Experimental treatment modalities for osteosarcoma
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General Introduction
Osteosarcoma; clinical presentation and epidemiology

Osteosarcoma is the most prevalent non-hematological primary malignant bone tumor. It is predominantly occurring in young adolescents, and drastically changes the lives of these patients. The approximate annual incidence of osteosarcoma is 2-3 cases per million persons a year with a peak of 8-11 per million at the age of 15-19 years. It affects about 1.4 times more often males than females (1). In older patients, osteosarcoma is relatively rare. In a fraction of osteosarcoma patients, prior radiation therapy for other cancer types is the causative agent. The interval between radiation therapy and the occurrence of osteosarcoma varies between 3 to over 50 years (2).

Patients usually present with pain and/or swelling of the involved region. Pain might be intermittent but gradually becoming persistent and severe. In approximately 10% of cases patients present with pathological fractures (1). Osteosarcoma predominantly originates in the metaphyses of long bones. The most common locations are the femur and the tibia (about 80% of all osteosarcoma patients). Other primary sites include humerus, pelvis, fibula, jaw and ribs. It is often a highly aggressive tumor that metastasizes primarily to the lung (3). Osteosarcoma of the head and neck usually arises in older patients and a minority of these patients develops systemic disease (4).

Osteosarcoma; biology and molecular abnormalities

In general, osteosarcoma demonstrates pronounced cell-to-cell variation and is characterized by complex chromosomal abnormalities (5). The tumor suppressor p53 is mutated in about 20% of osteosarcoma samples (6). The finding that patients with the Li-Fraumeni syndrome (p53 germ line mutations) are prone to develop osteosarcoma strongly supports an important role of p53 in the formation of osteosarcoma (7). In general p53 tumor suppressor capacities can be compromised either by point mutations in the p53 gene itself or by posttranscriptional inhibition of wild type p53 protein. The latter mechanism may be important for osteosarcoma cells because the major negative regulator of p53, mouse double minute 2 (MDM2) is predominantly amplified in osteosarcoma and soft-tissue sarcoma (8). The observation that MDM2 gene amplification might be
associated with increased risk for metastases strengthens the p53-osteosarcoma relationship (9).

The role of the Retinoblastoma (Rb) tumor suppressor in the pathogenesis of osteosarcoma is suggested by the observation that retinoblastoma patients, with the hereditary variant, having a constitutional Rb gene mutation are predisposed to osteosarcoma as second primary tumor (10, 11). Loss of heterozygosity, structural anomalies and subtle mutations in the Rb gene were present in 63%, 29% and 6% of tested osteosarcoma samples, respectively. Negative Rb-protein expression was observed in more than half of the osteosarcoma tumors (12).

Besides loss of tumor suppressing genes involved in cell cycle arrest and apoptosis (p53 and Rb), another hallmark of cancer is the acquired trait of being self-sufficient in growth signals. Expression of the epidermal growth factor receptor (EGFR) is present in high-grade osteosarcoma and is associated with improved prognosis (13).

Taken together, osteosarcomas present themselves with many genetic abnormalities that are shared among different cancer types. In the research described in this thesis, we aimed to exploit these abnormalities to design therapeutics that are specifically targeting cancer cells. Detailed analysis of the response of osteosarcoma cells to these newly developed therapeutics will be of great value to assess the therapeutic value of these new modalities not only for osteosarcoma but also for other cancers sharing similar genetic abnormalities.

**Classification**

Osteosarcoma is a primary malignant tumor in which tumor cells produce osteoid or bone. They are broadly subdivided into those predominantly occurring in the medullary cavity and those situated on the surface of the bone (table 1.1) (14). The most common osteosarcoma is conventional osteosarcoma, an intramedullary high-grade malignant tumor that can be divided into three major subtypes osteoblastic, chondroblastic and fibroblastic osteosarcoma. Unusual subtypes of conventional osteosarcoma are sclerosing osteoblastic, osteoblastoma-like, chondromyxoid fibroma-like, clear-cell, malignant fibrous-histiocytoma-like, giant cell rich and epithelioid osteosarcoma. Clinical significance of these subtypes is supported by the observation that a better response to chemotherapy was observed in patients with fibroblastic
osteosarcoma compared to chondroblastic osteosarcoma (15). Conventional, telangiectatic, small cell and high-grade surface osteosarcoma show a similar clinical course and are treated with the same treatment regimen. Low-grade central and parosteal (low-grade) osteosarcoma behave less aggressive and treatment consists of surgery alone. Periosteal osteosarcoma is an intermediate grade chondroblastic osteosarcoma with no consensus about the treatment whether surgery should be followed by chemotherapy (1, 16).

### Table 1.1 Classification of osteosarcomas

<table>
<thead>
<tr>
<th>Medullary osteosarcoma</th>
<th>Surface osteosarcoma</th>
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<tbody>
<tr>
<td>Conventional osteosarcoma</td>
<td>Parosteal osteosarcoma</td>
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<tr>
<td>Telangiectatic osteosarcoma</td>
<td>Periosteal osteosarcoma</td>
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<tr>
<td>Small cell osteosarcoma</td>
<td>High-grade surface osteosarcoma</td>
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<td>Low-grade central osteosarcoma</td>
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<td>Secondary osteosarcoma</td>
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### Treatment of osteosarcoma

Treatment of high-grade osteosarcoma (including conventional, telangiectatic, small cell and high-grade surface osteosarcoma) consists of preoperative (neoadjuvant) chemotherapy followed by surgery and postoperative (adjuvant) chemotherapy. Commonly used and active chemotherapeutic agents especially when combined are: doxorubicin, cisplatin, methotrexate and ifosfamide (3, 17-20). Their working mechanisms are discussed below.

### Chemotherapeutic agents

**Doxorubicin**: This anthracycline antibiotic agent is used for several types of solid tumors including osteosarcoma (21). Although it has been used for several decades as an anti-cancer agent in the clinic the exact working mechanism to induce tumor regression is uncertain (22). Several mechanisms to explain the cytostatic and cytotoxic properties have been reported, for example; interference with macromolecular biosynthesis, free radical formation, DNA adduct formation and DNA cross-linking, interference with DNA unwinding and strand separation and direct membrane effects, but the primary trigger is believed to be inhibition
of topoisomerase II that induces DNA damage (22). These events can lead to cell death or growth arrest predominantly in the G2/M cell cycle phase.

*Cisplatin*: Cisplatin belongs to the group of platinum based drugs. Nuclear DNA is the major target of cisplatin as it forms intra- or interstrand DNA cross-links or DNA-protein cross-links (23). These strands disrupt the normal 3-dimensional DNA structure and interfere with DNA replication and cell division resulting in cytotoxicity. The exact mechanism through which the DNA adducts induce the cytotoxic effect is poorly understood.

*Methotrexate*: Methotrexate is a folate antagonist and an important drug for the treatment of acute lymphoblastic leukemia, Non-Hodgkin lymphoma and osteosarcoma. It is thought to act primarily by inhibiting dihydrofolate reductase (DHFR). DHFR is a key enzyme in the thymidylate cycle and inhibition results in disrupted DNA synthesis and eventually to cell death (24, 25).

*Ifosfamide*: This chemotherapeutic agent belongs to the alkylating agents and is a prodrug. After administration, ifosfamide is activated by a liver cytochrome P450-catalyzed 4-hydroxylation reaction that forms cytotoxic metabolites (26). The predominant active metabolite is ifosfamide mustard that is capable of inducing DNA cross-linking thereby inducing the cytotoxic effect (27).

**European osteosarcoma study groups**

Several European osteosarcoma study groups have been formed. All aim to optimize chemotherapeutic regimens for osteosarcoma patients, but use different chemotherapeutic protocols.

The European Osteosarcoma Intergroup (EOI) compared in a randomized trial the use of a two-drug regimen (doxorubicin and cisplatin) with a three-drug regimen. The two-drug regimen appeared superior as defined by progression free survival (28). In two subsequent randomized trials, the two-drug regimen was compared with a multidrug regimen and with a two-drug intensified chemotherapy regimen. This did not result in increased progression free or overall survival (29, 30).

The co-operative German-Austrian-Swiss osteosarcoma study group (COSS) carried out several clinical trials. Based on the COSS-82 study in which postoperative salvage therapy failed to prove beneficial they concluded that chemotherapy should be as aggressive as possible upfront (31). This led to the design of COSS-86 protocol in which patients received a three-drug based regimen (doxorubicin, methotrexate and cisplatin) for low-risk patients and high-risk patients received the same therapy with an additional ifosfamide treatment.
Both groups showed similar results of overall free survival of about 70% (32). Furthermore they could not prove that higher dose intensities of these chemotherapeutic agents did result in better outcomes (33).

The Rizzoli institute in Bologna has treated patients with several preoperative chemotherapeutic protocols. From 1986 pre- and postoperative treatment consisted of at least three drugs (predominantly doxorubicin, cisplatin and methotrexate). Later they extended these protocols to four drugs pre- and postoperative. In these protocols they used high or low dose of doxorubicin, ifosfamide and methotrexate to assess effect on survival (34, 35).

The Scandinavian Sarcoma Group (SSG) has carried out three non-randomized preoperative chemotherapy trials. The first trial (SSG II) used the Memorial Sloan Kettering’s T-10 protocol consisting of preoperative high-dose methotrexate (36). Because only a minority of osteosarcoma patients were good responders the preoperative chemotherapy regimen was extended with cisplatin and doxorubicin (SSG VIII) (37). Later in a combined Italian/Scandanavian (ISG/SSG I) trial ifosfamide was added to the preoperative regimen that did not appear to improve outcome compared to the previous SSG VIII protocol (38).

Recently the EOI, the COSS, the SSG together with the Children’s Oncology Group formed EURAMOS (EURopean and AMerican OsteoSarcoma Group). This international group aims to conduct randomized controlled trials for the treatment of osteosarcoma.

Surgery

Several surgical approaches for the primary tumor exist such as limb-saving surgery, amputation or disarticulation and rotation-plasty. Previously, amputation or disarticulation was the most frequently performed surgical procedure for osteosarcoma (3, 39). Limb-saving surgery was considered riskful and reserved for patients with small tumors and tumors that did not extend beyond the cortex. Nowadays, limb-saving surgery is the most common used operation technique (3, 35, 40). This change in surgical policy was possible in the first place due to improved efficacy of chemotherapeutic agents resulting in a smaller tumor mass to be excised. Second, by the introduction of novel radiological imaging tools such as CT and MRI, the tumor is better visualized. It has been reported that limb-saving surgery plus chemotherapy resulted in the same outcome compared to amputation or disarticulation and chemotherapy (41, 42). However in patients
with inadequate surgical margins, especially with a poor response to chemotherapy the local recurrence rate is increased (43). Whether direct amputation would improved survival in these patients is controversial (44, 45). Together with the primary osteosarcoma tumor all detectable metastatic lesions are surgically removed when feasible.

Radiotherapy
Osteosarcoma is relatively resistant to radiotherapy and therefore radiotherapy is not part of standard treatment. Radiotherapy can provide local control of osteosarcoma tumor growth and pain reduction in patients with inadequate surgical resection or non-resectable tumors. A recent study reported a 5-year overall local control rate of 68% in patients receiving radiotherapy for osteosarcoma located in the face, spine, pelvis and trunk (46). Radiotherapy for osteosarcoma lung metastases is not advocated for, because it increases toxicity but does not appear to offer an additional effect over chemotherapy.

Prognosis
Before the introduction of chemotherapy in the early seventies, surgical treatment was the only option that resulted in a poor 5-year survival rate of approximately 20% (39). Chemotherapeutic agents like doxorubicin, methotrexate and cisplatin dramatically improved the 5-year survival to 50-70% (47-50).

Localized high-grade osteosarcoma at diagnosis
The vast majority of osteosarcoma patients present with localized disease without clinically detectable metastases (3). A good response to preoperative chemotherapy as determined by an increased degree of necrosis is an important positive prognostic factor (31, 51). Chemotherapy and surgery improved the 5-year survival rate to 50-70% in patients with osteosarcoma of the extremities (30, 32, 33, 51-53). Furthermore the location of the primary tumor is a significant prognostic factor. Especially patients with osteosarcoma in the axial skeleton have poor prognosis that is illustrated by the 5-yr survival rate of 15-27% (54, 55).
Metastatic disease at diagnosis
About 10-20% of the osteosarcoma patients present with clinically detectable metastatic disease at initial diagnosis (3, 56). Outcome of these patients is often poor with long-term survival rates between 10-30% (18, 56-58). Treatment consists of pre- and postoperative chemotherapy in combination with resection of the primary tumor as in osteosarcoma patients without metastases. Whenever feasible surgical resection of all metastases is performed. Patients without complete surgical resection of all tumor locations have a five-fold greater risk of dying (57). In the majority of patients these metastases arise in the lungs. In patients with lung metastases the number of metastases and bilateral distribution appear to be negative prognostic factors for survival (20, 57). Patients presenting with extra-pulmonary metastases and inadequate surgical resection (of all tumor locations) have an even poorer prognosis (18, 56, 57). However, a recent trial reported a 2-year survival rate of 58% in 12 patients with primary metastases to bones with or without lung metastases after treatment with methotrexate, cisplatin, doxorubicin combined with ifosfamide and etoposide and surgery (19). Longer follow-up and inclusion of more patients is necessary to recommend this chemotherapeutic regimen for these patients.

Recurrent osteosarcoma
Despite complete removal of the tumor and intensive chemotherapy 30-40% of patients with high-grade osteosarcoma will relapse (32, 35, 59). Short relapse-free interval, extra-pulmonary metastases and the number of lung metastases (>2) appear to be of negative prognostic value at relapse (59, 60). The lung is the predominant location of relapse involved in 80-90% of these patients in whom the majority of metastases is confined to the lung (59-61). Second-line therapy for these patients consists of surgical removal of the metastases in combination with chemotherapy. Although it is quite common to give chemotherapy, solid scientific evidence is lacking. The role of surgery is clearer and complete surgical removal of the metastases is critical. Patients without a complete surgical remission have a 5-year survival of 0%. This in contrast to a 5-year survival of about 40% in patients with complete surgical remission (59, 60, 62).

The front line of chemotherapeutic drugs consists of doxorubicin, cisplatin, methotrexate and ifosfamide that are employed over the last 20 years. The inclusion of additional chemotherapeutic drugs like, bleomycin, cyclophosphamide, actinomycin, vincristine or etoposide has not resulted in a
convincing prognostic benefit (63). No standard, second-line therapy exists for patients that relapse. Given the poor prognosis for many osteosarcoma patients and especially for those with non-operative metastases, new treatment strategies for osteosarcoma are warranted. In order to obtain more data about the treatment of relapsed osteosarcoma patients a database called EUropean RELapsed OSteosarcoma registry (EURELOS) has been formed.

**Novel treatment modalities for osteosarcoma**

Conventional chemotherapeutic agents have a broad working mechanism and thereby also damage or even kill non-target cells. The vast majority of all osteosarcoma treatment regimens consisted of conventional chemotherapeutic agents. However the observation that survival has reached a plateau with currently available chemotherapeutics prompted testing of new treatment modalities. To increase tumor specific cell kill, most new anticancer therapies act on specific receptors, ligands or enzymes and this is referred to as targeted cancer therapy. Some examples of these novel therapies for osteosarcoma (clinical and preclinical studies) are given below.

**Antiangiogenic agents**

Tumor progression of both the primary tumor and its metastases requires blood vessel formation. Vascular endothelial growth factor (VEGF) promotes neoangiogenesis. It activates endothelial cells and causes vascular endothelial cell migration and prevents apoptosis. A high percentage of osteosarcoma primary tumors and its metastases are VEGF positive correlating with poor prognosis (64). Anti-VEGF therapy with bevacizumab (a monoclonal antibody directed against VEGF) has shown therapeutic advance in colorectal cancer, but it has not been tested for osteosarcoma (65). Although relative few data regarding antiangiogenic therapy for osteosarcoma is available, some data are promising. For example Tsumeni et al. have shown in mouse experiments that pulmonary metastases could be suppressed by TNP-470, an angiogenesis inhibitor (66).
Insulin-like growth factor-1 therapy

The insulin-like growth factor-1 (IGF-1) mediates the adolescent growth spurt by bone growth and is present on osteosarcoma tumor cells. Adolescent growth spurt coincides with the peak incidence of osteosarcoma. This observation prompted research on the role of IGF-1 in the pathobiology of osteosarcoma. The finding that reduced IGF-1 levels after hypophysectomy in mice was associated with inhibition of local osteosarcoma tumor growth and metastatic behavior supported the assumed close relation between IGF-1 and osteosarcoma (67). In order to clarify if the same holds true in a patient based setting, OncoLar, an inhibitor of growth hormone release (downregulating IGF-1 production), was administered in a phase I study to 21 patients with metastatic or recurrent osteosarcoma. Although OncoLar treatment resulted in sustained 40-50% decrease in IGF-1 levels there was no objective tumor response (68).

Immunotherapy

Immunotherapy can be subdivided into passive and active immunotherapy. Passive immunotherapy can be accomplished by administrating anti-tumor antibodies. Active immunotherapy is based on manipulation of components of the immune defense system of a patient to generate anti-cancer auto-immunity. This can be achieved via administration of dendritic cells or vaccines after stimulation with tumor antigens (69). An example is the use of liposomal encapsulated muramyl tripeptide phosphatidyl ethanolamine (MTP-PE) that activates macrophages to become tumoricidal. Administration of this compound prolonged survival in animals with osteosarcoma lung metastases (70, 71). Based on these results this immune stimulatory agent was tested in a large randomized clinical trial by the Children’s Oncology Group for high-grade, intramedullary OS patients. The addition of MTP-PE to standard chemotherapeutic agents and subsequent surgical resection resulted in improvement in 3-year event free survival from 71% to 78%. This observation suggests that addition of MTP-PE to the chemotherapeutic regimen might improve survival of OS patients (72).

Moreover the EURAMOS-I trial, currently recruiting patients, tests if addition of interferon-alpha as maintenance therapy after the postoperative chemotherapy regimen results in better outcome in patients that responded good to pre-operative chemotherapy. The rationale of testing interferon-alpha comes from the observation by Strander et al. that interferon-alpha as adjuvant therapy resulted in increased survival (73).
Alternatively tumor specific monoclonal antibodies can be used. The human epidermal growth factor proto-oncogene (HER2) encodes for a transmembrane receptor belonging to the epidermal growth factor tyrosine kinase receptor family. Overexpression of HER2 correlated with poor prognosis in human cancers (74, 75). Inconsistent findings have been reported for HER2 overexpression in osteosarcoma (76, 77). More recently, Anninga et al. showed that membraneous HER2 overexpression is absent in human osteosarcoma (78). Treatment of osteosarcoma with the HER-2 monoclonal antibody trastuzumab (Herceptin®) is therefore not likely to be effective. This is supported by the observation that trastuzumab had no therapeutic efficacy on osteosarcoma cell lines (79).

A different example is the use of a human monoclonal antibody (105AD7) that mimics the complement regulatory protein CD55 and is capable of stimulating T-cell responses in vivo for the treatment of osteosarcoma. CD55 is often overexpressed by tumors to protect them from complement-mediated lysis. This antibody was administered to 28 osteosarcoma patients in whom it was well tolerated with evidence of clinical response in some cases (80).

**Restoration of the p53-tumor suppressor pathway**

The tumor suppressor p53 can induce cell cycle arrest, DNA repair and apoptosis in response to various types of stress. Abrogation of the p53-pathway is the most common disruption in cancers. A dysfunctional p53-pathway appears critical in the pathogenesis and progression of tumors as it fails to protect against sustaining mutations resulting in malignant transformation.

Restoration of p53 tumor suppressor capacities is an interesting approach to treat cancer. In cancer cells expressing mutant p53, the normal p53 gene can be introduced with e.g. a nonviral polyethylenimine vector (81), transferring-modified cationic liposome (82) or an adenoviral vector (Adp53; see next section adenoviral gene therapy). In tumor cells expressing wild type p53, p53-function might be hampered by p53-inhibitors. Of the p53-inhibitors that have been identified, MDM2 is regarded as the major negative regulator (83, 84). MDM2 exerts its action on different levels. MDM2 binds to p53 thereby inhibiting p53 transcriptional activity, it targets p53 for proteosomal degradation and it causes its nuclear export of p53 (83-86). MDM2 amplification is frequently observed in different tumor types with an overall frequency of 7% in the tested specimens but is especially prevalent in soft-tissue sarcomas (20%) and osteosarcomas (16%) (8). Recently, a potent and selective small molecule inhibitor of MDM2, Nutlin, was
discovered (87). This small molecule demonstrated \textit{in vitro} cell kill in wild-type but not p53-mutant cancer cells. Furthermore, \textit{in vivo} studies revealed tumor regression of subcutaneous osteosarcoma tumors (87). This new compound has not been tested in clinical trials yet, but pre-clinical results of this small-molecule for the treatment of osteosarcoma are promising.

\section*{Adenoviral gene therapy}

Adenoviral vectors for gene therapy can be divided into two different groups: replication-defective and replicating adenoviruses. Replication-defective adenoviruses are excellent tools to deliver foreign genes into mammalian cells (88, 89). Compared to other delivery vehicles they present some advantages including high efficiency of gene transfer, ability to infect non-dividing cells and relative safety of delivering adenoviruses to humans. Genes of interest can be for example IL-12 or p53 to eradicate the target cell via an immune response or apoptosis, respectively (88, 90). The notion that certain mutant viruses have the intrinsic property to selectively replicate in and kill tumor cells, extended the role of viruses from merely passive carriers to active “oncolytic agents” (91). Replicating adenoviruses can also be used as gene delivery tools, but have additional efficacy by their retained ability to replicate and thereby killing the infected cell.

\begin{figure}[h]
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\includegraphics[width=0.5\textwidth]{fig1.png}
\caption{Schematic representation of an adenovirus. Depicted is a single adenovirus (icosahedral capsid) with its binding moieties.}
\end{figure}

\section*{Cancer cell infection; re- and detargeting strategies}

The therapeutic effect of replication-defective and replicating adenoviruses is dependent on adequate tumor cell infection. Adenovirus serotype 5 is commonly used for gene therapy approaches. It contains three major proteins: hexon, penton base and fiber. The fiber can be subdivided into the knob, shaft and tail (figure 1.1). Both the fiber and penton base are involved in the virus cell-entry process. First, the adenoviral knob, which is located at the distal end of the fiber binds the native receptor for adenoviruses serotypes 2 & 5, the coxsackie and
adenovirus receptor (CAR) (92-95). Subsequently, the Arg-Gly-Asp (RGD) motifs of the penton base bind the integrins $\alpha_v\beta_3$ and $\alpha_v\beta_5$ that instigates internalization of the attached viral particle by the cell (96, 97) (figure 1.2). Recent reports have suggested that heparan sulfate proteoglycans (HSG) may also play a role in adenovirus cell-entry (98, 99). The viral particle is internalized through clathrin-mediated endocytosis (100). The virus is disassembled in the endosome and after breakdown of the endosome, the virus is translocated to the nuclear pore complex. It releases its genome in the nucleoplasm and viral genes or transgenes are expressed (94).

However, many cancer cells, including osteosarcoma, express low levels of the native adenovirus receptor (CAR) (101-104). Retargeting of adenoviral vectors to receptors abundantly expressed on osteosarcoma cells is therefore essential. Furthermore, proper adenoviral targeting requires that the virus should be designed to infect only (cancer) cells of interest with high efficiency. The primary receptor for adenoviruses is ubiquitously expressed on normal cells (92). Adenoviruses with expanded tropism via genetic modifications or adapter molecules often retain native tropism and infection of non-target tissues can thus still occur.

Fig. 1.2 Adenoviral cell entry: The adenoviral knob binds the coxsackie and adenovirus receptor (A) and subsequently the penton base binds to the integrins $\alpha_v\beta_3$ and $\alpha_v\beta_5$ (B) that instigates internalization of the attached adenovirus into the target cell (C). This viral endosome is located in the cell (D)
Detargeting of adenoviral vectors

A strategy to reduce infection of non-target cells is to administer the adenovirus directly into the tumor. Several clinical trials have been conducted using intra-tumor injected adenovirus as anti-cancer agent that suggested this is a safe and efficient delivery method (105, 106). Preclinical studies showed that this approach is feasible to induce tumor regression in subcutaneous primary osteosarcoma tumors (107). However, circulating adenoviruses are still detected after intratumoral injection. Wang et al. have shown that intratumoral injected adenovirus in a mouse model can reach the circulation via leaky tumor microvessels (108). Moreover, for osteosarcoma patients, besides local therapy, outcome is dependent on effective systemic treatment to control or eradicate metastatic cells. Systemic application of adenoviruses has some limitations originating from its promiscuous tropism. This results in uptake by normal cells predominantly in the liver (Kupffer-cells and hepatocytes) (109, 110). Therefore, ablation of native tropism might improve the efficacy/toxicity ratio in adenoviral therapy. Ablation of native tropism is predominantly achieved via specific mutations in the adenoviral genome (111-113). Mouse studies revealed that the biodistribution profile of an intravenously delivered adenovirus with ablated CAR-binding was not altered compared to the biodistribution profile of the parental adenovirus (114). Additional ablation of integrin binding was required to reduce infection of normal tissues (109, 115). This was nicely demonstrated by the observation that intravenous administration of an adenovirus unable to bind CAR and integrins resulted in a >700-fold reduction of transgene expression in the liver (109).

Moreover, the fiber of adenovirus type 5, which interacts with HSG, is important for adenoviral infection. Intravenous administration of a triply ablated (for CAR, integrin and HSG binding) adenovirus resulted in a 30,000-fold lower level of liver transduction compared to the conventional adenovirus (116). Theoretically, reduced uptake results in increased circulating adenovirus present for target cells however, distinct prolongation of circulating adenoviruses could not be clearly demonstrated in this study (117).

Retargeting of adenoviral vectors

Retargeting strategies of adenoviral vectors can be roughly divided into two categories: direct genetic modification of capsid proteins and conjugating adenovirus with adapter molecules.
Direct genetic modification of capsid proteins

Adenoviral tropism can be augmented via foreign peptides in the fiber (118, 119). For example, incorporation of the Arg-Gly-Asp (RGD)-containing peptide in the HI-loop of the fiber knob domain has been shown to expand adenoviral tropism towards integrins (107, 118, 120) (figure 1.3B). The RGD-motif was originally identified in tumor vasculature (121). Therefore, insertion of this motif into the fiber knob can potentially increase infection of the tumor neo-vasculature thereby augmenting the tumor cell kill.

A different approach is to exchange parts of the adenovirus fiber protein with that of different Ad serotypes or complete different viruses creating adenovirus carrying chimeric fibers (122-125). By switching the adenovirus fiber the original binding sites are replaced altering the tropism of the new designed adenovirus.

Adenovirus and adapter molecules

In contrast to genetic modifications of the capsid proteins, bispecific adapter molecules can be used to expand tropism to tumor cells. These bispecific adapter molecules are capable of binding the adenovirus fiber knob at one end and a cell-type specific protein at the other, for example a bispecific single chain antibody.
An advantage of this antibody-based approach is its excellent selectivity combined with versatility. A disadvantage of this approach is that the targeting moiety is not part of the adenoviral capsid. Therefore, when this approach is applied in replicating adenoviruses, it is of importance that during replication the targeting moiety is replenished.

**Cancer selective replication**

Replicating vectors have the advantage above non-replicating adenoviral vectors that they can overcome low transduction efficiency by allowing replication and subsequent spread to neighboring tumor cells (figure 1.4) (133). To limit viral replication and subsequent cell kill in non-target, normal tissues, replication should be restricted to cancer cells only (conditionally replicative adenoviruses; CRAds). Two different approaches to obtain this cancer selective replication have been tested. The first approach is based on placing early adenoviral genes under control of tumor specific promoters. The second strategy introduces deletions into early adenoviral genes to achieve cancer cell specific oncolysis.

![Fig. 1.4 Replicating adenoviral vectors. (A) cancer cell infection (B) viral replication. (C) Eventually viral replication will lead to cell lysis with spread of adenoviral vectors to neighbouring tumor cells.](image)

**Essential early adenoviral genes regulated by tumor specific promoters**

The adenoviral E1 genes are the first genes expressed after infection and key regulators of adenoviral replication. An approach to induce cancer selective replication is placing viral essential genes (like E1A) under transcriptional control of tumor specific promoters. This restricts viral replication to target tissues in
which this promoter is activated by specific stimuli. Some examples are prostate specific antigen for prostate cancer (134), α-fetoprotein for hepatocellular carcinoma (135) and MUC1 for breast cancer (136). For the treatment of osteosarcoma lung metastases and prostate cancer bone metastases a CRAd with E1A driving under the osteocalcin promoter has shown promising results (137, 138).

**Deletion of early adenoviral genes**

A second strategy for optimizing tumor selectivity is via introduction of deletions in adenoviral E1 genes to ablate functions required for viral replication in normal cells but not in cancer cells. One of the most tested CRAds is dl1520 (ONYX-015) that lacks the E1B-55kDa protein. It was designed to replicate in p53 mutant cancer cells (91). Recently it has been shown that loss of E1B-55kDa late viral RNA export, rather than p53 inactivation determined its selectivity (139). Several clinical trials with ONYX-015 using different administration routes (intratumoral, intravenous and intra-arterial) have been performed for several cancer types and metastases including head and neck cancer, metastatic colorectal cancer, advanced sarcomas and lung metastases (106, 140-143). These trials showed safety of the vector and minimal anti-cancer response. To improve the therapeutic effect in patients this CRAd was combined with chemotherapeutic agents, which resulted in objective tumor responses in some cases (144-146).

Another often applied attenuated vector is the conditionally replicating adenovirus Ad5-Δ24 also called dl922-947 (147, 148). This CRAd lacks 24 base pairs in the pRb-binding domain of E1A. The binding of E1A to pRb activates E2F. This induces S-phase progression and creates an environment suitable for viral replication. Mutated E1A in Ad5-Δ24 cannot bind to pRb and E2F is not activated. Therefore, this CRAd is limited to replicate in cancer cells with disrupted pRb-pathway in which E2F is constitutively active. It has been reported that the Ad5-Δ24 CRAd is more oncolytic compared to ONYX-015 (148-151). Insertion of the gene encoding p53 augmented the cell killing properties of this CRAd dramatically (152). However, cells expressing high levels of p53-inhibitors like MDM2 did not benefit from exogenous delivered p53 (153). Another strategy to increase the cell killing properties of CRAds is via insertion of toxic genes. Expression of a toxic product should be limited to target cells only and should not adversely affect viral replication. Linking transgene expression to adenoviral promoters activated late in the replication cycle can circumvent this (154).
Chapter 1

Aim and outline of this thesis

Over the last two decades several clinical trials for the treatment of osteosarcoma all have indicated that the 5-year survival rate has reached a plateau at 50-70%. Especially patients with poor response to chemotherapy or with detectable metastatic disease have a poor outcome. The aim of this study was to explore the use of virotherapy, chemotherapy and Nutlin for the treatment of (metastatic) osteosarcoma.

A previous study from our group has shown that CAR expression is low on primary osteosarcoma cells (103). Retargeting adenoviral vectors towards different receptors like EGFR and integrins is essential to achieve adequate adenoviral infection and thus therapeutic effect (103, 107). Intratumoral injection of an integrin-targeted CRAd in subcutaneous osteosarcoma tumors in nude mice resulted in a significant tumor growth delay strongly suggesting the suitability for CRAds as novel treatment strategy for local osteosarcoma (107).

In the first part of this thesis (chapter 2-4) we focus on enhancing the infection and cell killing effect of adenoviral gene therapy for osteosarcoma. In the second part of this study (chapter 5-8) we address the difficulties of systemic administration of adenovirus for the treatment of metastatic osteosarcoma.

In literature there is some discussion about CAR-expression on primary osteosarcoma cells and subsequently the need for retargeting adenoviral vectors for osteosarcoma treatment. In chapter 2 we analyze CAR expression on primary osteosarcoma cells and the effect of an adenoviral vector with augmented tropism on primary osteosarcoma cell infection.

A new treatment modality will likely be tested in addition to the existing therapy regimen. Previous reports have shown that chemotherapeutic agents in combination with CRAds resulted in additional or even synergistic combination effects. Based on these findings, we explore in chapter 3 the cell killing effect of a conditionally replicative adenovirus with doxorubicin or cisplatin on osteosarcoma cell lines and primary osteosarcoma cells.

In chapter 4 we address the question if restoring or increasing p53 levels in osteosarcoma cells can be used as therapy. We explore the use of non-replicating and replicating adenoviruses encoding p53, and of Nutlin, a potent and selective small molecule antagonist of MDM2, the major negative regulator of p53.

As discussed before, osteosarcoma patients with overt metastatic disease have a poor prognosis. The majority of all metastases are localized in the lungs. In
chapter 5 we explore the use of an intravenously delivered CRAd for the treatment of human osteosarcoma lung metastases in a nude mouse model. The cell killing effect of systemically delivered CRAds can be augmented by insertion of a toxic gene. In order to limit toxicity to non-target cells (normal cells) the toxic transgene should only be expressed in the cancer cells. Non-ablated CRAds can infect normal cells but replication is severely hampered. To increase the safety profile of these “armed” CRAds the toxic gene is placed under control of an adenoviral promoter, activated late in the adenoviral replication cycle (major late promoter). Expression of this transgene is thereby linked and restricted to the replication profile of the CRAd. In chapter 6 we tested this concept using a CRAd with a luciferase gene under control of the major late promoter.

Efficient systemic cancer treatment with CRAds is dependent on adequate delivery and infection. Previous studies have shown that ablation of native tropism resulted in reduced uptake of normal tissues like the liver after intravenous administration. Osteosarcoma primary cells are devoid of CAR, but do express the epidermal growth factor receptor (EGFR). Based on these findings, we designed an EGFR-targeted CRAd with ablative native tropism for CAR and integrin. In chapter 7 we tested its targeting profile on several cell lines, primary osteosarcoma cells and on human liver slices ex-vivo.

We extended this approach in chapter 8 and engineered an adenovirus that lacked all known adenovirus interaction sites with cellular receptors (ablated in CAR, integrin and HSG binding). We tested the targeting and detargeting properties of this virus on cell lines, liver slices ex-vivo and in vivo.

Finally in chapter 9 we summarize and discuss the results presented in this thesis.

References
General Introduction


General Introduction


