Tumor cells can't stand the heat

Boosting the effectiveness of hyperthermia in cervical carcinoma

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Publication date
2017
Document Version
Other version
License
Other

Chapter 1

General Introduction: Treating cervical carcinoma

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Cancer

Organ homeostasis is a controlled process whereby cell proliferation and cell death are tightly regulated. Normal healthy cells are equipped to respond to environmental factors. These factors may act as signals that modify diverse cellular characteristics such as cell shape, size, and division time. Cancer cells largely ignore these signals resulting in an unlimited cell division and a prolonged cell survival.

The precise mechanisms whereby tumor growth is initiated are unknown for the majority of cancers. Yet, there are certain risk factors associated with a higher cancer induction. The most common risk factor is smoking, which is associated with about 30% of all cancers; others factors are radon, alcohol, obesity and inherited genes (BRCA genes causing breast cancer are a well-known example) (Anand *et al*, 2008; Walker & Ho, 2012). Infections are not a common cause of cancer, but there are some specific tumors of which a high percentage (70%-99%) are caused by the Human Papillomavirus (HPV) such as carcinomas of the female uterine cervix, vagina, and vulva, and in both male and female in carcinoma of the anus and some specific tumors of the head and neck (Munoz *et al*, 2004; Saslow *et al*, 2012; Schiffman, 2007).

Carcinoma of the uterine cervix

The most common HPV-associated cancer is cervical carcinoma i.e. cancer of the lower region of the uterus (Figure 1). Each year, about 528,000 women are diagnosed with cervical carcinoma worldwide, which makes this the third most common cancer in women (www.cancer.gov), and it causes ~266,000

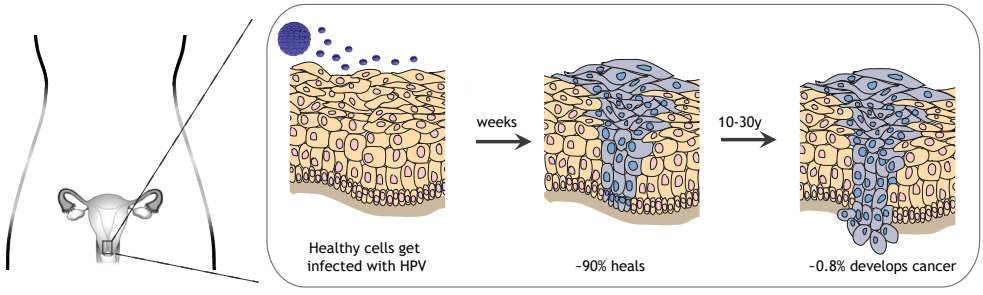

Figure 1: Cervical cancer. The cervix is the lower part of the uterus. A few weeks after exposure to HPV, many cells are infected. In approximately 90% of all cases the virus will be eliminated within a few weeks and only a small percentage of women who could not eliminate the virus will develop cervical carcinoma.

deaths per year (Saslow *et al*, 2007). Infections with HPV are not rare, as more than 70% of women will be carriers of HPV at some point during their life (Bosch *et al*, 2002; Munoz *et al*, 2004; Saslow *et al*, 2012; Schiffman, 2007). There are more than one hundred different types of HPV, of which two specific types are specifically associated with cervical cancer, i.e. the so called high-risk HPV type 16 (closely related to squamous cell carcinoma) and HPV type 18 (closely linked to adenocarcinomas and adenosquamous cell carcinoma) (Bosch *et al*, 1995; Lacey *et al*, 2001).

In approximately 90% of all cases, the immune system is capable of eliminating this virus within two years (Giuliano *et al*, 2011), which is shown in Figure 1. However, in some cases, typically 10 to 30 years after an infection with HPV, the virus can become active and may cause cell changes that can result into cancer (Pagliusi & Teresa Aguado, 2004).

DNA damage repair

Every day, in each cell, more than ten thousand DNA damages occur like base modifications, cross-links, mismatches and DNA breaks. Fortunately, mammalian cells are well equipped to cope with these damages (Ciccia & Elledge, 2010; Jackson & Bartek, 2009). Breaks can occur in one strand of the DNA, so-called single strand breaks (SSB) or in both DNA strands, so-called double strand breaks (DSB). These damages can be repaired by several pathways to maintain healthy cells (Figure 2). If DNA damage cannot be repaired, the cell will activate mechanisms to terminate itself. SSBs can be repaired by base excision repair (BER), nucleotide excision repair (NER) and mismatch repair (MMR). If the SSBs cannot be repaired, it can turn into a DSB, which are considered to be the most threatening lesions to the cell.

There are two major pathways to repair DSBs, non-homologous end joining (NHEJ) and homologous recombination (HR). Whereas NHEJ can take place during the entire cell cycle, but is considered error prone, HR is only active in the S-phase and G_2-phase of the cell cycle and requires a sister chromatid as a template to precisely repair the lesions and is therefore error-free. If one of these pathways is compromised, there is also a third pathway to repair DSBs, the so-called backup non-homologous end joining (b-NHEJ) (Iliakis *et al*, 2008).

These repair mechanisms will be explained in more detail in Chapter 2. It is not fully understood why or when one or the other repair pathway will be activated, but several factors are known. First, activation of a specific pathway depends on the type of DNA lesion. Second, as earlier mentioned, activation depends on the cell cycle phase wherein DNA damage occurs. As soon as DNA damage is recognized by the cell, the G_1, S or G_2 checkpoints can be activated to prolong a specific phase, leading

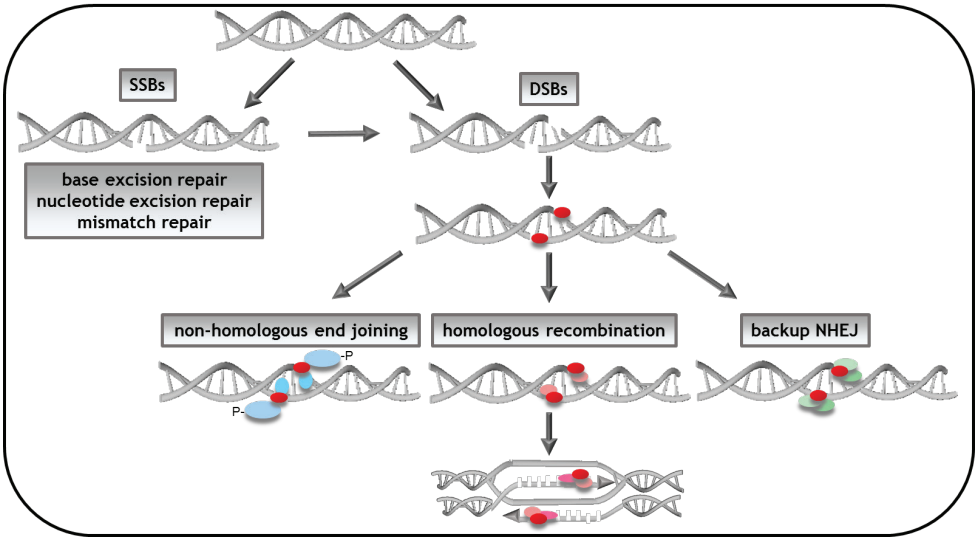

Figure 2: A simplified overview of DNA repair pathways. A break can be formed in one or in both DNA strands, called a single strand break (SSB) or double strand break (DSB) respectively. SSBs can be repaired via several pathways, like BER, NER and MMR. If the SSB is not restored, this can convert into a DSB. A DSB is recognized by the MRN complex (depicted in red), which recruits many different proteins of the NHEJ or HR to repair the damage. If one of the previous pathways is compromised, the b-NHEJ will repair the DSB.

to a cell cycle arrest. During this prolonged cell cycle phase, cells will try to repair the DNA damage. After successful repair, cells will resume their cell cycle, but if the damage is too severe, apoptosis might be induced (Allen *et al*, 2003; Rodrigue *et al*, 2006).

Cancer therapies

Surgery is the mainstay of treatment for women with early stage cervical cancer. In the Netherlands, radiotherapy plus chemotherapy is the first choice of treatment for women with locally or regionally advanced cervical cancer (Oncoline). Recurrences after surgery can be treated with radiotherapy or chemotherapy, and both can be combined with hyperthermia (Franckena *et al*, 2007). Both radiotherapy, chemotherapy and hyperthermia target the DNA of cancer cells. However, all cells have multiple mechanisms to restore the DNA damages as mentioned in the previous paragraph, which may counteract these therapies.

Most cancer therapies are designed to target rapidly dividing cells, and therefore mainly target tumor cells. Therapies can either damage DNA of

cancer cells and/or interfere with the DNA repair machineries. The three classical treatments for advanced cervical carcinoma are ionizing radiation, cisplatin-based chemotherapy and hyperthermia. In the last decade, there is also increasing interest in so-called targeted drugs, i.e. drugs that interfere at a specific point in one of the many molecular pathways involved in cancer proliferation, growth, survival and spread (Sawyers, 2004). Among the more promising targeted agents are PARP1-inhibitors, which are currently evaluated in clinical trials. Other interesting agents with clinical potential are inhibitors of heat shock proteins (HSP-inhibitors). The basic working mechanisms of these therapies will be explained in the next paragraphs.

Ionizing radiation

Ionizing radiation carries an amount of energy (>12.4 eV) sufficient to remove orbital electrons from atoms. These free electrons can interact either directly with the DNA or indirectly by creating free oxygen radicals which damage the DNA and thereby can create DNA SSBs and DSBs. SSBs are the least harmful to cells, but if they are at close proximity or repair pathways are impaired e.g. by a PARP1-inhibitor, SSBs can convert into DSBs. DSBs are the most harmful DNA lesions and if left unrepaired, they are potentially lethal to cells (Jackson

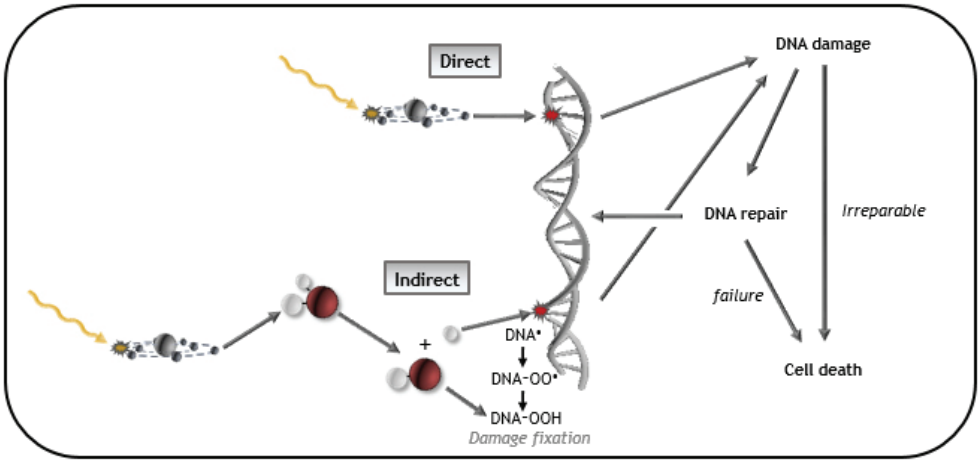

Figure 3: Ionizing radiation can cause DNA damage in a direct and in an indirect manner. A photon or charged particle with sufficient energy liberates an orbital electron that can directly damage the DNA. The second possibility, which happens in two-third of the cases, is again the interaction in which a photon liberates an orbital electron, that in turn interacts with a water molecule, creating an oxygen radical to induce DNA damage. These damages will recruit many proteins that try to repair the DNA. If this fails or if the damages are irreparable, they can accumulate and eventually the cell may die.

& Bartek, 2009). The physical radiation dose is expressed in gray (Gy); 1 Gy of ionizing radiation will inflict approximately 10,000 base damages, 1000 SSBs and 35 DSBs (Maalouf *et al*, 2011; Rothkamm & Lobrich, 2003). Radiotherapy has been proven to be very effective with over 50% of all cancer patients receiving this therapy, making this one of the most common treatments used worldwide (Begg *et al*, 2011; Delaney *et al*, 2005).

In this thesis we used either alpha particles (americium) or high energy photons (Cesium-137, γ-rays) for the *in vitro* experiments to irradiate cells. X-rays were used for the *in vivo* experiments and the patients studied in the retrospective study were treated by external high energy photon beam radiotherapy, and brachytherapy (Iridium-192). The DNA damage caused by these different types of radiation can be classified into two groups, namely direct and indirect ionizing radiation damage (Figure 3). Charged particles typically cause direct damage by transferring their energy directly through coulomb force to other atoms. This involves high linear energy transfer (LET). There is no intermediate step required to inflict damage, since the charged particle can interact and deliver all its kinetic energy to the DNA (Mettler, 2008; Phillips, 1997).
Indirect ionizing radiation damage requires an intermediate step, where a non-charged particle (e.g. a photon or a neutron) first interacts with an atom or molecule, like a water molecule in the neighborhood of the DNA. This

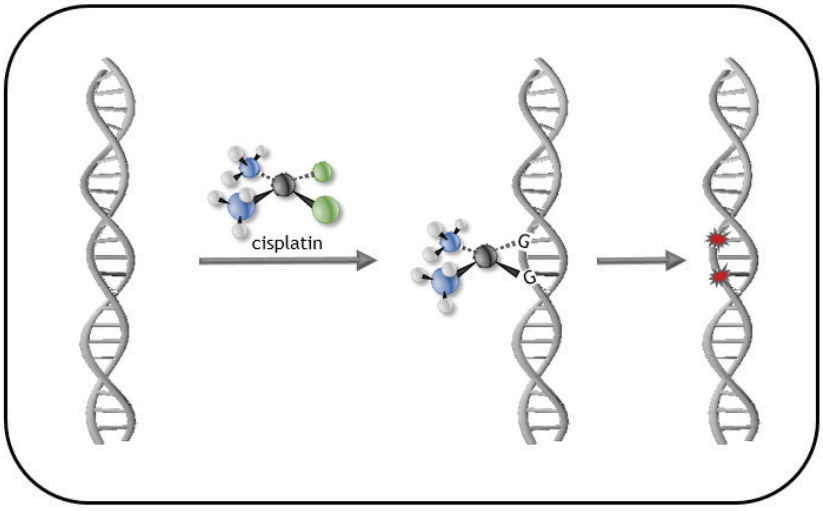

Figure 4: Cis-diamminedichloroplatinum(II) or cisplatin (cDDP) can induce DNA DSBs. When the chloride ligands (in green) dissociate from cisplatin inside cells due to the concentration in the extracellular fluid, cisplatin has a preference to bind to the guanine bases of the DNA. Cisplatin interferes with DNA replication and consequently, DNA DSBs can be induced (Cohen & Lippard, 2001).

interaction gives free oxygen radicals and DNA-derived free radical can be converted to irreparable peroxides, thereby fixating DNA-damage (Liu *et al*, 2015). This mechanism is responsible for two-third of the DNA damages, thus a dominant mechanism leading to DNA-damage in low-LET radiations like X-ray and γ-rays (Barcellos-Hoff *et al*, 2005; Hall, 1994; Riley, 1994; von Sonntag, 1987; Ward, 1988).

Relative biologic effectiveness (RBE) is the ratio of reference dose of radiation (usually 250 KeV X-rays) to a test dose of radiation (e.g. charged particles) which gives the same biological effect (Beck-Bornholdt, 1993). A higher RBE is related to a lower cell survival or more DNA damage, therefore RBE is helpful in understanding the potential biological effect of ionizing radiation. The RBE can differ for different types of ionizing radiation with similar absorbed doses, e.g. the action of direct ionizing radiation has a higher RBE than indirect action (Griffin *et al*, 1988). The RBE is not only influenced by the energy of the particles inducing DNA damages.

Oxygenation in (tumor) tissue is one of the most important factors associated with higher sensitivity to ionizing radiation. A better re-oxygenated irradiated area has a higher oxygen enhancement ratio (OER) than a hypoxic area, which is mainly caused by the indirect action of ionizing radiation (Barendsen *et al*, 1966). Moreover, fractionation of each lower doses (e.g. 25 times 2 Gy) ionizing radiation have a higher RBE than the accumulated doses given as a single dose (50 Gy) (Thames & Ang, 1998).

Alkylating agents (e.g. cisplatin)

Radiotherapy is often combined with chemotherapeutic agents that typically increase the induction of DNA damage, or interfere with the repair of the radiation-induced DNA damage (Boeckman *et al*, 2005; Sears & Turchi, 2012). Cis-diamminedichloroplatinum(II) or cisplatin (cDDP) is one of the oldest cytostatic drugs and the most commonly used in combination with radiotherapy. By binding to guanine bases, cisplatin can form cross-links (s-s bonds) to the DNA (Figure 4). These s-s bonds between cisplatin and the DNA strand prevent correct DNA replication, resulting in breaks in the DNA strand(s) (Baik *et al*, 2003; Sarmah & Roy, 2014). Moreover, cisplatin is not only able to induce breaks in the DNA, it can also disrupt NHEJ (Boeckman *et al*, 2005; Diggle *et al*, 2005; Myint *et al*, 2002; Turchi *et al*, 2000).

Hyperthermia

Hyperthermia is a therapy involving heating of the tumor above the normal physiological body temperature. In clinical setting, mild hyperthermia means heating the tumor for approximately 1 hour at 41-42.5°C. Mild hyperthermia is only marginally effective against cancer as a single modality, but is a potent sensitizer of both radiotherapy and chemotherapy. Radiotherapy plus hyperthermia is indicated as an alternative to cisplatin in cervical cancer patients in whom cisplatin is contraindicated, for instance because of renal or neural toxicity. Furthermore, in patients with a recurrent tumor after previous radiotherapy, it is an option to treat the recurrent tumor with cisplatin and hyperthermia to prevent side effects from a high cumulative radiation dose (Franckena *et al*, 2009).

On a macroscopic level, heating the tumor causes vasodilatation, which improves the blood flow, and thereby increases oxygen levels resulting in higher effectiveness of radiotherapy (Iwata *et al*, 1996; Shakil *et al*, 1999; Song *et al*, 2001; Song *et al*, 1997). Furthermore, due to vasodilatation and better tumor perfusion, chemotherapeutic agents can better penetrate the tumor (Song *et al*, 2001; Thrall *et al*, 2006). At a molecular level, it has been shown that hyperthermia can inhibit DNA repair in tumor cells by temporarily downregulating the BRCA2 protein and thus inhibiting repair by HR (Krawczyk *et al*, 2011). This leads to an accumulation of DNA DSBs and therefore fewer cancer cells will survive this therapy.

Hyperthermia has been applied in the clinic since the early 1980s and hyperthermia may be the most effective radiosensitizer known. The potential of thermoradiotherapy (i.e. radiotherapy combined with hyperthermia) has been shown in several large randomized clinical trials, in which the overall survival is significantly better after a therapy with hyperthermia compared to radiotherapy alone. Van der Zee et al. reported 27% of the cervical cancer patients had a three year survival if treated with radiotherapy alone vs 51% for patients treated with radiotherapy combined with hyperthermia. Results of a multicentre randomized clinical trial in locally advanced cervical cancer reported hyperthermia combining with chemoradiotherapy increased complete response rates with ~10% compared to chemoradiotherapy without hyperthermia (Harima *et al*, 2016). A systematic review by Datta and colleagues reports a 22% greater complete response rate in patients with locally advanced cervical cancer if patients received radiotherapy with hyperthermia, compared to radiotherapy alone (Datta *et al*, 2016b). Also for head and neck tumors, the addition of hyperthermia to radiotherapy increased complete response rates with ~25% (Datta *et al*, 2016c) and for locoregional recurrent breast cancers with ~22% (Datta *et al*, 2016a). Despite these convincing results both in pre-clinical and clinical settings, hyperthermia is not a standard therapy.

PARP1-inhibitor

In the last decade, PARP1-inhibitors have found their way into clinical trials (Underhill *et al*, 2011). Poly (ADP-ribose) polymerase-1 (PARP1) is one of the critical enzymes required in DNA replication; deficiency of PARP1 may lead to SSBs (Sugimura *et al*, 2008). In the absence of PARP1, these SSBs are generally repaired by HR, in which BRCA1/2 proteins are essential (Bryant *et al*, 2005). In tumor cells without properly functioning BRCA proteins, PARP1-inhibitors cause potentially lethal damages; this therapeutic effect of PARP1-i is already clinically exploited (Bryant *et al*, 2005; Dedes *et al*, 2011; Eppink *et al*, 2012; Farmer *et al*, 2005; Helleday, 2011; Krawczyk *et al*, 2011). The use of PARP1-inhibitors is thus mainly used in patients with a hereditary deficiency of BRCA or with tumors that are BRCA-deficient for other (unknown) reasons (mainly cancers of the ovary and breast) (Rouleau *et al*, 2010; Schreiber *et al*, 2015).

However, as hyperthermia has the ability to temporarily downregulate BRCA2 and thereby inhibit HR, the combination of PARP1-inhibitor and hyperthermia creates a possibility to induce this synthetic lethal effect also in BRCA-proficient tumor types (Figure 5).

A major advantage of PARP1-inhibitors is their selectivity. Theoretically they can only induce potential lethal damages in cells that have insufficient HR, which are the BRCA-deficient tumors cells (Fong *et al*, 2009), or in tumor

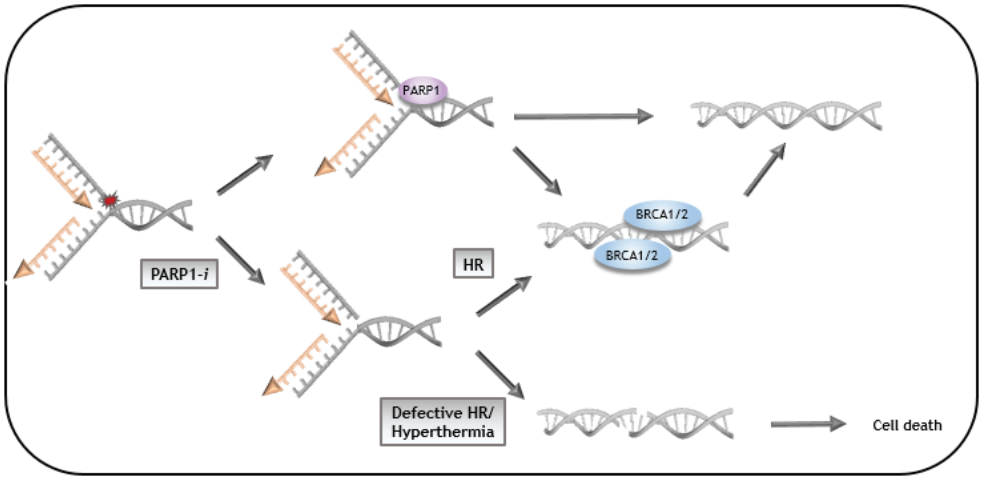

Figure 5: Working mechanism of PARP1-inhibitors. During replication PARP1 is essential. In the absence of PARP1, SSBs can occur, which are generally repaired by HR to repair the DNA break. However, if HR is not functional due to BRCA mutations, or because the pathway is compromised by hyperthermia, these DSBs can be potentially lethal to cells.

cells that are locally treated with hyperthermia. The effectiveness of PARP1-inhibitor in cells with functional BRCA, either homo- or heterozygous, was ~1000-fold lower than in mutated BRCA cells (Farmer *et al*, 2005). PARP1-inhibitors are predicted to have a significantly reduced effect on normal cells compared to either BRCA1/2 mutated cells, or to the hyperthermia targeted cells with consequently compromised BRCA proteins. In conclusion: PARP1-inhibitors may have a great potential for future clinical use.

HSP90-inhibitor

The molecular chaperone protein heat shock protein 90 (HSP90) regulates protein folding, prevents misfolding and assists in the function of various proteins (Morimoto *et al*, 1997; Whitesell & Lindquist, 2005). HSP90 is therefore essential for cell survival. Cellular stress, for example heat shock, causes expression of HSP90 proteins. High levels of HSP90 are found in multiple solid and hematologic tumors, and have been correlated with poor prognosis in breast cancers (reviewed by (Den & Lu, 2012)). Inhibition of HSP90 destabilizes proteins, causing protein degradation via the proteasomal pathway (Sepp-Lorenzino *et al*, 1995). Therefore, inhibition of HSP90, seems a promising target in anti-cancer treatments, particularly when combined with the strategies already discussed which aim at inducing DNA damage or blocking DNA repair pathways. Several HSP90-inhibitors are currently under investigation in phase I and II clinical trials (Banerji *et al*, 2005; Neckers & Neckers, 2005).

Combinational treatment

As already described in the previous paragraph, hyperthermia and PARP1-inhibitors have potential in many different tumor types, more specifically in BRCA wild-type tumors, when applied together or combined with radiotherapy and chemotherapy. Since PARP1-inhibitors are most effective in cells without functional BRCA, the combination with hyperthermia is close to a selective therapy, minimizing severe side-effects. However, several phase I trials testing PARP1-inhibitors reported dose limitation by severe myelosuppression and elevated hepatic transaminases (Underhill *et al*, 2011). Therefore, combining hyperthermia and PARP1-inhibitors with either radiotherapy or cisplatin can possibly lower the dosage of PARP1-inhibitors and or cisplatin to reduce the normal tissue toxicity associated with these two modalities without losing the effectiveness of treatment outcome. Combinational treatments may be a clinically effective strategy to induce more severe and permanent damage in cancer cells, without (severe) side-effect.

1

Outline of this thesis

The five-year overall survival of women with cervical carcinoma is (only) 65% with the present standard treatment of chemoradiation. In this thesis, the aim is to investigate new strategies involving hyperthermia to improve treatment outcome. Unfortunately, hyperthermia is somewhat overshadowed by scepticism. That scepticism is not justified as the many clinical trials performed since the 1980s prove that hyperthermia clinically works. Without the induction of (severe) normal tissue toxicity, hyperthermia may be one of the best radiosensitizers and chemosensitizers available. These factors are compelling reasons for patients to choose hyperthermia over therapies that cause severe side-effects, to enjoy a better quality of life during the treatment period and thereafter. A better understanding of the biological mechanisms of hyperthermia will probably facilitate the acceptance of hyperthermia as a clinical modality. Furthermore, improving and optimizing current clinical set ups are required to get even better results.

First, **PART I** of this thesis provides insight in how hyperthermia works. To this end a review on the effects of hyperthermia on DNA repair pathways was compiled (**Chapter 2**). In this review the complexity of the effects of hyperthermia become clear, as hyperthermia triggers multiple pathways, which contribute to is clinical efficacy. In **Chapter 3** we describe an experimental follow up inspired by the review, for which experiments were designed to confirm the review outcomes, showing that, at molecular level, hyperthermia does not only inhibitor DNA repair by HR, but that more molecular mechanisms are disabled by hyperthermia. Furthermore, PARP1-inhibitors have been studied extensively and have been found to induce synthetic lethality when administered to BRCA-deficient patients. Combining this knowledge with the fact that hyperthermia temporarily downregulates BRCA2, it makes sense to speculate that hyperthermia may be applicable to more tumor types for which PARP1-inhibitors can be successfully applied, even including BRCA wild-type tumors.

Cervical carcinoma reponds very well to hyperthermia in terms of tumor control and patient survival. The experiments described in **Chapter 4** reveal an important new mechanism why this is the case. HPV is found in virtually all cervical carcinoma cells; one of the surviving strategies of HPV is to disable the function of p53. In this chapter we describe that hyperthermia is able to re-activate p53.

In **PART II** of this thesis, potential improvements in the application of hyperthermia in combination with radiotherapy are described, studying specifically the sequence and time interval between hyperthermia and radiotherapy. Regarding sequence, in Europe normally first radiotherapy is

applied and then hyperthermia, in the United States it is generally performed in the opposite order. The rationale for Europe's model, is to first induce DNA damages with radiotherapy after which hyperthermia can interfere with the repair of these DNA lesions. In the United States the aim is to first increase blood flow and thus re-oxygenate the hypoxic tumors by hyperthermia, and thereby sensitizing the tumor to radiotherapy. In **Chapter 5,** we describe experiments in different cervical carcinoma cell lines and on a xenograft model, looking at cell survival, cell distribution, apoptosis and DNA repair proteins, to explain the possible differences in sequence of hyperthermia and radiotherapy.

In **Chapter 6** we describe an investigation of the time interval between radiotherapy and hyperthermia and its effect on clinical outcome. To this end, a retrospective study was performed in cervical cancer patients treated with radiotherapy and hyperthermia, complemented with experiments on patient biopsies that provide an explanation of observed clinical differences of *in vitro* DNA-damage response.

Eventually, in **PART III** multimodality options were investigated for patients who cannot receive radiotherapy e.g. because the tumor is located in a previously irradiated area. The possibility of applying cisplatin with hyperthermia and the addition of PARP1-inhibitor *in vitro* and *in vivo* was explored in the experiments described in **Chapter 7**. Next, to improve quality of life after cisplatin-based chemotherapy, it was investigated whether a PARP1-inhibitor can lower the cisplatin dose without diminishing the effectiveness. This is described in **Chapter 8**. In **Chapter 9** of this thesis, the combination of hyperthermia and a small molecule inhibitor of heat shock protein (HSP90-inhibitor) is explored to boost the effectiveness of hyperthermia.

Furthermore, a literature search was carried out to investigate whether hyperthermia has the ability to target all tumor cells, even the subset of therapy-resistant cells that remain alive even after high doses of radiotherapy and chemotherapy. It is crucial to eliminate this subset of cells as they have the property to induce metastasis. These hypotheses are discussed in **Chapter 10**. Finally, in **Chapter 11** the findings of this thesis are summarized and discussed in the context of what is known about hyperthermia and what the future perspectives may be.

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