of 158 amino acids and two zinc binding domains (25). Due to lack of enzymatic activity, E6 protein needs to bind to other cellular proteins to exert its function. The first protein reported to interact with E6 was an E3 ubiquitin ligase E3A (UBE3A) also called E6-associated protein (E6AP) (26, 27). E6AP is a 860 amino acid large protein with a C-terminal region, recognized as the homologous to E6AP carboxyl-terminus (HECT) (28). The E6 binding region is localized to a central 18 amino acid α-helical epitope (29-31). E6AP can be utilized by E6 to target proteins that are normally not recognized by E6AP, such as p53, as well as to accelerate protein turnover (32, 33). Following interaction of E6AP and E6, E6AP half-life decreases due to self-ubiquitination and degradation (34). As a consequence, p53 half-life is reduced from several hours to 20 minutes (35) (Fig. 1A). A recent study reported that in E6AP knock-out mice a complete loss of the oncogenic potential of E6 was observed, indicating that E6AP is absolutely required for E6 to cause cervical cancer (36). The majority of cervical cancers are still wild-type p53 probably due to HPV E6-mediated inactivation of p53, while only a very small subset of cervical cancers is HPV negative and expresses mutant p53 (37-42).
Therefore, strategies to interfere with E6-induced p53 degradation in order to restore p53 functionality offer an interesting therapeutic option for cervical cancer.

A recent study demonstrated that as a consequence of HPV16 E6-induced p53 inactivation, two kinases, namely serum and glucocorticoid-regulated kinase 2 (SGK2) and p21-activated kinase 3 (PAK3), played a new crucial role in cells proliferation and viability. When the cells lost both p53 and either SKG2 or PAK3, cell death occurred. The kinases did not show similar function in non-infected human foreskin keratinocytes expressing wild-type p53 (43). Hence, a novel therapeutic strategy may be based on the synthetic lethal interaction between loss of p53 and drugs targeting SGK2 or PAK3 in HPV-positive cervical cancer.

IRRADIATION AND CISPLATIN INDUCE CELLULAR RESPONSE IN CERVICAL CANCER

Cellular damage induced by irradiation is initiated by damage to the cell membrane or as clustered damage within the nuclear DNA. Thereafter, DNA damage results in transcription of numerous cellular proteins, such as p53, c-MYC, c-FOS/ c-Jun and nuclear factor-kappa B (NF-κB). These proteins are in turn responsible for the transcription of cytokine-, growth factor-, and cell cycle-related genes (44).

The role of wild-type p53 in response to chemotherapy and radiation was comprehensively reviewed by Lu and El-Deiry (45). In vivo wild-type p53 is an essential factor for radiosensitivity in a variety of normal tissues (46-49). Moreover, an in vivo study showed that p53 null mice are resistant to radiotherapy. In both E6 and E7 transgenic mice response of the epidermis to radiation is diminished. It has been suggested that p53-dependent as well as p53-independent pathways are involved, since the effects in E6 and E7 transgenic mice are stronger than in p53-knockout mice (50). Wild-type p53 is considered to function as a checkpoint protein coordinating DNA repair with cell cycle progression or apoptosis. Following irradiation, the wild-type p53 level in murine colon, thymus and spleen increases and leads to cellular G1 arrest. The role of wild-type p53 in G2 arrest remains elusive. The effect of p53 is exerted through its transcriptional activation of p21\textsuperscript{Waf1}, a protein that is involved in cell cycle arrest, and of proteins involved in a pro-apoptotic response, such as p53-upregulated modulator of apoptosis (PUMA), NOXA, Bak and Bcl-2–associated X protein (Bax). Additionally, the function of p53 depends on ataxia telangiectasia mutated (ATM), a protein kinase located upstream of p53 in the DNA damage signalling pathway (45). Irradiation-induced DNA double-strand breaks recruit ATM, implying that ATM might be a key sensor protein of the DNA damage and gets activated by DNA double-strand breaks. P53 is directly phosphorylated by ATM. In addition, ATM also phosphorylates other proteins that exert their effect on p53 stabilization, like checkpoint kinase 2 (Chk2), Mdm2 and the Mdm2 homolog Mdmx. ATM deficiency leads to delayed and inefficient p53 stabilization (51, 52).

The level of p53 accumulation depends on the extent of DNA damage. This implies that
the dosage of irradiation influences the fate of the cells, i.e. whether the cell goes into reversible cell cycle arrest (at lower dose) or into apoptosis (at higher dose) (45). Radiation induces p53 expression and p53-dependent transcription of p21Waf1 in human cervical cancers biopsies (53, 54). DNA damage by chemotherapeutic drugs, such as cisplatin, also results in elevated p53 levels and enhanced p53-dependent transcription (55-57). DNA is the preferential and cytotoxic target for cisplatin, which results in the formation of monoadducts, intrastrand crosslinks, and interstrand crosslinks. The crosslinks are cytotoxic, since they lead to DNA double strands breaks (58). Cisplatin decreases HPV E6 and/ or E7 mRNA levels in HPV16-positive SiHa and CaSki cervical cancer cells and HPV18-positive HeLa, C4-I, C4-II and SW756 cells (59-62) (Table 1). This can explain the observed p53 activation in cervical cell lines following a genotoxic insult, such as cisplatin (60, 63), resulting in cell cycle arrest or apoptosis (60, 62, 64). Additionally, irradiation also decreases E6 mRNA levels in SiHa, CaSki and HeLa cells. Yet, the E6 mRNA levels recover quickly to normal. When irradiation is combined with cisplatin, E6 mRNA downregulation is maintained for a longer period (61). The prolonged decrease in E6 levels may explain chemoradiation efficacy in most HPV-positive cervical cancer patients.

Mechanisms that are responsible for the effect of cisplatin on the radiosensitivity of tumor cells generally include inhibition of potentially lethal or sublethal DNA damage repair, an increase in the radiosensitivity of hypoxic cells (65), and prolonged p53-restoration (60). Irradiation can also change intracellular signaling by modification of the activity of tyrosine kinases, mitogen-activated protein (MAP) kinases, stress-activated protein (SAP) kinases, and rat sarcoma (RAS)-associated proteins. Radiotherapy activates the epidermal growth factor receptor, thereby propelling quiescent cells into S phase, thus sensitizing these cells to radiation (66). Additionally, MAP kinases are suggested to play an important role in the cisplatin-induced activation of apoptosis in cervical carcinoma cells. Suppression of extracellular signal-regulated kinase (ERK), an important kinase in the MAP kinase pathway, induces cisplatin resistance in HeLa and SiHa cells (67). Activation of Bak via MAP kinases may explain the pro-apoptotic effect of this signaling pathway after cisplatin-induced DNA damage (68). Chemo- and radioresistance in cervical cancer patients have been associated with hypoxia in those tumors (69-71). Under hypoxic condition, E6 degrades the tumor suppressor cylindromatosis (CYLD), a NF-κB pathway inhibitor, thereby inducing NF-κB activation (72). NF-κB is a known stimulator of cancer angiogenesis, proliferation, survival, invasion, and metastasis (73) and its tumor expression actually correlates with higher tumor stages in cervical cancer (74).

Summarizing, p53 plays a central role in determining the fate of damaged cells. Since p53 expression levels are tightly controlled by HR HPVs E6, normal response to genotoxic insult is strongly decreased in HPV infected cancer cells. Restoring the apoptosis machinery is a rational approach to improve current treatment (Fig. 1B illustrates the effect of chemoradiation on HPV-positive cervical cancer). Moreover, targeting E6 may suppress pro-survival of cervical cancer cells at the same time.
Figure 1. HPV-mediated changes in molecular pathway and the effect of chemoradiation in cervical cancer. (A) HR HPV integration leads to aberrant expression of E6 and E7 oncogenes. E7 decreases pRb, thereby inducing E2F and p14. This leads to Mdm2 inhibition and p53 increase. To counteract the p53 upregulation, E6 binds to E6AP and degrades p53 via the proteasome system. The disruption caused by E6/E7 propels the infected cell into proliferation. (B) Irradiation and cisplatin induce DNA damage, ATM signaling, p53 activation and apoptosis induction via the intrinsic pathway. In HPV-positive cervical cancer, p53-dependent signaling is disrupted by E6/E7, which leads to reduced apoptosis. Cisplatin decreases E6/E7 expression.
IRRADIATION AND CISPLATIN ACTIVATE THE INTRINSIC AND EXTRINSIC APOPTOTIC PATHWAY IN CERVICAL CANCER

Both chemo- and radiotherapy are considered to induce apoptosis mainly via the intrinsic (mitochondrial) apoptotic pathway (see Fig. 1B). The intrinsic apoptotic pathway is activated after p53 response on cellular damage, leading to a cascade of events involving mitochondria, which leads to cell death. Activation of the extrinsic apoptotic pathway reduces the importance of p53 in executing apoptosis. TNF superfamily of cytokines, like TNF, Fas ligand (Fasl) and TRAIL, known as “death ligands”, are able to induce apoptosis in tumor cells by binding with their cell membrane receptors, thus utilizing the extrinsic pathway. TRAIL apoptotic signal is transmitted via the death inducing signalling complex (DISC) consisting of death receptors 4 (DR4) and 5 (DR5), Fas-associated death domain (FADD), and caspase-8 and -10 that leads to activation of initiator caspases 8 and 10 and finally results in cleavage of effector procaspases, such as caspase-3, into active caspases. Next, caspase-3 will cleave death substrates and induce apoptosis (see Fig. 2B). The anti-apoptotic protein FLICE-inhibitory protein (FLIP) can compete with caspase-8 to form an inactive complex (75, 76). Given the fact that the extrinsic apoptotic pathway can induce cell death independent of p53, exploiting this route is of particular interest in HPV-related cervical cancers. In addition, active caspase-8 cleaves the Bcl-2 interacting domain (Bid), which can trigger the intrinsic apoptotic pathway by activation of caspase-9 and finally caspase-3 (75, 76).

A variety of changes in tumor suppressor proteins (e.g. p53, ATM), enzymes (e.g. protein kinase C), cell membrane components (e.g. sphingomyelin-ceramide), nuclear transcription factors (e.g. NF-κB), cytokines (e.g. interferon, TNF-α), and regulators or effectors of apoptosis (e.g. Bcl-2, Bax, cytochrome C, caspases and increased Fas expression) occur in response to radiotherapy. Thus, both the intrinsic as well as extrinsic pathway can be activated by radiotherapy in cervical cancer (77-81). Cisplatin has also been suggested to activate both pathways in HeLa cells. This involves Fas and caspase-8 upregulation, Bcl-2 downregulation, cytochrome c release and downstream caspase activation (82). Cisplatin and anti-Fas monoclonal antibody induced more apoptosis than cisplatin alone in cervical cancer cells (83). Furthermore, cisplatin also raised DR5 mRNA and protein expression and enhanced caspase-dependent apoptosis at least in wild-type p53 ovarian cancer cells (84).

RATIONAL TARGETS IN CERVICAL CANCER CELLS FOR COMBINATION THERAPY

First, we discuss direct inhibition of HR HPV E6/E7 with various methods. Afterwards, strategies to interfere in E6/E7 cellular functionality like inhibition of the proteasome pathway or histone deacetylases (HDACs) are reviewed. In addition, the extrinsic apoptotic pathway as possible alternative therapeutic target for apoptosis induction is evaluated.
E6/E7 Targeting in Cervical Cancer Models

E6/E7 Inhibitors

HPV viral proteins E6 and E7 disrupt p53 and pRb functions and thereby possibly decrease sensitivity to chemoradiation. Many efforts to target these proteins in order to overcome resistance have been undertaken, as reviewed by Bernard (85). The most common approach applied in vitro is targeting the E6/E7 transcripts by short interfering RNA (siRNA), antisense oligodeoxynucleotides or ribozymes (see Fig. 2A) (85-87). E6 and E7 RNA interference alone inhibited growth of human cervical carcinoma xenografts in mice (88-91) (Table 1).

Effect of E6/E7 downregulation by siRNA combined with cisplatin varied between different studies. E6/E7 RNAi enhanced cisplatin sensitivity in HeLa (92), while E6 knockdown alone with different RNAi sequences had no impact on cisplatin sensitivity in this cell line (93). E6 siRNA restored p53 expression in SiHa and CaSki cells, and E7 siRNA induced pRb stabilization in CaSki. The combination of E6/E7 siRNA and cisplatin induced subadditive toxicity in both cell lines (94). Simultaneous targeting of E6/E7 by ribozymes sensitized CaSki cells to cisplatin and irradiation (95), while E6 antisense enhanced cisplatin efficacy in C41 cells (96).

An alternative approach is small molecules targeting the E6-E6AP interaction (reviewed by (97)). Two HPV16 and 18 E6 binding peptides were able to abrogate E6-E6AP formation and prevent p53 degradation in protein mixtures (98). Other small peptides that showed specific binding to HPV16 E6 proteins were tested in cervical cancer cell lines. The peptides showed higher binding affinity to E6 compared with E6AP, resulting in p53 stabilization. The stabilized p53 is functionally active, as indicated by increased expression of the p53 target genes p21 and PUMA. The peptides are HPV16 specific, since suppressed colony formation was only observed in HPV16 SiHa and MRI-H186 cell lines, but not in HPV18 HeLa cells or osteosarcoma cell line U2OS. In addition, apoptosis was seen in SiHa but not in HeLa cells (99). Furthermore, several chemical compounds, binding to E6, inhibited the E6-E6AP interaction, thereby preventing p53 degradation in ectopic E6 expressing cells (100).

Direct targeting of p53 is another option to rescue p53 from E6-mediated degradation. A small molecule called reactivation of p53 and induction of tumor cell apoptosis (RITA) has been reported to bind directly to p53 at the N-terminal region (101). In HeLa and CaSki cells RITA inhibited the E6AP-p53 formation, thereby rescuing p53 from proteasomal degradation. The p53 is functional since the expression of downstream pro-apoptotic targets like Bax, NOXA and PUMA, increased, leading to apoptosis. HeLa cells treated with RITA also entered cell cycle arrest at G2, due to the p53-dependent repression of mRNA levels of G2-associated cell cycle genes such as CyclinB1, CDC25C and CDC2. Additionally, RITA repressed the growth of cervical cancer xenografts in mice (102).

Since E7 is involved in the cell cycle control, inhibiting E7 oncogenic properties should
be directed at E7 or the cellular proteins targeted by E7. Recently a small peptide targeting HPV16 E7 has been shown to bind and degrade E7, thereby restoring pRb regulatory function of E2F. The peptide, thus, induced G1-phase arrest and suppressed proliferation of SiHa cells in vitro and inhibited SiHa tumor growth in mice (103).

Cdk2 is an interesting downstream target of E7 due to its central role in driving HPV-infected cells through unscheduled proliferation. Several small molecules with Cdk2 inhibitory effect have been tested in cervical cancer cells. So far these drugs have not been tested in cervical cancer patients yet (21, 104). However, the Cdk inhibitors currently available are not Cdk2 specific, which might result in undesirable toxicities due to the off-target effects (105). Additional research to develop more specific Cdk inhibitors is therefore considered essential.

**Proteasome Inhibitors**

Proteasome inhibitors act by inhibiting the ubiquitin-proteasome protein degradation. Since HPV E6 targets p53 for degradation through the ubiquitin-proteasome system, proteasome inhibitors are good candidates to increase wild-type p53 expression in cervical carcinoma cells (Fig. 2A). Recently, it has been shown that the proteasome inhibitor MG132 increases p53 protein levels and transcriptional activity in human cervical cancer cell lines. MG132 sensitized SiHa and HeLa to TRAIL-receptor and Fas-mediated apoptosis through DRs upregulation and caspase-8 activation (106). Additionally, MG132 increased SiHa radiosensitivity under hypoxic conditions (107). Bortezomib (PS341, Velcade®) that specifically target chymotrypsin-like activity of the proteasome (108), sensitized SiHa to radiotherapy via inhibition of the NF-κB pathway (109). Treatment with bortezomib resulted in the accumulation of the hypoxia-inducible factor HIF-1α but strongly reduced HIF-1α transcriptional activity under hypoxic conditions thus decreasing the production of vascular endothelial growth factor (VEGF).

In mice bearing SiHa xenograft, human plasma VEGF levels actually decreased by approximately 90% during treatment with bortezomib, indicating a reduced hypoxia response in tumors. Furthermore, bortezomib induced more caspase-3 cleavage in these xenografts (110) (Table 1). Bortezomib is clinically used for multiple myeloma and more ubiquitin system inhibitors are currently being studied (108). Bortezomib combined with radiotherapy was well tolerated in advanced solid tumors (111). A phase I trial with bortezomib and chemoradiation in a broad range of cancer types, including cervical cancer has been completed (112).

Other protease inhibitors with specific chymotrypsin-like proteasomal activity such as lopinavir and succinyl-alanine-alanine-proline-phenylalanine chloromethyle ketone (AAFP-CMK) have been tested in both HPV-positive and HPV-negative cell lines. Lopinavir, a HIV protease inhibitor, suppressed chymotrypsin-like proteasomal activity in cervical cancer cells SiHa and C33A-E6 (C33A transfected with HPV16 E6), thereby increasing p53 levels (113, 114). AAFP-CMK was able to inhibit cell growth in SiHa as well as in C33A cells (115). AAFP-CMK
may be an interesting therapeutic drug for cervical cancer and probably for cervical dysplasia as well since it showed selective targeting of HPV-positive raft cultures (116).

**Histone Deacetylase (HDAC) Inhibitors**

Besides DNA methylation, gene promoter regions are regulated by acetylation, phosphorylation and methylation of the histone proteins present in the nucleosomes of the chromatin. The histone acetylation increases the distance between DNA and histones, thereby enhancing the promoter binding to transcription factors. Adversely histone deacetylation decreases the accessibility of many promoters. Histone acetylation is reversible and mediated by histone acetyltransferases (HATS), whereas HDACs remove the acetyl groups. Different classes of HATS and HDACs have been identified in both the nucleus and the cytoplasm, affecting both histone and nonhistone proteins. Together HATS and HDACs control diverse functions ranging from gene expression, ageing to apoptosis (117-119). In HPV-positive cervical cancer cells, E7 binds to HDACs (120). The E7-HDAC association prevents HDAC binding to E2F promoter, which in turn enhances E2F expression and proliferation (Fig. 2A). Additionally, HDAC inhibitors can compete with E6 for p53 binding sites. A study in cervical cancer cells showed that p53 became hyperacetylated following treatment with HDAC inhibitors trichostatin A or valproate and was thus rescued from E6-mediated degradation (121) (Table 1).

HDAC inhibition resulted in both p53-dependent and p53-independent apoptosis in cervical carcinoma cells (Fig. 2B). Treatment with HDAC inhibitor induced apoptosis via E2F-mediated activation of pro-apoptotic isoforms of p73, a protein that is structurally related to p53, results in mitochondrial potential membrane breakdown and cytochrome c release (122, 123). In addition, the HDAC inhibitor apicidin decreased E6/E7 mRNA and protein levels in SiHa cells (124). A combination of bortezomib with the HDAC inhibitors trichostatin A or vorinostat showed synergestic effect in HeLa, SiHa, CaSkI and ME180 cells. Bortezomib treatment enhanced p53 levels, while the combination bortezomib plus trichostatin A induced massive caspase-3 cleavage. In mice, bortezomib as well as trichostatin A each slowed HeLa xenografts growth. When combined this resulted in an even stronger tumor growth inhibition (125). Given the capacity of HDAC inhibitors to hamper HPV replication and restoring p53 stability, they are potentially another interesting option to be added to chemoradiation in cervical cancer.
## Table 1. Summary of E6/E7 Targeting Agents in Preclinical Cervical Cancer Models

<table>
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<th>Study type</th>
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<td>HeLa</td>
<td>18</td>
<td>↓ E6/E7 mRNA</td>
<td>In vitro</td>
<td>(93)</td>
</tr>
<tr>
<td>Antisense deoxynucleotides</td>
<td>C4-I, C4-II, HeLa</td>
<td>18</td>
<td>↓ E6/E7 mRNA</td>
<td>In vitro/in vivo</td>
<td>(96)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>↑ p53 protein</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ribozymes</td>
<td>CaSki</td>
<td>16</td>
<td>↓ E6/E7 mRNA + protein</td>
<td>In vitro</td>
<td>(95)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>↑ p53/pRb protein</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Proteasome inhibitors</td>
<td>MG132</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>SiHa</td>
<td>16</td>
<td>↑ p53/p21 protein</td>
<td>In vitro</td>
<td>(106)</td>
</tr>
<tr>
<td></td>
<td>HeLa</td>
<td>18</td>
<td>↑ p53/p21 protein</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bortezomib</td>
<td>CaSki</td>
<td>16</td>
<td>↑ p53 protein</td>
<td>In vitro/in vivo</td>
<td>(125)</td>
</tr>
<tr>
<td></td>
<td>HeLa</td>
<td>18</td>
<td>↑ p53 protein</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HDAC inhibitors</td>
<td>Valproate</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>CaSki</td>
<td>16</td>
<td>↑ E6/E7 mRNA</td>
<td>In vitro</td>
<td>(121)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>↑ p53 mRNA + protein</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>↑ p53 hyperacetylation</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>SiHa</td>
<td>16</td>
<td>~ E6/E7 mRNA</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>HeLa</td>
<td>18</td>
<td>~ E6/E7 mRNA</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>↑ p53 mRNA, ~ p53 protein</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>~ p53 mRNA, ~ p53 protein</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>~ p53 mRNA, ~ p53 protein</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>↑ p53 mRNA, ~ p53 protein</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sodium butyrate</td>
<td>HeLa</td>
<td>18</td>
<td>↓ pRb protein</td>
<td>In vitro</td>
<td>(122)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>↑ p73 activation</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

1 Only single agent treatments are listed, combination treatments are discussed in the text.
2 p53, p21 and pRb expression, p73 activation and p53 hyperacetylation can serve as read-outs for changes in E6/E7 functionality.
3 ↑ indicates increased level, ↓ indicates decreased level, ~ indicates similar level.
Figure 2. Targeting HR HPV E6/E7 to enhance treatment efficacy of chemoradiation and TRAIL-pathway targeting anti-cancer drugs. (A) The mechanism of direct targeting of E6/E7 using strategies based on siRNA, antisense deoxynucleotides, ribozymes, small molecules or HDAC inhibitors and indirect inhibition of E6 effects using proteasome inhibitors in cervical cancer. (B) E6/E7 are inhibited by siRNA* (*including antisense deoxynucleotides, ribozymes), small molecules and HDAC inhibitors, thereby releasing p53 to take part in chemoradiation-induced activation of the intrinsic pathway. HDAC inhibitors acetylate p53, thereby rescuing it from E6-mediated degradation. HDAC inhibitors also enhance mitochondrial outer membrane permeabilization via pro-apoptotic p73 isoforms. Proteasome inhibitors increase p53 accumulation and exert additional effects on the extrinsic pathway by increasing TRAIL-DRs membrane expression, caspase-8 and caspase-3 activation. RhTRAIL and agonistic DR-antibodies bind to TRAIL-DRs, thereby activating the caspase-8, which in turn activates caspase-3, either directly or indirectly, through cleavage of Bid and activation of the intrinsic (mitochondrial) pathway, resulting in apoptosis.
TRAIL, DR4 and DR5-selective TRAIL Variants and Agonistic DR4 and DR5 Antibodies to Activate the Extrinsic Pathway in Cervical Cancer

TRAIL and TRAIL Receptor Expression in Cervical Cancer

Five TRAIL receptors have been identified, two death receptors DR4 and DR5 and three decoy receptors namely DcR1, DcR2, and osteoprotegrin (75, 76). TRAIL and their receptors are expressed in many normal epithelial cells (126) as well as tumor cells, including those of cervical cancer origin. HPV16-positive SiHa cells and HPV18-positive HeLa cells are positive for both DR4 and DR5 (106, 127). Another study, only taking into account DR5 expression, found DR5 staining in HPV16-positive CaSki, HPV18- en HPV45-positive MS-751, HPV39-positive ME-180, HPV-negative HT-3, and HPV-negative C33A human cervical cancer cells (128). The characteristics and HPV status of the human cervical cancer cell lines mentioned in this article are summarized in Table 2. The expression of the DRs is increased in cervical cancer compared to normal tissue (128-130), while the promoters of the DcRs in cervical cancers are often methylated (131). Additionally, elevated TRAIL and DR levels occur during viral infections such as HIV-1 infection in lymphoid tonsillar tissue (132), in macrophages and T cells (133), during reovirus infection in epithelial cells (134). Currently no data concerning TRAIL or DRs after HPV infection are available yet. Down-regulation of either E6 or E7 resulted in an enhanced cell surface expression of DR4 and DR5 in SiHa (135). This may be due to the reactivation of p53, which is known to induce DR4 and DR5 expression (76). The abundant presence of DRs in cervical carcinoma does, however, pose the receptors as interesting targets for innovative therapy modalities in cervical cancer (128-130).

Recombinant Human TRAIL for the Treatment of Cervical Cancer

Untagged trimeric recombinant human TRAIL (rhTRAIL) is especially of interest, since it is cytotoxic to cancer but not to normal cells, with 50% of the cancer cell lines being sensitive for rhTRAIL. Human cervical cancer cell lines have been exposed to rhTRAIL, and sensitivity to rhTRAIL-induced apoptosis has been analyzed. Although transfection with HPV16 E6 has been reported to decrease FADD and procaspase-8 levels, thereby protecting the transfected cells from TRAIL- and Fas-induced apoptosis (136-138), cervical cell lines showed a wide range of sensitivity to rhTRAIL. CaSki was found to be very sensitive, HeLa intermediate sensitive and SiHa resistant to rhTRAIL (106, 127). Furthermore, rhTRAIL at concentrations that were effectively inducing apoptosis in cervical cancer cells, was not toxic to normal cervical cells in culture (139).

In mice, rhTRAIL proved to be a potent apoptosis inducer in a variety of human tumor xenografts, including lung cancer, colorectal cancer and non-Hodgkin’s lymphoma. No data are available for rhTRAIL sensitivity of cervical cancer xenografts. Numerous preclinical in vitro and in vivo studies have shown that combinations of “classic” chemotherapy or radiotherapy
with rhTRAIL exhibit synergistic effects. In addition rhTRAIL can overcome resistance to irradiation or chemotherapeutic agents (75, 76). We recently showed that irradiation marginally enhanced p53 but strongly enhanced DR4 and DR5 membrane expression. Furthermore, irradiation treatment induced activation of the intrinsic pathway and sensitized HeLa cells to rhTRAIL-induced apoptosis (140).

Combination of rhTRAIL and HDAC inhibitors or proteasome inhibitors have shown efficacy in cervical cancer cells. The HDAC inhibitor sodium butyrate sensitized HeLa cells to TRAIL-induced apoptosis by reducing cellular FLIP (c-FLIP) levels (141). RhTRAIL in combination with the proteasome inhibitor MG132 induced high levels of apoptosis in HeLa and SiHa. MG132 increased DR4 and DR5 expression and restored p53 functionality. The combination treatment increased caspase-8 and caspase-3 activation as well as X-linked inhibitor of apoptosis protein (XIAP) inactivation (106). An interesting question therefore is whether such a combination is effective against cervical cancer but not toxic to normal cervical tissue. This made us test rhTRAIL in combination with the proteasome inhibitor MG132 on ex vivo normal and preneoplastic human cervical samples. Single treatment minimally induced apoptosis and the combination was effective in both normal and preneoplastic human cervical samples. The apoptotic effect, however, was clearly more distinctive in the preneoplastic cervical samples suggesting a therapeutic window MG132 treatment resulted in p53 rescue, as shown by the increase in p53 expression in the preneoplastic cervical samples (142).

Recombinant human (rh)APO2L/TRAIL is being tested in phase I and II studies for combination therapies in non-cervical cancer patients. Results of clinical phase I studies with rhAPO2L/TRAIL (dulanermin, Genentech) showed that there was no dose-limiting toxicity with doses up to 15 mg/kg, given intravenously for 5 consecutive days every three weeks. Two patients with metastatic chondrosarcoma had a partial tumor response for longer than 6 months (143). RhAPO2L/TRAIL has a serum half-life in humans of 23-41 min. Non-small-cell lung cancer patients were treated with rhAPO2L/TRAIL combined with paclitaxel, carboplatin and bevacizumab (144). Preliminary results showed no benefit from adding up rhAPO2L/TRAIL to standard treatment with carboplatin and paclitaxel (with or without bevacizumab) (145).

**Death Receptor-Selective TRAIL Variants**

In addition to rhTRAIL targeting all TRAIL receptors, new variants have been developed that bind DR4 and DR5 with stronger affinity (146). Targeting of a specific death receptor might lead to better tumor-specific therapies, since the competitive binding of TRAIL to DcRs can be avoided. The TRAIL variant specific against DR5 showed a reduced binding to DcR1 up to >20-fold less compared to rhTRAIL, while the binding to DcR2 was ~80% less compared to rhTRAIL. Furthermore, the TRAIL variant still exhibited a preference for DR5 when both DR4 and DR5 were present (147). DR4- as well as DR5-selective TRAIL mutants have been
tested in HeLa cells. DR5-selective TRAIL variants, including D269H were more effective apoptosis inducers than wild-type TRAIL at lower concentrations. The DR4-selective TRAIL variant induced less the least apoptosis in the cells (148). The DR5-selective TRAIL variant E195R/D269H has been tested on DR5 expressing human ovarian cancer xenografts. It showed slightly better activity than rhTRAIL and was in combination with cisplatin clearly superior over rhTRAIL in mice bearing xenografts. The cisplatin sensitizing effect was indicated by p53 and DR5 membrane expression enhancement. The combination treatment induced higher caspase-3 activation (84). TRAIL variants have not yet been tested in the clinic.

**Agonistic DR4 and DR5 Antibodies**

Another interesting option to specifically target DR4 and DR5 is the application of DR specific human agonistic antibodies. Five monoclonal antibodies are currently being investigated in clinical trials, one against DR4, mapatumumab (TRM-1; HGS-ETR1) (Human Genome Sciences, Rockville, MD, USA), and four against DR5, lexatumumab (HGS-ETR2) (Human Genome Sciences), tigatuzumab (CS-1008), a humanized version of TRA-8 antibody (Sankyo, Tokyo, Japan), conatumumab (AMG 655) (Amgen, Thousand Oaks, CA, USA) and PRO95780 (Genentech, Inc, South San Francisco, CA) (75, 149).

In HeLa cells, the anti-DR4 antibody mapatumumab combined with irradiation induced much more apoptosis than single treatments. This effect was due to irradiation-induced p53 stabilization and DR4 membrane expression increase. The sensitizing effect of irradiation can also be mediated via DR5 in HeLa cells as seen for the combination of irradiation and a non-clinical agonistic DR5 antibody (140) and the combination of cisplatin and the anti-DR5 antibody lexatumumab (150). The anti-DR5 antibody TRA-8 combined with chemoradiotherapy and radiotherapy caused synergistic effects in vitro and tumor growth inhibition in cervical cancer xenografts bearing mice (128). In ex vivo cervical tumor specimens, the combination of TRA-8 and cisplatin induced more cell death than with either agent alone. Cisplatin increased DR5 expression, thereby rendering the cervical cancer specimens more sensitive to TRA-8 as indicated by cleaved caspase-8 as well as caspase-3 (151).

A number of studies have been performed with the agonistic antibodies and indicate that antibodies have a longer half-life time (about ~18 days) (152) compared to rhAPO2L/TRAIL (23-41 minutes) (144). The agonistic DR4 antibody mapatumumab showed limited toxicity in phase I-II studies. Stable disease was the best treatment response described for a number of often heavily pretreated solid tumor types (152-155). Mapatumumab can be safely combined with cisplatin and gemcitabine as well as carboplatin and paclitaxel in advanced solid tumors cases (156, 157). A phase I study with the agonistic DR5 antibody PRO95780 in solid tumor and non-Hodgkin’s lymphoma patients showed that the antibody is well tolerated up to 20 mg/kg (158). Preliminary results in non-small cell lung cancer patients showed that adding PRO95780 to paclitaxel/carboplatin/bevacizumab did not increase treatment efficacy (159).
A phase I study with tigatuzumab (CS-1008) in lymphoma and solid malignancies showed that the drug is well tolerated up to 8 mg/kg (160). Tigatuzumab is currently tested in phase I studies in colorectal cancer. Several phase II studies are investigating tigatuzumab for combination therapies in solid tumors, including platinum-based chemotherapy (76). Clinical trials with conatumumab showed that as single agent conatumumab is well tolerated up to 20 mg/kg (161, 162). Combinations of conatumumab with other drugs like paclitaxel, carboplatin, the proteasome inhibitor bortezomib or the HDAC inhibitor vorinostat have been initiated (163). Till now, no data on DR-antibodies treatment in cervical cancer patients, either alone or combined with irradiation or chemoradiation, are available.

**Table 2. Summary of the HPV status and characteristics of the human cervical cancer cell lines**

<table>
<thead>
<tr>
<th>Cell lines</th>
<th>HPV status</th>
<th>Origin</th>
</tr>
</thead>
<tbody>
<tr>
<td>HeLa</td>
<td>18</td>
<td>adenocarcinoma</td>
</tr>
<tr>
<td>SiHa</td>
<td>16</td>
<td>squamous cell carcinoma</td>
</tr>
<tr>
<td>CaSki</td>
<td>16</td>
<td>small intestine metastasis of squamous cell carcinoma</td>
</tr>
<tr>
<td>HT-3</td>
<td>HPV-negative</td>
<td>lymph node metastasis of carcinoma with p53 and pRb mutation, carcinoma type not specified (164)</td>
</tr>
<tr>
<td>MS-751</td>
<td>18 and partial 45</td>
<td>lymph node metastasis of squamous cell carcinoma</td>
</tr>
<tr>
<td>ME-180</td>
<td>greater homology to HPV- 39 than HPV-18</td>
<td>omentum metastasis of squamous cell carcinoma</td>
</tr>
<tr>
<td>C33A</td>
<td>HPV-negative</td>
<td>squamous cell carcinoma with p53 and pRb mutation (164)</td>
</tr>
<tr>
<td>C4-I</td>
<td>18</td>
<td>squamous cell carcinoma</td>
</tr>
<tr>
<td>C4-II</td>
<td>18</td>
<td>squamous cell carcinoma</td>
</tr>
<tr>
<td>SW756</td>
<td>18</td>
<td>squamous cell carcinoma</td>
</tr>
<tr>
<td>MRI-H186</td>
<td>16</td>
<td>carcinoma, type not specified (99)</td>
</tr>
<tr>
<td>SKG-II</td>
<td>18</td>
<td>squamous cell carcinoma (165, 166)</td>
</tr>
<tr>
<td>CaLo</td>
<td>18</td>
<td>squamous cell carcinoma (121, 167)</td>
</tr>
</tbody>
</table>

*Based on information available online at ATCC-LGC Standards site (168) unless otherwise specified*

**CONCLUSIONS AND FUTURE PERSPECTIVES**

Several studies are available that aimed at evading the negative effects of HR HPV E6 and E7 on treatment efficacy in cervical cancer cell lines, xenografts and human cervical cancer specimens. In preclinical studies, direct targeting of the E6/E7, inhibition of the proteasomal degradation pathway, inhibition of HDAC and targeting of death receptors showed promising results, either as monotherapy or in combination with irradiation or cisplatin in cervical cancer models (see Fig. 2B). Yet, the administration route of siRNA, ribozymes or antisense deoxynucleotides against E6/E7 remains problematic. Therefore, small peptide ligands or peptidomimetics that target either E6-E6AP binding, p53, E7 or E7 downstream target Cdk2
could be even more attractive candidates. However, those small molecules have not reached
the clinics yet. Another option would be to develop drugs that target specific kinases based on
synthetic lethal interaction principle.

Combination strategies of interest for cervical cancer as discussed above have already en-
tered the clinic. The rationale of adding proteasome inhibitors or HDAC inhibitors to current
standard regime is that these drugs target E6/E7-oncogenic activities and thus may enhance
treatment response. Especially targeting E6 would benefit chemoradiation due to p53 stabili-
zation. Furthermore, the extrinsic pathway can be exploited for combination strategies with
chemoradiation, applying rhTRAIL and agonistic DR antibodies that have entered phase I/
II trials (Fig. 2B). Several interactions are to be expected. In the first place, irradiation and
cisplatin sensitize cervical cancer cells to rhTRAIL or DR antibodies via upregulation of DR4
or DR5 membrane expression. Secondly, TRAIL-DR targeting drugs not only activate the ex-
trinsic pathway, but can also induce activation of the intrinsic pathway, resulting in a syner-
gistic apoptotic effect of the combination as indicated by higher caspase-3 activation in pre-
clinical studies. Till now, rhTRAIL or agonistic DR antibodies have not been combined with
chemoradiation in clinical phase I/II studies. A clinical trial with the agonistic DR4 antibody
mapatumumab and chemoradiation in cervical cancer patients is accruing in our institution
(ClinicalTrials.gov Identifier: NCT01088347) (169). In order to closely monitor the effect of
chemoradiation and mapatumumab tumor biopsies will be taken at different time points dur-
ing the trial, and molecular markers for apoptosis will be studied. Novel combinations with
targeted drugs such as proteasome inhibitors or HDAC inhibitors and TRAIL death receptor
ligands for the treatment of cervical cancer can be envisioned in the near future. Chemoradia-
tion, however, will continue to be the main component of all combinations (Fig. 2B).

ACKNOWLEDGMENTS

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ANTICANCER DRUGS AIMED AT E6 AND E7 ACTIVITY IN HPV-POSITIVE CERVICAL CANCER

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