Targeting chondrosarcoma and osteosarcoma cell metabolism : the IGF pathway and beyond

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Chapter 1

General introduction and outline of this thesis

Primary bone cancers are a heterogeneous group of cancers that originate from the bone. They account for less than 0.2 % of all cancers (1). In the Netherlands, 228 primary bone cancers have been diagnosed in 2016 (2). This heterogeneous group of cancers differ in terms of etiology, clinical manifestation and prognosis. Here, we focus on the two most common subtypes: osteosarcoma and chondrosarcoma.

Osteosarcoma

Osteosarcoma is the most frequent primary malignant bone tumour. This tumour is characterized by the production of osteoid matrix. The incidence of osteosarcoma has a bimodal distribution, with the first peak around puberty and the second, smaller peak, observed above the age of 60. The incidence is 5.0 (4.6-5.6) per million 0-19 year olds (3, 4). The second peak in incidence is likely to represent a secondary malignancy, as half of the osteosarcoma patients above 60 years suffer from Paget disease of the bone (5). In addition, osteosarcoma is the most common radiation-induced sarcoma (5-7). Most primary osteosarcomas occur in the metaphysis of the long bones, in particular the distal femur (30%), the proximal tibia (15%) and the proximal humerus (15%), where bone growth is particularly active, especially at the pubertal age of onset of this tumour (4, 5).

Treatment of osteosarcoma consists of pre-operative and postoperative chemotherapy in combination with surgical resection of the tumour. Limb salvage has largely replaced amputation for local tumour control. Several chemotherapeutic agents have been tested in clinical trials, in which the currently used combination of doxorubicin, cisplatin and methotrexate was found to be the most effective (8). Despite the harsh chemotherapeutic regime, about 45% of the patients develop distant metastases, most often in the lungs (85%) (8, 9). Since the introduction of the chemotherapy in the 1980s survival has reached a plateau of 65-70% (10, 11). Therefore, new treatment options for osteosarcoma are desperately needed.

Osteosarcoma can be divided in several subtypes (5, 12). The focus of this thesis is on conventional osteosarcoma. Conventional osteosarcoma can be further

subdivided, based on the predominant matrix, in the osteoblastic (76-80% of the cases), the chondroblastic (10-13%) and the fibroblastic (10%) variant (5, 13, 14). Less common subtypes of conventional osteosarcoma are the giant cell rich, osteoblastoma-like, epithelioid, clear cell and chondroblastoma-like subtype (5). Although the proportion of good histological responders, defined as >90% chemotherapy induced tumour necrosis, differs significantly between the conventional osteosarcoma subtypes, there is no difference in the overall survival (14).

Osteosarcoma cell of origin

The cell of origin of osteosarcoma is not well defined (15). However, two candidate progenitor cells are discussed in literature. The first hypothesis is that osteosarcoma arises from mesenchymal stem cells (MSCs). MSCs have the capability to differentiate into multiple lineages, such as myocytes, adipocytes, chondrocytes and osteocytes. These lineages of differentiation are respectively initiated by MyoD, PPARγ, SOX9, and osterix/Runx2 (16, 17). The capacity of MSCs to differentiate in multiple lineages resembles the histological spectrum observed in osteosarcoma (18). In addition, it has been suggested that the timing of the mutation can explain the different stages of differentiation observed in osteosarcoma, where an early genetic or epigenetic alteration may result in the development of an highly aggressive, undifferentiated osteosarcoma and vice versa (17, 19). Further evidence favouring the theory that osteosarcoma arises from MSCs comes from *in vitro* and *in vivo* studies, and from observations in the clinic. *In vitro*, our group and others have shown that mouse MSCs can undergo spontaneous transformation after long time culturing *in vitro* (18, 20-23) and that these MSCs formed osteosarcomas upon *in vivo* grafting (18). However, human MSCs do not show spontaneous *in vitro* transformation, even after prolonged culturing (24-27). In addition, publications reporting spontaneous human stem cell transformation have been retracted (28, 29) and can most likely be attributed to cross-contamination. Clinical observations also provide evidence favouring the mesenchymal stem cell theory, as osteosarcoma formation is found in patients following bone-marrow transplantation for unrelated diseases (30, 31). In addition, the existence of primary extraskeletal osteosarcoma supports this theory as in this rare subtype, osteosarcoma arises in tissues where no osteoblasts are present. The second theory hypothesizes

that osteosarcomas arise from osteoblast-committed cells. Evidence for this theory stems mostly from studies in mice that show that osteoblast specific knockout of p53 (and Rb) can induce murine osteosarcoma (32-36). More specifically, Walkley *et al*. and Berman *et al*. made an osterix-cre-mediated deletion of p53 and pRb, which resulted in osteosarcoma formation (32, 35). As mentioned above, osterix is a transcription factor that is required for osteoblast differentiation and bone formation (37). Interestingly, p53 (and Rb) knockdown in undifferentiated mesenchymal cells also results in the formation of several sarcomas, among which osteosarcoma is the most frequent one (33). One study by Quist *et al*. compared osteosarcoma formation in Prx1-Cre, Collagen-1α1-Cre and Osteocalcin-Cre conditional disruption of p53 and Rb to transform undifferentiated mesenchyme, preosteoblasts and mature osteoblast, respectively (36). The Prx1 and Collagen-1α1 had a near complete penetrance of osteosarcoma, whereas only 44% of the osteocalcin-cre mice mice developed osteosarcomas (36). Although there seems to be increasing evidence supporting the first hypothesis, the differentiation state of the progenitor cells remain a point of debate among those who study this disease.

The IGF pathway

The IGF pathway has two closely related ligands: IGF1 and IGF2. IGF2 is an imprinted gene, only expressed from the paternal allele; IGF1 is mainly produced by the liver upon stimulation by the growth hormone (GH). The GH is produced by the pituitary gland and under control of the hypothalamus, as the hypothalamus produces the growth hormone releasing hormone (GHRH) and the growth hormone-inhibiting hormone (GHIH) (also known as somatostatin) which balance determines the amount of GH that is released. Besides the function of IGF1 and IGF2 as endocrine hormones, they can also act in autocrine and paracrine manners (38). In the circulation, six IGF binding proteins (IGFBPs) can bind IGF1 and IGF2 (39). IGFBPs are expressed in many peripheral tissues, except for IGFBP1 which is mainly produced by the liver (40). These binding proteins increase the half-lives of the IGF ligands, but they also interfere in the interaction between the IGF ligands and their receptors. IGFBP3 is the most abundant protein, which forms a ternary complex with insulin-like growth factor acid-labile subunit (IGFALS) and accounts for 80% of all IGF binding.

IGF1 and IGF2 can bind to the IGF1 receptor (IGF1R). Unlike IGF1, IGF2

can additionally bind to the insulin receptor (IR) with high affinity (Figure 1). Two monomeric isomers of the IR have been identified: IRA (lacking exon 11) and IRB (with exon 11). These alternative splice variants differ in the C-terminus, where they influence receptor-ligand interaction (41). IGF2, in addition to binding to the IR and the IGF1R, can bind the IGF2 receptor (IGF2R). The IGF2R does not confer intracellular signalling but functions as a decoy receptor to decrease the availability of IGF2 to the IGF1R (42). Upon ligand binding, the IGF1R can form homodimers or hybrid receptors with the IR. The α chains of the IGF1R (which are located extracellular) induce tyrosine autophosphorylation of the β chains (which span the membrane) (43). This phosphorylation recruits the downstream signalling protein insulin receptor substrate (IRS) to the cell membrane (43). Phosphorylation of IRS creates binding sites for SH2 domain of various proteins, which finally leads to the activation of the PI3K/AKT/mTOR pathway and the Ras/Raf/ERK signalling pathways (43). Both these pathways are involved in many cellular functions, such as cell growth, proliferation and differentiation (44).

Indications for IGF pathway involvement in osteosarcoma genesis

In normal development, IGF1R signalling plays a key role in the growth and development of bone. This is illustrated in mice, as IGF1R knockout mice are approximately 55% smaller compared to IGF1R wildtype mice (45). Interestingly, in dogs, the size of different breeds is dependent on IGF1 plasma levels (46). Due to the role of the IGF pathway in normal bone development, the IGF pathway has been studied for decades in osteosarcoma. Interestingly, the peak incidence of osteosarcoma correlates with the increased levels of GH and IGF ligands in puberty (Figure 2). Furthermore, two studies suggest that height at diagnosis is a risk factor of osteosarcoma (47, 48). In addition, a few cases of osteosarcoma in patients with acromegaly are reported (49) and patients with a congenital IGF1 deficiency seem protected against cancer (50). Multiple GH treated patients presented with osteosarcoma (51, 52).

Strikingly, a recent study identified mutations in IGF signalling genes in 7% and IGF1R amplification in 14% of osteosarcoma cases (53). These clinical observations combined with the role of the IGF pathway in normal bone development strongly suggest a role for the IGF pathway in osteosarcoma genesis.

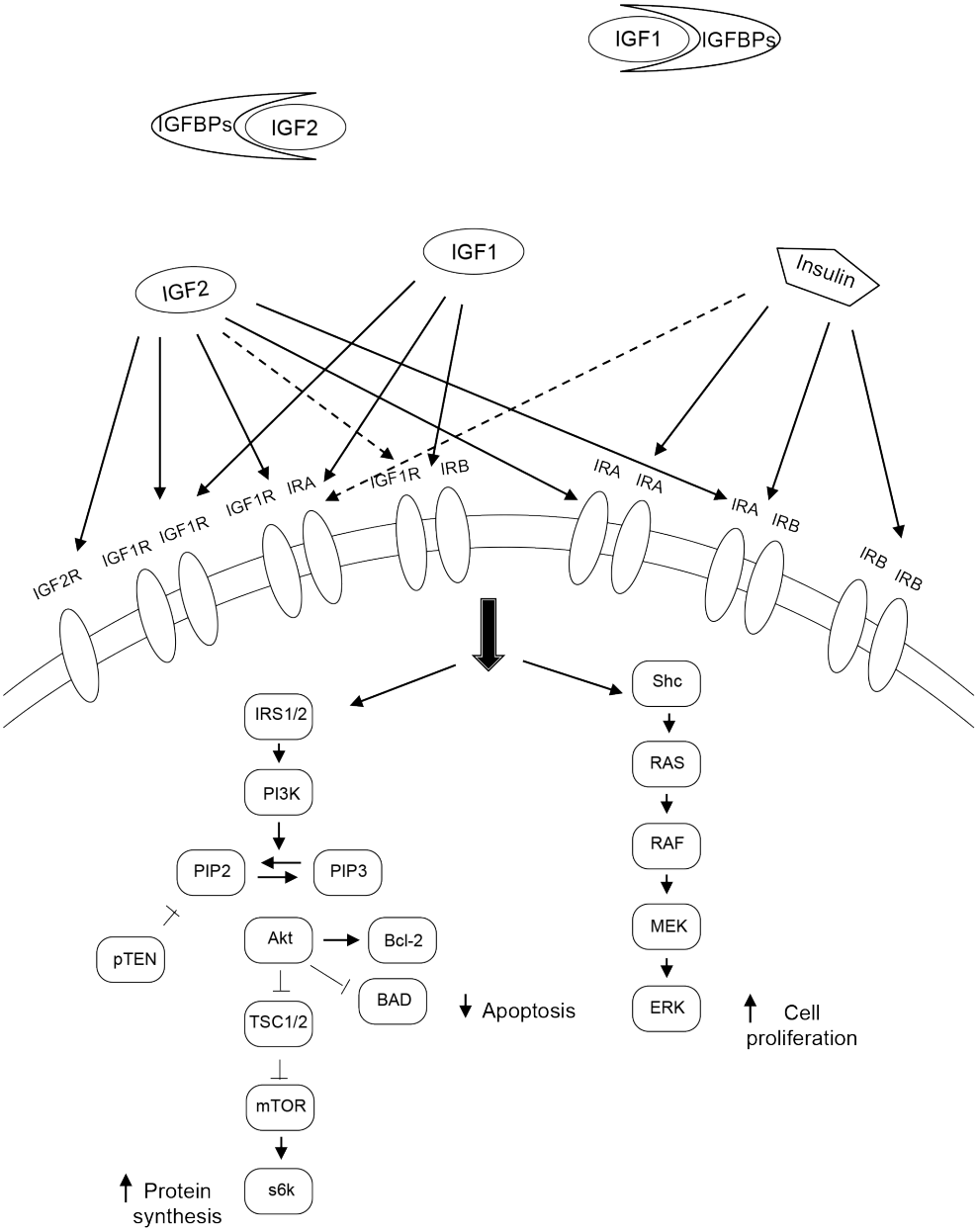

Figure 1. The components of the IGF pathway, adapted from van Maldegem *et al*. (54).

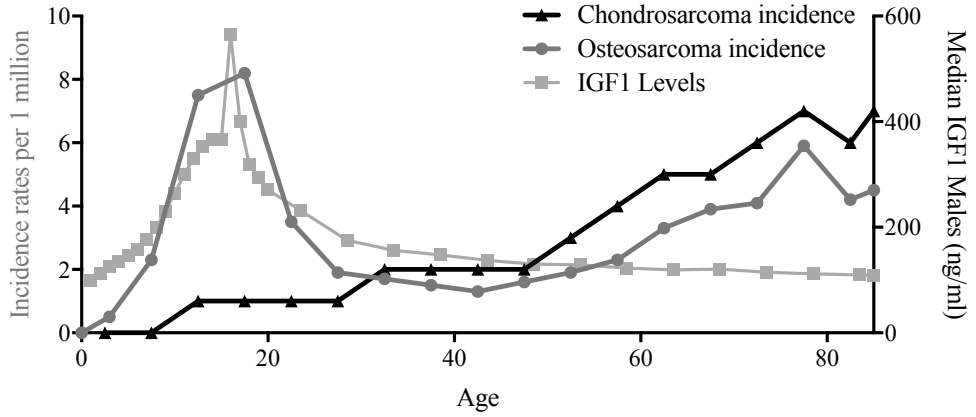

Figure 2. The peak in IGF1 levels correlates to the peak incidence in osteosarcoma. Osteosarcoma incidence rates, chondrosarcoma incidence rates and IGF1 levels were derived from Mirabello *et al*. (55), WHO Classification of bone tumours 2013 and the Mayo Clinic, respectively.

Chondrosarcoma

Chondrosarcoma is the second most common type of malignant bone tumour. It accounts for 20% of all bone cancers and is characterized by the production of cartilage. It is most common in the 3rd to 6th decade of life (Figure 2), and most frequently occurs in the bones of the pelvis, the ribs, and bones of the extremities. In rare cases, they occur in the small bones of the hands and feet, or in the skull (56). Conventional chondrosarcoma is the most common subtype that accounts for 85% of the cases (57). This subtype can be histologically subdivided into atypical cartilaginous tumour (ACT), grade II and grade III chondrosarcomas (58). The ACT, previously known as grade I, accounts for 61% of the cases. The first line treatment is curettage with local adjuvant treatment, resulting in a 5-year survival rate of 95%. Grade II (36%) and grade III (3%) chondrosarcomas have a worse 5-year survival of 86% and 58%, respectively, due to the occurrence of metastases (59-61). These tumours are treated by *en bloc* resection with wide resection margins to prevent local recurrence. Chondrosarcoma patients with unresectable disease, due to tumour location, tumour size or extensive metastatic disease, have a 5 year survival of only 2% (62, 63). These chondrosarcoma patients slightly benefit from doxorubicin-based chemotherapy, which increases the 3 year survival from 8% to 26% (62).

Chondrosarcoma either arises in the metaphysis or epiphysis of the bones, in which case it is called a central chondrosarcoma, or it arises on the surface

of the bone, in which case it is called a peripheral chondrosarcoma. Central and peripheral differ in aetiology, demonstrated by their different genetic background (Figure 3).

Central chondrosarcomas

In addition to primary chondrosarcoma, where tumours arise without a precursor lesion, secondary chondrosarcomas can also arise, in which the tumour arises from a benign precursor lesion. In case of a central chondrosarcoma, this benign precursor lesion is the enchondroma (Figure 3). Enchondromas show a predilection for the small bones of the hands and feet, and occur over a wide age distribution (64). Ollier disease and Maffucci syndrome, where patients present with multiple enchondromas, suggest the presence of an underlying genetic alteration. Indeed, gain of function mutations in *isocitrate dehydrogenase 1 and -2 (IDH1 and -2)* have been identified as driver mutations of enchondromas (65-67). 52%- 87% of the enchondromas harbour an *IDH1/2* mutation (65, 66).

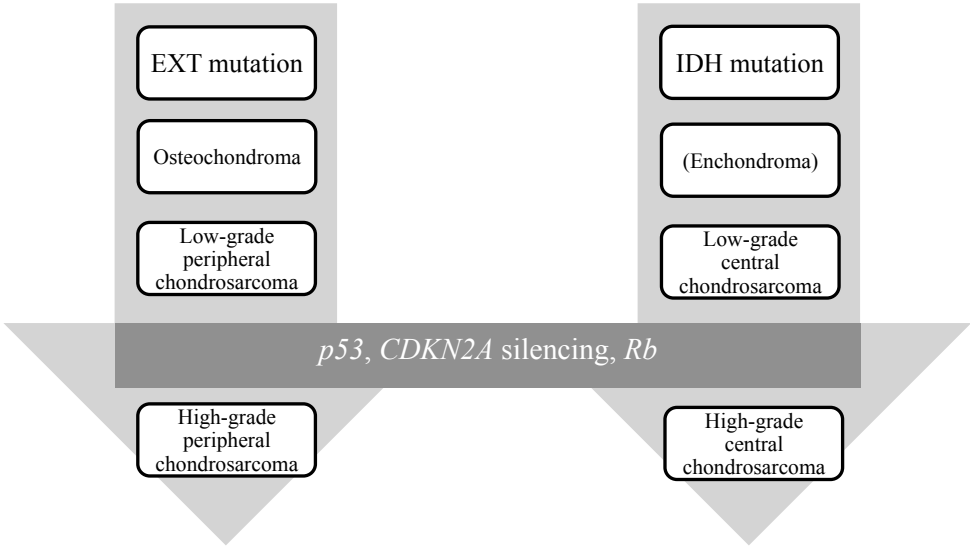

Figure 3. Chondrosarcoma genetics. *EXT* mutations have been identified as potential driver mutations of osteochondromas, the benign precursor lesions of peripheral chondrosarcomas. *IDH1/2* mutations can give rise to enchondromas, which can than develop into central chondrosarcomas. However, central chondrosarcomas can also occur without the presence of a benign precursor lesion. Mutations in *p53* and *retinoblastoma (Rb)* and *CDKN2A* silencing have been identified in the development of low-grade to high-grade lesions.

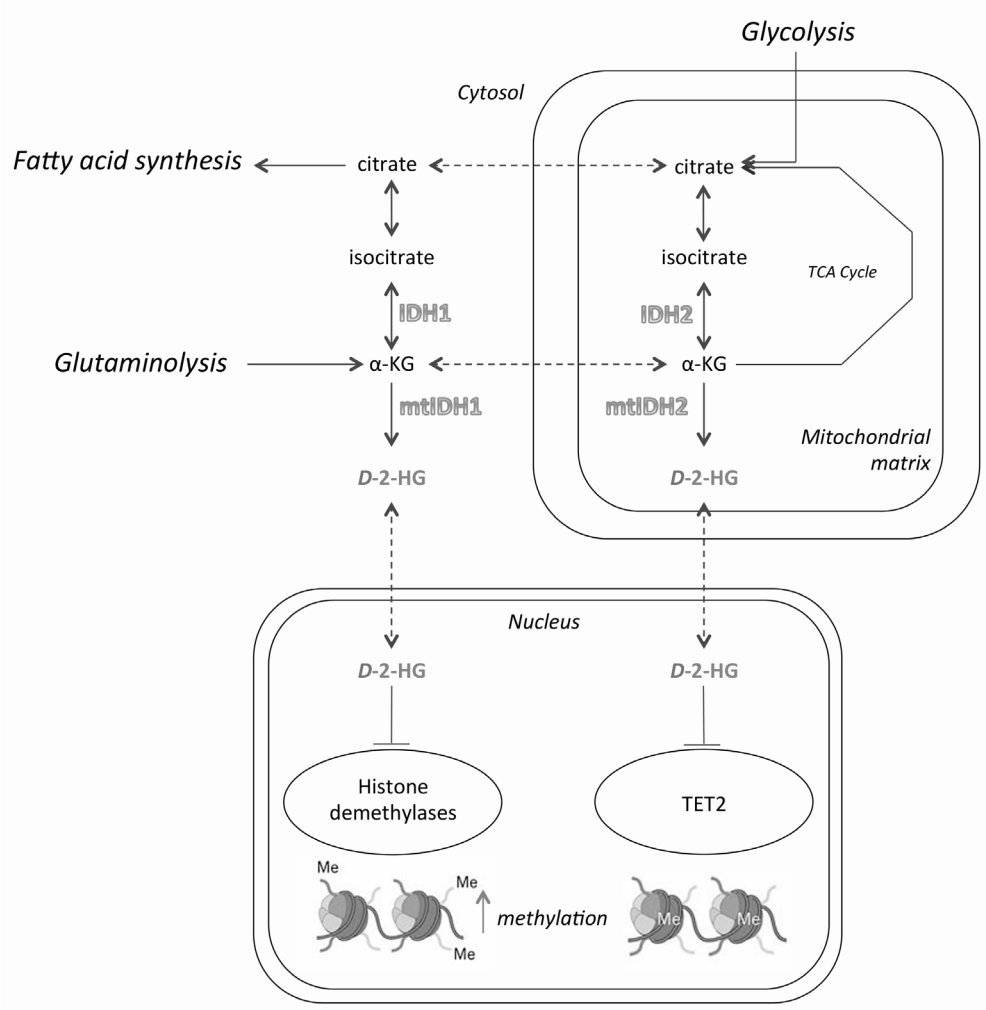

Figure 4. Schematic representation of the cellular consequences of the *IDH1* and the *IDH2* mutation. Mutations in *IDH1/2* result in the production of *D*-2-HG. *D*-2-HG inhibits histone demethylases and TET2. TET2 catalyses the conversion 5-methylcytosine to 5-hydroxymethylcytosine, the first step in DNA demethylation.

IDH1 and IDH2 are essential enzymes in cell metabolism, as they convert isocitrate to α-ketoglutarate (α-KG) in respectively the cytoplasm and the mitochondria (Figure 4). The mutant enzyme acquires the activity to convert α-KG to *D*-2-hydroxyglutarate (*D*-2-HG), an oncometabolite that competitively inhibits the α-KG dependent enzymes by the high structural similarities (68). More specifically, *D*-2-HG inhibits histone demethylases and tet methylcytosine dioxygenase 2 (TET2)(68). TET2 is essential

for the conversion of the modified genomic base 5-methylcytosine to 5-hydroxymethylcytosine, the first step in cytosine demethylation. An aberrant methylation phenotype is observed in *IDH1/2* mutated gliomas (69-72), leukaemia's (73) and enchondromas (66).

Peripheral chondrosarcomas

Peripheral chondrosarcomas always arise secondary from osteochondromas. Osteochondromas are benign lesions, characterized by bony projections with a mature hyaline cartilaginous cap. They are most commonly located adjacent to the growth plate of the long bones (Figure 3) (74). Malignant transformation from a solitary osteochondroma to a secondary peripheral chondrosarcoma only occurs in 1% of the cases. Multiple osteochondromas are found in approximately 15% of the patients (75). When this is the case a patient has the hereditary autosomal dominant syndrome 'Multiple Osteochondromas' (MO), which is caused by a germline mutation in the *exostosin-1* (*EXT1*) or – *2* (*EXT2*) gene (74-76). In the 10-15% of the multiple osteochondroma patients where no point mutation in *EXT1* or *EXT2* can be identified, somatic mosaicism with large genomic deletions is likely the underlying mechanism for osteochondroma genesis(77, 78). Somatic homozygous deletions of *EXT1* can also be found in sporadic osteochondromas (74, 75). Strikingly, secondary peripheral chondrosarcomas no longer contain the *EXT* mutations, suggesting that these arise from the wildtype *EXT* cells present in the lesion (79).

EXT1 and EXT 2 are essential for heparan sulfate synthesis as they catalyse the elongation of the heparan sulfate chains. Heparan sulfate is a proteoglycan that regulates Indian Hedgehog (IHH) signalling in the growth plate (80, 81). IHH acts together with the parathyroid hormone-related protein in a negative feedback mechanism that regulates the balance between chondrocyte proliferation and differentiation in the growth plate (81-83). When IHH signalling is impaired due to a mutation in *EXT1* or *EXT2*, this results in less chondrocyte differentiation and increased chondrocyte proliferation, thereby causing osteochondroma genesis.

Rare chondrosarcoma subtypes

Dedifferentiated chondrosarcoma accounts for 10% of chondrosarcoma

patients (84). It is a highly malignant variant, reflected by the 28% 10-year survival that is seen in patients diagnosed without metastasis (85). Patients that are diagnosed with metastasis have an even worse prognosis, as only 10% survives the first two years (85). Dedifferentiated chondrosarcoma has a typical histology, as two clearly distinct compartments can be observed. The first is a well-differentiated cartilage tumour, usually an enchondroma or a low-grade conventional chondrosarcoma. This component is juxtaposed to a dedifferentiated, high-grade non-cartilaginous sarcoma (86). The dedifferentiated component determines patient prognosis, and is therefore the focus of research that aims to identify new therapeutic strategies.
In addition to genetic alterations that are present in many cancer types, such as *p53* mutation, *PTEN* deletion, *MYC* amplification and *MDM2* amplification (86-88), mutations in *IDH1/2* have been identified in 54% of the dedifferentiated chondrosarcomas (86).

Mesenchymal chondrosarcoma is a rare aggressive subtype in which distant metastasis can be identified even after 20 years (89-91). It accounts for 3% of all chondrosarcoma cases, and has a 10-year survival of 43% (92). Small round undifferentiated cells with islands of differentiated cartilage histologically characterize this subtype. In addition to its occurrence in the skeleton (mainly in the craniofacial bones, the ribs and the vertebrae), 20-30% of the cases present with primary soft tissue localization (64, 89). Tumour localization in the jaw is a bad prognostic factor, as well as presentation with metastases, while a young age is potentially a good prognostic factor (93, 94).

In contrast to dedifferentiated chondrosarcomas, no mutations in *IDH1/2* have been identified. Furthermore, mutations in *p53*, deletions in *p16* and *MDM2* amplifications are rare events (86, 95, 96). Interestingly, mesenchymal chondrosarcomas carry the HEY1-NCOA2 fusion gene (97). Furthermore, in a case where this fusion was absent, an IRF2BP2-CDX1 fusion gene has been identified (98). This demonstrates that mesenchymal chondrosarcoma has a distinct genetic background.

Clear cell chondrosarcoma is a low-grade variant of chondrosarcoma, with a mortality of ~15% (99). It is the most rare subtype, as it accounts for only 2% of

all chondrosarcoma cases. While metastases are rare, clear cell chondrosarcomas recur locally after curettage in 86% of the cases (100, 101). Interestingly it has a male: female ratio of 2.6:1, and is therefore the only subtype that has a sex preference (100). It derived its name by the histological presentation, showing clear cells that are surrounded by hyaline cartilage (99). It is most commonly located at the epiphyseal ends of long bones, and in line with conventional chondrosarcoma, the best treatment option is *en bloc* resection with clear margins (99, 100). Clear cell chondrosarcomas have widespread chromosome gains and losses, often with hemizygous involvement of the *p16* locus. No alterations in *IDH1/2*, *p53* and *MDM2* have been reported (86).

Chondrosarcoma cell of origin

Originally, it was believed that chondrosarcoma originates from remnants of the growth plate cartilage. Although the chondrosarcoma cell of origin remains uncertain, it is now widely supported that chondrosarcomas most likely originate from MSCs undergoing chondrogenic differentiation (102). The formation of chondroid callus during bone facture healing demonstrates the lifetime presence of these cells in bones. Furthermore, the existence of extraskeletal chondrogenic neoplasms suggests that these cells are even present outside the skeleton (103). A strong argument favouring the MSC as the cell of origin for central chondrosarcoma is provided by a study from our group, in which elevated levels of *D*-2-HG (the oncometabolite induced by an *IDH1/2* mutation) blocked osteogenic differentiation and variably promoted chondrogenic differentiation of MSCs (104). For secondary peripheral chondrosarcoma, compelling evidence comes from studies in zebrafish and mice. Chondrocytes in *Ext2*-null zebrafish have been shown not to undergo terminal differentiation, and pre-osteoblasts failed to differentiate towards osteoblasts (105). In mice, *Ext1* inactivation in chondrocytes resulted in osteochondromas formation, the precursor lesion of secondary peripheral chondrosarcomas, thereby demonstrating that osteochondromas originate from proliferating chondroctyes in the growth plate cartilage (106, 107).

There are striking parallels between normal cartilage growth and differentiation and cartilage tumours (108). Histologically, distinct chondrosarcoma subtypes show high similarities with different zones of chondrocyte differentiation in the

normal growth plate (106). In addition, an analyses that evaluated expression levels of chondrogenesis-relevent genes demonstrated that grade I tumours cluster with chondrocytes, while grade III tumours cluster with earlier stages of MSCs (107). Due to these parallels, increasing our understanding of normal cartilage development will increase our understanding of chondrosarcoma genesis.

Targeting chondrosarcoma cell metabolism

Already in 1924, Otto Heinrich Warburg observed that cancer cells metabolize glucose in a different way than normal differentiated cells: while normal cells use mitochondrial oxidative phosphorylation to produce their energy, tumour cells mainly produce their energy via glucose fermentation, even in the presence of sufficient oxygen (110, 111). This phenomenon, later called "the Warburg effect", was the first indication that cancer cell metabolism differs from normal cell metabolism. The aerobic glycolysis used by cancer cells is an inefficient way to generate adenosine 5'-triphosphate (ATP), but might be required to allow the production of nucleotides, amino acids and lipids, which are essential for cell proliferation (112). Despite the fact that the Warburg effect was identified almost a decade ago, relatively little progress had been made in increasing the understanding of how altered cell metabolism contributes to carcinogenesis. However, the recent identification of mutations in TCA cycle enzymes in several cancers, among which mutations in *IDH1* and *IDH2*, renewed the interests in cancer cell metabolism as this demonstrates that enzymes involved in cell metabolism play an important role in tumourigenesis. In 2011, Hanahan and Weinberg recognized the reprogramming of energy metabolism as a hallmark of cancer (113). The identification of mutations in TCA cycle enzymes opened new therapeutic possibilities.

When AGI-5198 was identified as a specific IDH1 mutant inhibitor (selective for the R132H mutation) (114), the field of chondrosarcoma immediately recognized its potential as chondrosarcoma therapy. However, our group has shown that treatment with AGI-5198 does not influence the tumorigenic properties of chondrosarcoma cells (115). In *IDH2* mutant leukaemia, an initial proliferation burst followed by cellular differentiation was observed upon inhibition of the IDH2 mutant enzyme (116, 117). Furthermore, in *IDH1* mutant glioma, the initially described impaired cell proliferation upon IDH1 mutant inhibition (114) could not be confirmed in other studies (118, 119).

In contrast to gliomas (120-122), the *IDH1/2* mutation status does not correlate with chondrosarcoma survival, and does not cause loss of 5-hydroxymethylcytosine or altered histone modifications in central chondrosarcomas (123). This suggests that while the *IDH1* or *-2* mutations are an early event in tumorigenesis, at later stages other processes involved in chondrosarcoma progression render these cells independent of the mutant IDH1/2 enzymes. Therefore, we propose to target the altered metabolism caused by the *IDH1/2* mutations as therapeutic strategy for chondrosarcoma.

One pathway that is linked to cell metabolism and has been suggested to play a role in chondrosarcoma progression is the IGF pathway (124). IGFBP3, the binding protein that accounts for 80% of the IGF1 and IGF2 binding, is lower expressed in enchondromas than in normal growth plate cartilage, and expression levels decrease with increasing grades of chondrosarcoma (125). Furthermore, it has been described that IGF1 induces migration of chondrosarcoma cell lines, which can be blocked by an IGF1R antibody (126). Together with collaborators from the Ludwig Center at Dana-Faber/Harvard, our group performed functional profiling of Receptor Tyrosine Kinases in chondrosarcomas (127). IGF1Rβ and IRβ hypermethylation was identified in 1 out of 5 cell lines tested; an IGF1R/IR inhibitor could inhibit proliferation of this cell line (127). Taken together, these studies suggest a potential role for the IGF pathway in chondrosarcoma development, migration and proliferation.

It has been described that the *IDH1/2* mutation influences nicotinamide adenine dinucleotide (NAD^+) synthesis. More specifically, metabolic profiling of glioblastoma cells upon mutant IDH1 inhibition revealed nicotinamide phosphoribosyltransferase (NAMPT) as a therapeutic target for *IDH1/2* mutated tumours (128). An increased sensitivity for NAMPT inhibitors correlated with a decreased expression of nicotinic acid phosphoribosyltransferase (NAPRT) caused by an increased methylation of the NAPRT promoter (128). NAMPT and NAPRT are rate-limiting enzymes of the primary salvage pathway and the Preiss-handler pathway, respectively, which are involved in NAD^+ synthesis. Tumour cells depend on these pathways for their rapid NAD turnover, as they lack expression of key enzymes in the de novo synthesis of NAD^+ from tryptophan (129-131).

Another main metabolic pathway that has been identified as being altered by the *IDH1/2* mutations is glutaminolysis (132-137), an important energy-producing pathway in tumour cells. Glutamine, a non-essential amino acid, can subsequently be converted to glutamate and α-ketoglutarate by glutaminase and glutamate dehydrogenase, respectively, thereby fuelling the TCA cycle and the *D*-2-HG production in *IDH1/2* mutated cells. *IDH1/2* mutated cells are more sensitivity to several compounds that interfere with glutaminolysis compared to *IDH1/2* wildtype cells, such as CB-839, metformin and phenformin. CB-839 is an inhibitor of glutaminase that decreases *D*-2-HG levels and inhibits growth of *IDH1/2* mutated acute myeloid leukaemia cells (138). An endogenous heterozygous knock-in of the R132H *IDH1* mutation sensitizes breast epithelial cells to metformin, the first in line antidiabetic drug, and its lipophilic analogue phenformin (133). Therefore, inhibition of NAD^+ synthesis or glutaminolysis potentially leads to synthetic lethality with the mutated IDH1/2 enzyme in chondrosarcoma.

Aim and Outline of the thesis

The aim of this thesis was to develop new therapeutic strategies by identifying cellular pathways that are essential for chondrosarcoma and osteosarcoma cell survival.

Chapters 2, 3 and 4 are focussed on osteosarcoma. Although osteosarcoma is a rare disease, many preclinical experiments with osteosarcoma cells have been published. In **Chapter 2**, a systemic analysis of the literature on osteosarcoma *in vitro* studies is given to determine how the quantity of these studies developed over time. The downside of this huge number of studies is discussed from a researcher's perspective. **Chapter 3** reports on the analyses of genome-wide gene expression data, where overexpression of the IGF pathway in osteosarcoma is identified compared to osteoblasts and MSCs. Osteosarcoma cell lines were treated with an IGF1R/IR dual inhibitor.[1] In **Chapter 4**, a commentary on a similar transcriptional profiling study is given. An overview of where we are with IGF1R-directed therapy, where we need to go and how we get there is presented.

1. The gene set analysis described in Chapter 3 was performed by dr. M.L. Kuijjer. E.F.P. Peterse performed the Western blotting and the Proliferation assays.

Chapters 5, 6 and 7 are focussed on chondrosarcoma. In **Chapter 5**, the potency of targeting the IGF pathway in chondrosarcoma is explored, as previous studies suggested the IGF pathway as a potential therapeutic target. IGF1R expression levels are evaluated in cartilage tumours, and expression of IGF1R signalling mediators and sensitivity for IGF1R inhibition is evaluated in chondrosarcoma cell lines. In chapters 6 and 7, metabolic pathways altered by the *IDH1/2* mutation are explored as therapeutic strategy. **In Chapter 6**, interference in NAD^+ synthesis pathway is evaluated, as vulnerability for NAD^+ depletion is reported for *IDH1/2* mutant cells. Chondrosarcoma cell lines were treated with NAMPT inhibitors, and NAPRT expression and methylation was evaluated in cartilage tumours using qRT-PCR and genome-wide methylation arrays. **Chapter 7** describes a preclinical study evaluating the effect of interfering in chondrosarcoma cell glutaminolysis. The expression of glutaminase, the first essential enzyme of glutaminolysis, is compared between low-grade and high-grade chondrosarcomas to see if this pathway is involved in chondrosarcoma progression. In addition, chondrosarcoma cell lines are treated with the glutaminase inhibitor CB-839, and the compounds metformin, phenformin and chloroquine that all inhibit glutaminolysis at different levels. In **Chapter 8**, results described in chapters 2 to 7 are discussed and future perspectives are given.

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Chapter 2

In vitro studies of osteosarcoma: A researcher's perspective of quantity and quality

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Abstract

Osteosarcoma is a primary malignant bone tumour, for which no improvement in survival rate has been made since the nineteen seventies. We set out to systemically identify the *in vitro* studies performed in the past two decades describing potential future therapies. Strikingly, we obtained a total of 5282 PubMed hits on this subject. The amount of publications has increased almost exponentially over the past few years. Studies from Chinese institutes are mainly responsible for this huge increase, accounting for 53% of the publications in 2015. Approximately 1/3 of all drugs described in the past three years could be classified as traditional medicine. Furthermore, it struck our attention that even though in such studies multiple cell lines are essential to represent the heterogeneity in patients, many studies were performed with only one or two cell lines, i.e. U-2 OS or MG-63. These cells are fast growing, facilitating rapid experimental application but also boosting drug responsiveness. This probably explains why so many *in vitro* studies have been published for this relatively rare disease. Furthermore, it illustrates the current publication pressure, especially in China.

1. Introduction

Osteosarcoma is the most frequent primary malignant bone tumour. It has a peak incidence between 10 and 14 year of age and only 30% of all osteosarcomas occur in individuals aged > 40 (1). Osteosarcoma is a rare disease, with an annual incidence rate of approximately 4.4 per 106 for people aged 0–24 years (1). Several subtypes of osteosarcoma can be distinguished, of which conventional high-grade central, or intramedullary osteosarcoma, is by far the most common (75% of the cases). Osteosarcoma is characterized by the production of osteoid matrix and is located mostly at the metaphysis of long bones. In addition to surgery patients receive intensive pre- and postoperative chemotherapy (2). Although neo-adjuvant chemotherapy has markedly improved outcome, since its introduction in the 70ties survival has reached a plateau of about 60–70% (3). Especially osteosarcoma presenting with metastases at diagnosis has a particular poor outcome. Therefore, new treatment options are needed. As osteosarcoma is a rare disease, international collaborations are essential for the conduction of clinical trials. The European and American osteosarcoma study group (EURAMOS), started its first trial in 2005, in which 2260 patients from 326 centres across 17 countries were enrolled

(2). This largest osteosarcoma study to date could be achieved by a committed collaboration between four well established study groups. However, due to the absence of consensus and regional differences in compound approval, a second study has not emerged yet, which is especially discouraging now this successful worldwide network has been established (2).

2. Almost exponential increase in amount of osteosarcoma *in vitro* studies

To see how the quantity of osteosarcoma *in vitro* studies developed overtime, we set out to systematically identify all the drugs that have been tested on osteosarcoma cells *in vitro* in the past two decades. A PubMed search strategy was compiled. To our surprise, we got a total of 5282 hits in PubMed. It struck our attention that the number of publications on treatment of osteosarcoma cell lines with various compounds has increased almost exponentially (Figure 1A). After importing these PubMed hits in EndNote X7, we obtained an indication of how the amount of publications developed in China, the USA, India, Australia, Canada and Europe (defined as countries that are in the Schengen Area and/or the European Union) in the past few years by searching for these countries in the authors address box. The majority of the publications came from China, Europe or the USA. The amount of publications from the USA showed a minor increase, from 45 in 1996 to 73 in 2015, whereas the amount of publication from Europe increased substantially from 35 in 1996 to 176 in 2015. Strikingly, the amount of publications from China rose from 1 to 359, thereby mainly being responsible for the huge increase in publications observed in the last few years. China has been the leading country since 2012, and 53% of the publications from 2015 involved Chinese institutes; this is a much higher proportion than reported for other research areas such as haematology research (4). We read all the abstracts of the hits of the past three years (n=1755), and categorized them based on non-osteosarcoma (n=297, many studies on other cancers metastasizing to the bone), no *in vitro* study (n=179), no drugs study (n=596, many studies performed siRNA knockdown only) and no inhibitory effect (n=126), leaving us with 560 studies from the last three years, a relatively large number when considering the rareness of this disease. Interestingly, approximately 1/3 of the compounds could be classified as traditional medicine, of which over 80% was published by Chinese authors. For example, we encountered several compounds such as

Evodiamine, a chemical extracted from the *Tetradium* genus of plants (5) and crude extracts of *Rheum palmatum* L., the root of Chinese rhubarb (6).

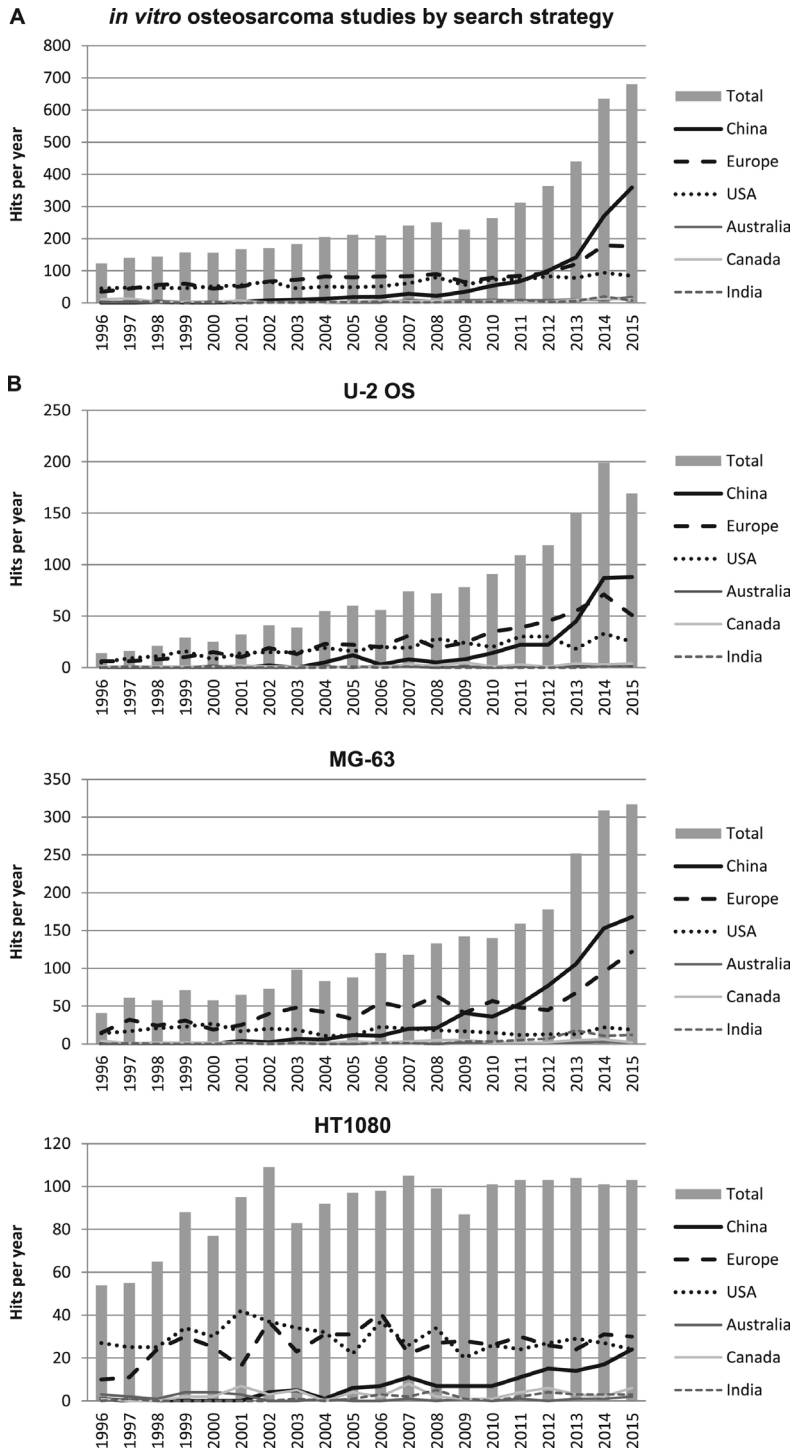

Figure 1. The amount of hits from PubMed searches per year. A: The amount of osteosarcoma *in vitro* studies greatly increased in the past few years, with an increased proportion of articles with Chinese authors. B: Amount of PubMed hits per year for U-2 OS (osteosarcoma), MG-63 (osteosarcoma) and HT-1080 (fibrosarcoma) demonstrates that the exponential increase in *in vitro* studies is not identified in other sarcomas.

3. The use of a single cell line

Remarkably, many studies were performed with only one cell line, either U-2 OS or MG-63. Due to the high heterogeneity of osteosarcoma, studying a panel of cell lines instead of a single cell line is essential for eventual clinical applicability. U-2 OS and MG-63 are both ATCC cell lines established in 1964 and 1977 respectively. Searching PubMed for U-2 OS (and U2OS/U2-OS/U-2-OS) and MG-63 (and MG63) identified respectively 1449 and 2564 publications from 1996 to 2015, while the combination only resulted in 163 PubMed hits. Determining the amount of publications per year and per country for these cell lines as described above further demonstrated the almost exponential increase in publications with osteosarcoma cell lines attributable to studies from Chinese institutes (Figure 1B). To compare these results with another ATCC sarcoma cell line with a comparable amount of total publications, we performed an identical search for HT-1080 (fibrosarcoma), in which we did not see this trend (Figure 1B), demonstrating that this is not a trend observed in all ATCC sarcoma cell lines. U-2 OS and MG-63 are ubiquitously applied also outside the osteosarcoma field for general cell biology studies because they are among the few human cell lines that are relatively susceptible to transfection. In addition, these cells grow rapidly facilitating experimental application but also boosting drug responsiveness (7). This probably explains why so many *in vitro* studies have been published for this relatively rare disease.

4. A researcher's perspective

There may be a jewel hidden in this avalanche of studies, but it is difficult to discern due to the huge quantity of papers using questionable designs. If *in vitro* studies are performed, researchers should always include a panel of cell lines to represent the tumour heterogeneity in osteosarcoma patients. As a researcher, it is impossible to keep track of all the research that is being published,

which may lead to missing promising therapeutic targets. The publication of these relatively easy executable studies is obviously fed by the incentive of "publish or perish", but it keeps scientists occupied with often irrelevant work, it usurps financial budgets and obscures relevant investigations. Other major drivers of the enormous increase in research papers is the substantial increase in the number of academic journals (8). Aggressive editor's requests to submit manuscripts to new journals keep filling a scientist's mailbox, which can be quite disturbing. This trend to prevail quantity over quality is occupying precious time from editors and reviewers. Currently, the Science Citation Index is used for medical career evaluation in the majority of large Chinese hospitals, resulting in a huge pressure of Chinese medical doctors to publish articles and an increase in number of publishing scientists (9, 10). Therefore, it is essential that the evaluation system on research performance will be changed with the focus shifted from quantity to quality as was recently again advocated that bibliometrics are warping science (11). Our analysis of *in vitro* osteosarcoma studies illustrates the effect of the increased publication pressure since the convenience of osteosarcoma cell lines renders them into low hanging fruit but results in studies with limited scientific value, which constrains solutions for this deadly disease that affects young patients.

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Chapter 3

IR/IGF1R signaling as potential target for treatment of high-grade osteosarcoma

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Abstract

Background: High-grade osteosarcoma is an aggressive tumor most often developing in the long bones of adolescents, with a second peak in the 5th decade of life. Better knowledge on cellular signaling in this tumor may identify new possibilities for targeted treatment. Methods: We performed gene set analysis on previously published genome-wide gene expression data of osteosarcoma cell lines (n=19) and pretreatment biopsies (n=84). We characterized overexpression of the insulin-like growth factor receptor (IGF1R) signaling pathway s in human osteosarcoma as compared with osteoblasts and with the hypothesized progenitor cells of osteosarcoma – mesenchymal stem cells. This pathway plays a key role in the growth and development of bone. Since most profound differences in mRNA expression were found at and upstream of the receptor of this pathway, we set out to inhibit IR/IGF1R using OSI-906, a dual inhibitor for IR/IGF1R, on four osteosarcoma cell lines. Inhibitory effects of this drug were measured by Western blotting and cell proliferation assays. Results: OSI-906 had a strong inhibitory effect on proliferation of 3 of 4 osteosarcoma cell lines, with IC_{50}s below 100 nM at 72 hrs of treatment. Phosphorylation of IRS1, a direct downstream target of IGF1R signaling, was inhibited in the responsive osteosarcoma cell lines. Conclusions: This study provides an *in vitro* rationale for using IR/IGF1R inhibitors in preclinical studies of osteosarcoma.

Background

High-grade osteosarcoma is the most prevalent primary malignant bone tumor. The disease occurs most frequently in children and adolescents at the site where proliferation is most active, ie the metaphysis adjacent to the epiphyseal plate (1). The 5-year overall survival of osteosarcoma patients has raised from 10-20% to about 60% after the introduction of preoperative chemotherapy in the 1970s. However, about 45% of all patients still die because of distant metastasis. No additional treatments have been found that can increase survival significantly, and administering higher doses of preoperative chemotherapy does not result in improved outcomes (2, 3). Better knowledge on cellular signaling in high-grade osteosarcoma may identify

new possibilities for targeted treatment of this highly aggressive tumor.

We have previously described the roles of bone developmental pathways Wnt, TGFβ/BMP, and Hedgehog signaling in osteosarcoma, but unfortunately so far could not identify suitable targets for treatment (4, 5). In addition to these signal transduction pathways, insulin-like growth factor 1 receptor (IGF1R) signaling plays a key role in the growth and development of bone. Aberrant signaling of this pathway has been implicated in various cancer types, among others sarcomas (6, 7). Key players of insulin-like growth factor (IGF) signaling are the ligands IGF1, IGF2, which are circulating polypeptides that can be expressed in endocrine, paracrine, and autocrine manners, and the tyrosine kinase receptor IGF1R, which forms homodimers, or hybrid receptors with the insulin receptor (IR) (8). IGF1R and IR/IGF1R hybrids are activated by both IGF1 and -2, which trigger autophosphorylation of IGF1R and subsequent downstream signal transduction. A second IGF receptor, IGF2R, can bind IGF2, but does not confer intracellular signaling, thereby diminishing the bioavailability of IGF2 to IGF1R (9). Autophosphorylation of IR/IGF1R receptors recruits the signaling proteins insulin receptor substrate (IRS) and Src homology 2 domain containing transforming protein (Shc) to the cell membrane, which get phosphorylated and subsequently activate the downstream PI3K/Akt and Ras/Raf/ERK signaling pathways, both of which are known to be important in cancer. These pathways ultimately act on several biological processes, such as transcription, proliferation, growth, and survival (9-11). Interestingly, treatment targeted against IGF1R signaling has shown to be effective in a subset of Ewing sarcoma, another bone tumor that manifests at young age (12).

The role of the IGF1R pathway in growth has been illustrated in studies of knockout mice. It was shown that IGF1 null mice are 40% smaller than littermates, while IGF1R null mice are approximately 55% smaller (13). In dogs, the size of different breeds was demonstrated to be dependent on IGF1 plasma levels (7). Additionally, a specific IGF1 SNP haplotype was described to be common in small breed dogs and nearly absent in giant breeds (14). Interestingly, large and giant dog breeds are more prone to develop osteosarcoma (15), which in dogs is biologically very similar to the

human disease (16). Two recent studies on human osteosarcoma suggest a positive correlation between patient birth-weight and height at diagnosis and the development of the disease (17, 18). Involvement of some members of IGF1R signaling in osteosarcoma has been described (as has been reviewed in Kolb *et al*. (19), but the activity of this pathway remains to be determined.

We have analyzed genome-wide gene expression in high-grade osteosarcoma cell lines and pretreatment biopsies, and observed significantly altered activity of genes involved in IGF1R signaling when compared to profiles of mesenchymal stem cells and osteoblasts. Specifically, upstream inhibitors of IGF1R signaling were found to be downregulated in osteosarcoma, and low expression of these genes correlated with worse event-free survival. We inhibited IR/IGF1R signaling with the dual IR/IGF1R inhibitor OSI-906. This showed inhibition of phosphorylation of IRS1 and of strong inhibition of proliferation in 3/4 osteosarcoma cell lines. Interestingly, the cell line which could not be inhibited with OSI-906, 143B, has a k-ras oncogenic transformation, which is a component of the Ras/Raf/ERK pathway, one of downstream effectors of IGF1R signaling. These results suggest that IR/IGF1R signaling may be an effective targeted for treatment of high-grade osteosarcoma patients.

Methods

Cell culture

The 19 high-grade osteosarcoma cell lines that were used in this study were characterized and are described in Ottaviano *et al*. (20). The 12 mesenchymal stem cell and 3 osteoblast cultures were previously described (21). MSCs have been previously (22) characterized through FACS analysis and have been tested for their ability to be committed under proper conditions towards adipogenesis, chondrogenesis and osteogenesis as described in Bernardo *et al*. (23). Osteoblast cultures were derived from MSCs which were treated to undergo osteogenic differentiation. Cell line DNA was short tandem repeat profiled to confirm cell line identity with use of the Cell ID system of Promega (Madison, WI). For Western blotting experiments, cells were maintained in RPMI 1640 (Invitrogen, Carlsbad, CA), supplemented with 10% fetal bovine

serum (F7524, Sigma-Aldrich, St. Louis, MO) and 1% glutamax (Gibco 35050, Invitrogen, Carlsbad, CA).

Microarray experiments, preprocessing, and data analysis

For genome-wide gene expression analysis, we used Illumina Human-6 v2.0 BeadChips. Microarray experiments and data preprocessing are described in Kuijjer *et al*. (21). Previously deposited genome-wide gene expression data of mesenchymal stem cells (MSCs) and osteoblasts can be found in the Gene Expression Ombinus (GEO accession number GSE28974 and GSE33382, respectively). Data from osteosarcoma cell lines have been published before (24), but since we normalized and processed all raw data together, we deposited normalized values in the Gene Expression Omnibus (GEO, accession number GSE42351, superseries accession GSE42352). Data from the 84 high-grade osteosarcoma pretreatment biopsies have been previously published (GEO accession number GSE33382) (21). Ethical guidelines of the individual European partner institutions were followed and samples and clinical data were handled in a coded fashion and stored in the EuroBoNeT biobank. We determined significant differential expression between osteosarcoma cell lines (n=19) and mesenchymal stem cells (n=12), and between osteosarcoma cell lines and osteoblasts (n=3) using Bioconductor (25) package *LIMMA* (26) in statistical language R (27). Probes with Benjamini and Hochberg false discovery rate-adjusted P-values <0.05 were considered to be significant. Gene set analysis was performed on KEGG pathways (28) (Release 63.0, July 1, 2012) using R-package globaltest (29). For each analysis, the top 15 significant KEGG pathways were returned. All returned pathways had a Benjamini and Hochberg false-discovery rate-corrected P-value $<1 \cdot 10^{-5}$. To visualize differential expression in the IGF1R pathway, we performed Core analyses using Ingenuity Pathways Analysis (IPA, Ingenuity Systems, www.ingenuity.com).

Antibodies and reagents

Rabbit monoclonal and polyclonal antibodies against IGF1R and IRS1, respectively (both 1:1,000) were obtained from Cell Signaling (Danvers, MA). Rabbit polyclonal antibody against phospho-IRS1 (Y612, 1:1,000) was purchased from Biosource, Invitrogen (Carlsbad, CA). A mouse monoclonal

antibody against α-tubulin from Abcam (Cambridge, UK) was used as a loading control (1:3,000). Secondary antibodies (both 1:10,000, BD Transduction Laboratories, Lexington, KY) were horseradish peroxidase (HRP) conjugated polyclonal goat-anti-rabbit IgG for components of the IR/IGF1R pathway, and HRP conjugated polyclonal goat-anti-mouse for α-tubulin. OSI-906 was purchased from Selleck Chemicals LLC (Houston, TX).

Western blotting

Osteosarcoma cell lines OHS, KPD, SAOS2, and 143B were treated with 0.5% DMSO or with 1 μM OSI-906 for 3 hrs, and were subsequently lysed using Mammalian Protein Extraction Reagent (Thermo Scientific 78503), to which Halt Phosphatase and Protease Inhibitor Cocktails (Thermo Scientific 78420 and 78418, respectively) were added according to the manufacturer's protocol. Concentrations of cell lysates were determined using the BioRad DC Protein Assay Kit (Biorad, Hercules, CA). Per sample, 20 μg of protein was loaded on SDS-PAGE gels. Lysate of HepG2-A16 cells transfected with IR and stimulated with insulin, containing 10 μg of protein, was taken along as a positive control. Western blotting was performed as described in Schrage *et al*. (30).

Proliferation assays

OSI-906 was diluted in DMSO and stored at -20°C. OHS, SAOS2, KPD, and 143B cells were plated in 96 wells plates, using 4,000, 2,000, 12,000, and 2,000 cells per well, respectively. After 24 hrs, OSI-906 was added in triplicate at different concentrations of 0 nM, 0.01 nM, 0.1 nM, 1 nM, 10 nM, 100 nM, 1 μM, and 10 μM. The inhibitor was incubated for 72 hrs and 96 hrs, in different experiments. The results shown are representative results from at least three independent experiments. Cell proliferation reagent WST-1 (Roche) was incubated for 2 hrs and subsequently measured using a Wallac 1420 VICTOR2 (Perkin Elmer, Waltham, MA). Data were analyzed in Graphpad Prism 5.0 (www.graphpad.com). Relative IC_{50}s were calculated using results from the different concentrations up to the highest dose where toxicity was not yet present.

Results

Enrichment of IGF1R signaling in high-grade osteosarcoma

Genome-wide gene expression data were of good quality for all cell lines. *LIMMA* analysis resulted in 7,891 probes encoding for differentially expressed (DE) genes between osteosarcoma cell lines and MSCs, and 2,222 probes encoding for DE genes between osteosarcoma cells and osteoblasts. We tested the global expression patterns of KEGG pathways using globaltest (29) and determined the intersection of the pathways most significantly different in osteosarcoma cell lines as compared with MSCs, and of osteosarcoma cell lines as compared with osteoblasts. This approach resulted in five significantly affected pathways – insulin signaling pathway, oocyte meiosis, ubiquitin mediated proteolysis, progesterone-mediated oocyte maturation, and glycerophospholipid metabolism. Details of the globaltest are shown in Table 1. IGF1R signaling is involved in three out of the five detected KEGG pathways (insulin signaling pathway, oocyte meiosis, and progesterone-mediated oocyte maturation). Interestingly, a globaltest on mRNA expression of previously published pretreatment biopsies (21) compared with normal bones (31) also returned insulin signaling as the most significantly affected pathway (data not shown). Notably, there is no specific IGF1R signaling pathway in the KEGG database (28). Because of the over-representation of IGF1R signaling, and because of its known role in cancer, we decided to study expression of members of this pathway in detail.

Differentially expressed genes of the IGF1R pathway

To determine which genes have the most specific up- or downregulation in osteosarcoma, we combined lists of significantly differentially expressed genes of osteosarcoma cell lines (n=19) and a previously published set of osteosarcoma pretreatment biopsies (n=84, GEO accession GSE33382) in comparison with mesenchymal stem cells (n=12) and osteoblasts (n=3) by four-way Venn analysis of all significantly affected probes with the same direction of fold change (upregulated or downregulated in all four analyses). We identified *IGFBP4* and *GAS6* as the most downregulated genes in osteosarcoma (average log fold changes of -4.43 and -4.29, respectively). *IGFBP2* was also present in the top 20

Table 1. Globaltest results.

KEGG pathway	Analysis	adj*P*	Statistic	Expected	Std.dev
Insulin signaling pathway	OScellvsOB	$1.01 \cdot 10^{-7}$	26.34	4.76	1.92
	OScellvsMSC	$3.07 \cdot 10^{-15}$	35.12	3.33	1.78
Oocyte meiosis	OScellvsOB	$2.70 \cdot 10^{-7}$	37.45	4.76	2.9
	OScellvsMSC	$5.04 \cdot 10^{-16}$	53.7	3.33	2.84
Ubiquitin mediated proteolysis	OScellvsOB	$3.21 \cdot 10^{-7}$	22.88	4.76	1.75
	OScellvsMSC	$5.04 \cdot 10^{-16}$	37.99	3.33	1.89
Progesterone-mediated oocyte maturation	OScellvsOB	$7.16 \cdot 10^{-7}$	34.26	4.76	2.71
	OScellvsMSC	$1.34 \cdot 10^{-15}$	55.35	3.33	2.77
Glycerophospholipid metabolism	OScellvsOB	$1.40 \cdot 10^{-6}$	27.13	4.76	2.25
	OScellvsMSC	$2.25 \cdot 10^{-15}$	55.86	3.33	2.82

The top five significant pathways with aberrant expression in osteosarcoma cell lines versus osteoblasts (OScellvsOB) and osteosarcoma cell lines versus mesenchymal stem cells (OScelssvsMSC). adjP: FDR-adjusted p-value, Statistic: test statistic of the globaltest, Expected: expected test statistic of the globaltest, Std.dev: standard deviation under the null hypothesis.

results from this four-way analysis. In addition, *IGFBP3* and *-7* were significantly downregulated, and *IGF2BP3* was significantly upregulated in three out of the four analyses. Both *IGFBP4* and *GAS6* show high variability in expression in osteosarcoma cell lines and biopsies (Figure 1A). Patients of whom biopsies had very low expression of these genes had poor event-free survival profiles (log-rank test for trend, $P = 0.01$ for *IGFBP4* and $P = 0.04$ for *GAS6*, Figure 1B). To visualize mRNA expression of the IGF1R signaling pathway members, we used Ingenuity Pathways Analysis on *LIMMA* toptables from osteosarcoma cells as compared with mesenchymal stem cells and from osteosarcoma cells as compared with osteoblasts (Figure 2). As can be seen in this figure, overlap of differentially expressed genes between these analyses was detected upstream of IGF1R.

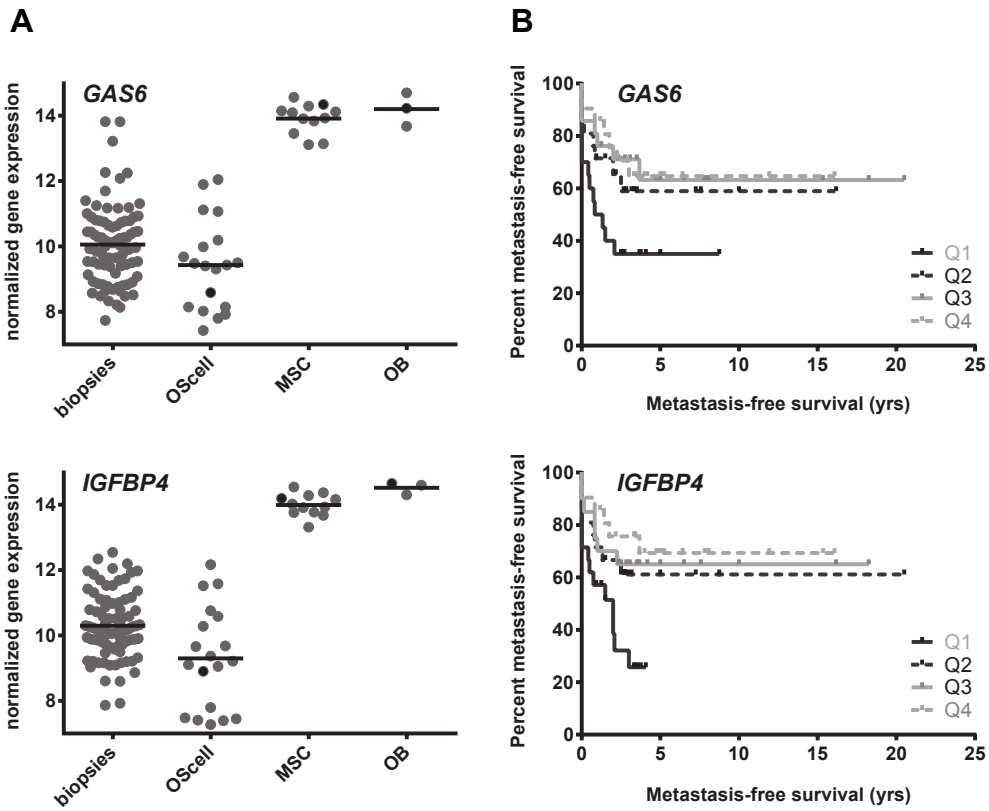

Figure 1. mRNA expression of *GAS6* and *IGFBP4*. **A:** Normalized gene expression levels of *GAS6* and *IGFBP4* in osteosarcoma biopsies, cell lines, mesenchymal stem cells (MSCs), and osteoblasts (OB). Expression of both proteins is considerably higher in the controls (FDR-adjusted P<0.001 for both genes in all four analyses). **B:** Kaplan-Meier curves depicting metastasis-free survival in years for 83 high-grade osteosarcoma patients (for 1/84 patients, we did not have follow-up data available), based on quartiles of mRNA expression of the genes of interest.

OSI-906 inhibits phosphorylation of IRS1

Gene expression levels of IGF1R and IRS1 were validated at the protein level by Western blot analysis (data not shown). To determine the activity of IR/IGF1R signaling, we performed Western blot analysis on cell lysates of OHS, KPD, SAOS2, and 143B, using antibodies against IRS1 and phosphorylated IRS1, before and after treatment with OSI-906, a selective small molecule dual kinase inhibitor of both IR and IGF1R, as IRS1 is a direct downstream target of IGF1R. An inhibition of intrinsic IRS1 phosphorylation at Y612 was detected after treatment with OSI-906 in all cell lines (Figure 3), indicating that this inhibitor could affect signaling downstream IGF1R in osteosarcoma cells.

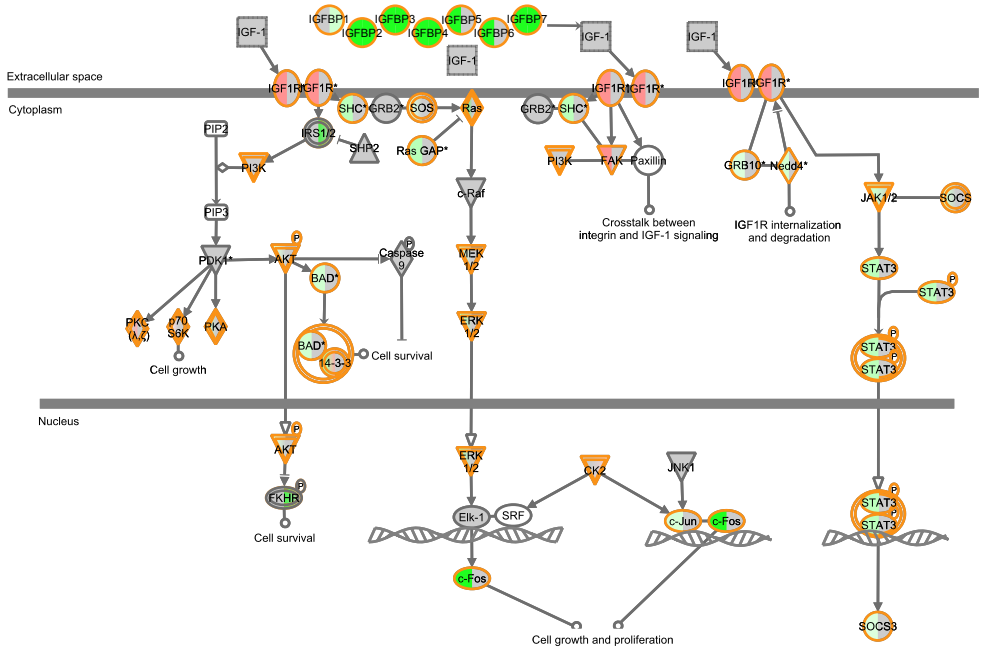

Figure 2. Ingenuity pathways analysis canonical pathway IGF1 signaling. This figure shows the IGF1 signaling pathway, with significantly upregulated genes in red, downregulated genes in green, and genes that did not meet our criteria for significance in gray. The left part of the symbols shows the analysis of osteosarcoma cell lines as compared with mesenchymal stem cells, the right part as compared with osteoblasts. Most consensus in gene expression is found upstream IGF1R signaling, in the expression of the IGF binding proteins.

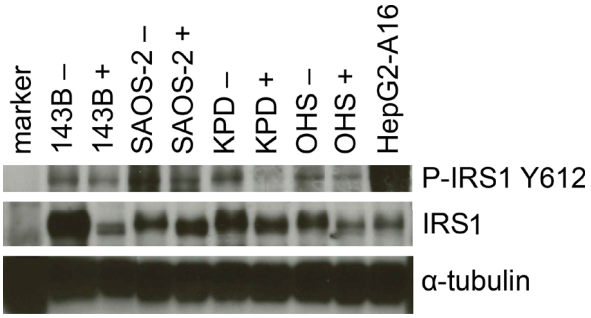

Figure 3. Validation of IR/IGF1R downstream signaling. Western blot of IRS1 and p-IRS1 of lysates of untreated (–) osteosarcoma cell lines OHS, KPD, SAOS2, and 143B, and of these cells treated for 3 hrs with 1 μM of OSI-906 (+).

OSI-906 inhibits proliferation of 3 of 4 osteosarcoma cell lines

In 3 of 4 osteosarcoma cell lines tested, inhibition with OSI-906 was dose-dependent (Figure 4). Except for a toxic response at the maximum dose of 10 μM (data not shown), there was no effect on 143B. Because of this toxicity, relative IC_{50}s were determined using measurements until 1 μM. OHS, SAOS2, and KPD had an IC_{50} of 25 nM, 92 nM, and 90 nM at 72h, respectively, and of 37 nM, 57 nM, and 23 nM at 96h of inhibition, respectively. At 1 μM OSI-906, approximately 60% of proliferation of OHS, SAOS2, and KPD cells was inhibited, while 143B proliferation was not inhibited (Figure 4).

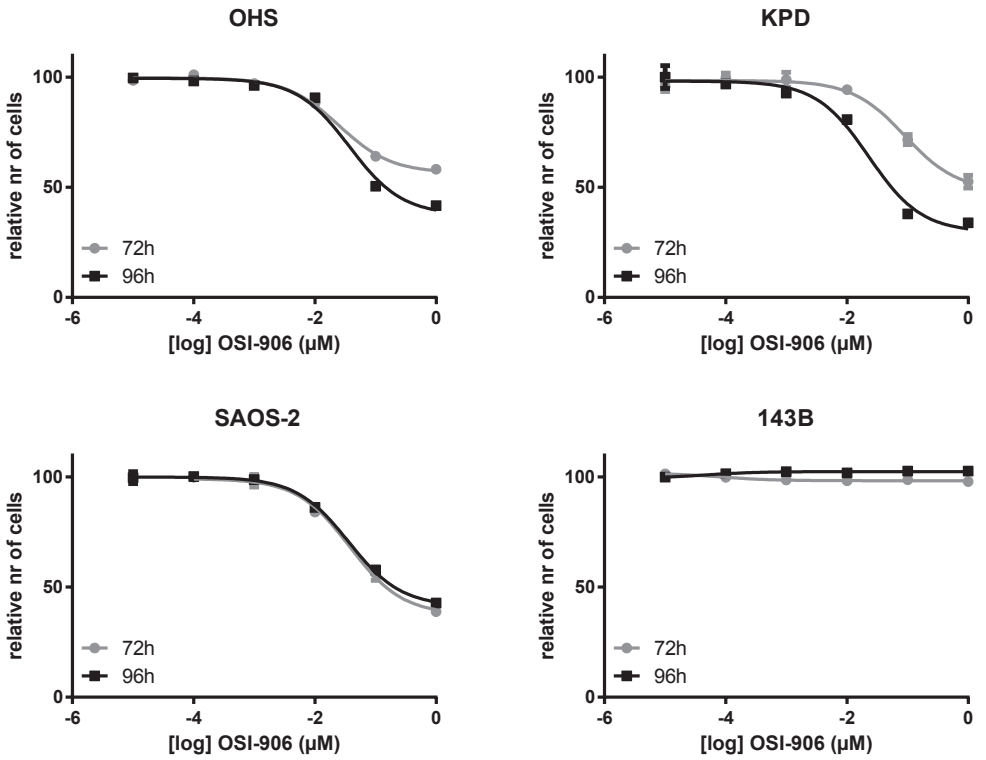

Figure 4. Inhibition of osteosarcoma cell lines with OSI-906. Osteosarcoma cell lines were inhibited with different concentrations of OSI-906, for 72 (gray line) or 96 (black line) hours. OHS, KPD, and SAOS2 showed a dose-dependent inhibition, while 143B did not respond to OSI-906.

Discussion

Genome-wide gene expression and subsequent gene set analysis on osteosarcoma cell lines and biopsies indicated increased insulin-like growth factor signaling in high-grade osteosarcoma as compared with the hypothesized osteosarcoma progenitors, which is currently the best control, since there is no benign precursor and no certainty of the normal counterpart of osteosarcoma. Because IGF1R signaling can be exploited as a therapeutic target, and osteosarcoma patients are in severe need of new therapies, we examined mRNA expression of members of this signaling pathway in detail. *IGFBP4* and *GAS6*, which code for proteins that inhibit IGF1R signaling, showed the highest significant downregulation (log fold changes <-4) in a four-way analysis, in which osteosarcoma pretreatment biopsies or cell lines were compared with osteoblastic cultures (n=3) or MSCs (n=12). Insulin-like growth factor binding proteins (IGFBPs) generally inhibit IGF1R signaling by competitively binding IGFs, but can under certain circumstances also stimulate IGF1R signaling (32). IGFBP4 is a negative regulator of IGF signaling in various tissues, including bone (33). GAS6, or growth arrest-specific 6, was shown to inhibit the growth promoting effects of IGF signaling and to stimulate differentiation in the chondrogenic cell line ATDC5 (34). Both *IGFBP4* and *GAS6* expression have previously been shown to be downregulated in osteosarcoma cell lines (*IGFBP4* in MG63 (35), *GAS6* in MG63 and SAOS2 cells (36)). Next to *GAS6* and *IGFBP4*, *IGFBP2* was also significantly downregulated in all four analyses, with log fold changes of approximately -3. IGFBP2 generally inhibits IGF action and may play a role in IGF2-induced osteoblast differentiation (33). *IGFBP3* was highly downregulated in three out of four analyses, and has been shown to elicit anticancer effects by inhibiting IGF1R signaling in Ewing sarcoma (37). IGFBP7 activity has not yet been reported in sarcoma, but has been associated with e.g. hepatocellular carcinoma (38). Interestingly, *IGF2BP3* was highly overexpressed in 3 of 4 analyses. This binding protein can bind IGF2 mRNA, thereby probably activating the translation of IGF2 (39). Overexpression of *IGF2BP3* has been reported in several cancer types (40, 41). Figure 2 shows that differential expression is most pronounced in upstream regulators of IGF1R, while downstream components, such as *SHC* and *FOS*, are slightly

downregulated, although for most genes this only holds when compared with mesenchymal stem cells, and not with osteoblasts. This may be caused by negative feedback loops, triggered by the active IGF1R signaling pathway. These results suggest that, in osteosarcoma, the IGF1R signaling pathway can be inhibited at the level of the receptor. We therefore validated protein levels of IGF1R and of IRS1, a direct downstream component of IGF1R and IR signaling using Western blotting. IGF1R and IRS1 protein levels correlated fairly well with mRNA expression levels. Most importantly, phosphorylated IRS1, which is a measure for pathway activity, was detected in all four osteosarcoma cell lines, indicating that IGF1R signaling is active in osteosarcoma, and is possibly regulated upstream of IGF1R. Accordingly, targeting this receptor may be an effective way to inhibit this pathway.

OSI-906 is a selective small molecule dual kinase inhibitor of both IR and IGF1R (42). We specifically chose to treat osteosarcoma cells with a dual inhibitor, because the insulin receptor can activate the same downstream signaling pathways as IGF1R, therefore providing cells a way to circumvent single inhibition of IGF1R. This has formerly been demonstrated in osteoblasts (43) and in Ewing sarcoma cells (44). In fact, this dual inhibitor has been shown to cause enhanced inhibition of the Akt signaling pathway when compared with a selective monoclonal antibody against IGF1R, which could inhibit IR/IGF1R hybrids, but not IR homodimers (45). OSI-906 is currently being tested by OSI Pharmaceuticals in a Phase III trial in adrenocortical carcinoma and in a Phase I/II clinical trial in ovarian cancer. Treatment of osteosarcoma cells with OSI-906 at physiological levels leads to decreased phosphorylation of IRS1 at Y612. Inhibition of IRS1 at Y612 after treatment with OSI-906 was previously reported by Buck *et al*. in direct complementation breast cancer cells for IGF1R-IGF2 and IR(A)-IGF2 (45). Interestingly, we also detected a small shift in the size of p-IRS1 on the Western Blot, indicating that multiple phosphorylation groups are removed after treatment with OSI-906. Surprisingly, total IRS1 levels were highest in 143B, and were downregulated after treatment with OSI-906 in this cell line, although this had no effect on cell growth in this line, as opposed to the three others, which showed low IC_{50}s. Proliferation of 143B was only inhibited most likely unspecifically at high and toxic levels of the drug. The

143B cell line is a derivative of the osteosarcoma cell line HOS, transformed by a *KRAS* oncogene. Constitutive activation of the Ras/Raf/ERK pathway can explain why proliferation of this cell line cannot be inhibited by OSI-906. Of the cell lines that were responsive to OSI-906, KPD and OHS showed that treatment of 96 hrs was most effective, while SAOS2 already reached maximum inhibition at 72 hrs.

IGF1R signaling has been previously modulated in sarcoma in preclinical and clinical models. Several phase I and II clinical trials including treatment with IGF1R monoclonal antibodies are currently being conducted in sarcoma, especially in Ewing sarcoma (an overview of these trials is given in Olmos *et al*. (46)). Monoclonal antibodies against IGF1R have modest activity against Ewing sarcoma, as was observed in a phase I/II study of figitumumab (partial response in 14.2% of all subjects) (47) and in a phase II study using R1507 (complete/partial response rate of 10%) (48). Results of a phase II study of ganitumab in subjects with Ewing sarcoma and desmoplastic small round cell tumors were published very recently, and reported clinical benefit in 17% of all patients (49). Preclinically, treatment with different monoclonal antibodies against IGFR1 has been performed in osteosarcoma xenograft models, in which a response was detected in at least 60% of all cases studied (50-52). However, no objective responses were observed in phase I trials testing monoclonal antibodies (47, 53, 54), although 2 of 3 patients treated with R1507 had prolonged stable disease (53). Clinical data using dual IGF1R/IR inhibitors osteosarcoma is still very limited (55). Because resistance to highly specific IGF1R inhibitors may develop through IR (44), blocking both IGF1R and IR with a dual kinase inhibitor will most likely lead to better inhibition of downstream IRS1 signaling. We thus expect clinical outcomes to improve for osteosarcoma patients treated with dual IGF1R/IR inhibitor OSI-906. The effects of combination of OSI-906 with chemotherapeutics in osteosarcoma still need to be assessed before such a treatment can be clinically tested.

Phosphorylated IRS could be used as a biomarker in order to determine whether patients would respond to IGF1R inhibition. Patients with tumors exhibiting an activating mutation in downstream pathways will most likely not respond to IGF1R inhibition. Further research needs to be performed in order to assess

these candidate biomarkers for response to treatment. The IGF1R pathway acts on several biological mechanisms that promote tumor progression – mitogenesis, protection from apoptosis, malignant transformation, and metastasis (6). It is therefore possible that inhibiting these pathways with a dual IR/IGF1R kinase inhibitor, such as OSI-906, may reduce tumor sizes, as well as osteosarcoma metastasis, the leading cause of death in these patients.

Conclusions

Using gene set analysis of genome-wide gene expression data of high-grade osteosarcoma biopsies and cell lines, we detected an over-representation of IGF1R signaling. Specifically, different upstream inhibitors of IGF1R signaling, eg several IGF binding proteins, were downregulated. As this indicated the IGF1R receptor as a potential target for treatment of osteosarcoma, we set out to inhibit this receptor in four osteosarcoma cell lines. We used OSI-906, a selective small molecule dual kinase inhibitor of both IR and IGF1R, since the insulin receptor can activate the same downstream signaling pathways as IGF1R, thereby providing a way to circumvent single inhibition of IGF1R. Treatment with OSI-906 resulted in inhibition of phosphorylation of IRS1 Y612, a direct downstream target of IGF1R, and in strong inhibition of proliferation in 3 of 4 osteosarcoma cell lines. The non-responsive cell line, 143B, has a k-ras oncogenic transformation, and may therefore not respond to this treatment. In conclusion, we have shown that IGF1R signaling is active in osteosarcoma, and that dual inhibition of IR/IGF1R inhibits downstream signaling and proliferation of these cells. Responsiveness to this treatment may be evaluated by Western blotting against phosphorylated IRS. This study provides an *in vitro* rationale for using dual IR/IGF1R inhibitors in preclinical studies of osteosarcoma.

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Chapter 4

CORR Insights®: Transcriptional profiling identifies the signaling axes of IGF and transforming growth factor-β as involved in the pathogenesis of osteosarcoma

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Where are we now?

Targeted therapeutics are needed to improve the prognosis of patients with osteosarcoma.Since the introduction of chemotherapy in the 1980s, 5-year survival for the often young patients with osteosarcoma has plateaued at about 50% to 60%. For patients with metastases, the outcomes remain particularly poor. By performing a serial analysis of a gene expression experiment comparing osteosarcomas with normal osteoblasts and mesenchymal stem cells, the authors identified the insulin-like growth factor (IGF) pathway and the transforming growth factor-β (TGF-β) pathway as possible targets for therapy. Since the effects of stimulation or inhibition of the TGF-β pathway on osteosarcoma cell proliferation is debatable (1) and the developed TGF-β pathway inhibiting agents elicit unwanted effects (2), we will focus on the possibility of targeting the IGF pathway in osteosarcoma.

The current paper emphasizes that the IGF pathway plays an important role in osteosarcoma pathogenesis, which is supported by other preclinical studies showing reduced proliferation in the majority of osteosarcoma cell lines (3) and xenografts (4) upon IGF1 receptor (IGF1R) inhibition. In addition, the peak incidence of osteosarcoma correlates with the increased levels of growth hormone and IGF ligands in puberty, and it has been described that the expression of several IGF pathway members (IGF1R, IGF1, growth arrest-specific 6, and IGF binding proteins 4 [IGFBP4]) correlates with osteosarcoma prognosis (3, 5). However, clinical trials demonstrate that only a small subset of osteosarcoma patients respond to IGF1R antibodies (6). This illustrates the general trend with IGF1R inhibitors. Although preclinical models have shown promising results, evidence for their efficacy in large-scale randomized controlled trials is lacking. As a consequence, pharmaceutical companies have discontinued the production of all IGF pathway targeting agents.

Where do we need to go?

The current paper emphasizes the importance of IGF signaling in the development of osteosarcomas. Despite the fact that only a subset of patients with osteosarcoma are sensitive to IGF1R inhibition, the strong preclinical

rationale deserves further clinical and translational exploration. Predictive biomarkers to identify the subset of patients that will benefit from IGF-directed therapy should be identified. The most obvious biomarker would be expression of the IGF1R, but a Phase 2 trial with Cixutumumab (IGF1R antibody) and Temsirolimus (mTOR inhibitor) in patients with bone and soft tissue sarcomas showed that IGF1R expression does not correlate with therapeutic response (7). However, the search for biomarkers is ongoing, and several other biomarkers have been proposed, such as mutated Kirsten rat sarcoma viral oncogene homolog (KRAS) and phosphorylated extracellular signal-regulated kinases (ERK) (8).

Besides the identification of predictive biomarkers, the efficacy of alternative methods to target the IGF pathway should be explored, such as tyrosine kinase inhibitors that target both the IGF1R and the insulin receptor, given its crossreactivity. Once *in vitro* studies identify the best approach to target the IGF pathways, and once biomarkers have been identified, the next step would be small clinical trials to reevaluate the efficacy of IGF pathway inhibitors in patients with osteosarcoma. These clinical trials must incorporate a strong translational research program. Therefore, we should convince pharmaceutical companies to resume the production of IGF pathway inhibitors, especially the IGF1R/insulin receptor dual inhibitors.

How do we get there?

One clinical trial with Linsitinib (a dual IGF1R insulin receptor inhibitor) is currently being performed in Ewing sarcoma, where IGFBP3 is downregulated as a consequence of the Ewing sarcoma breakpoint region 1-Friend leukemia virus integration 1 fusion gene (EWSR1-FLI-1) (9) resulting in overactivity of the IGF pathway. Interestingly, IGF1R inhibition seems promising in a subset of patients with Ewing sarcoma (6). This EuroSARC study, led by Prof. Dr. Hassan from the University of Oxford, aims to identify predictive biomarkers by incorporating a strong translational research program in this clinical trial. The biomarkers that will be identified in the EuroSARC study should be tested in other tumors including osteosarcoma, and as mentioned above, new trials for osteosarcoma, similar to this trial in Ewing sarcoma, should

be conducted. The clinical trials that have been performed in osteosarcoma thus far have two major limitations. First, all the studies involved IGF1R monoclonal antibodies. It is known that signaling via the insulin receptor can circumvent IGF1R inhibition (10). Indeed, it was shown in osteosarcoma cells that dual inhibition of IGF1R and insulin receptor is more effective than IGF1R inhibition alone (11). Therefore, results from clinical studies with dual inhibitors are warranted. Second, in the clinical trials conducted so far, IGF1R-targeting antibodies were administered as a monotherapy to patients that failed complete remission upon chemotherapy. IGF1R inhibitors should be tested in combination with chemotherapeutic agents, as it has been described that the ligand IGF2 is upregulated in patients in response to chemotherapy (12) and that in osteosarcoma cell lines IGF1R inhibition can enhance the effect of doxorubicin and radiotherapy (13).

The current paper strengthens the view that the IGF pathway can be an effective target for therapy in osteosarcoma, thereby emphasizing the need for the industrial facilitation of clinical trials with IGF1R/insulin receptor dual inhibitors combined with chemotherapy, in which a strong translational program is embedded for predictive biomarker identification in osteosarcoma patients.

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Chapter 5

No preclinical rationale for IGF1R directed therapy in chondrosarcoma of bone

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Abstract

Background: Chondrosarcoma is a malignant cartilage forming bone tumour for which no effective systemic treatment is available. Previous studies illustrate the need for a better understanding of the role of the IGF pathway in chondrosarcoma to determine if it can be a target for therapy, which was therefore explored in this study. Methods: Expression of mediators of IGF1R signalling and phosphorylation status of IRS1 was determined in chondrosarcoma cell lines by qRT-PCR and western blot. The effect of activation and inhibition of IGF1R signalling on downstream targets was assessed by western blot. Ten chondrosarcoma cell lines were treated with OSI-906 (IGF1R and IR dual inhibitor) after which cell proliferation and migration were determined by a viability assay and the xCELLigence system, respectively. In addition, four chondrosarcoma cell lines were treated with a combination of doxorubicin and OSI-906. By immunohistochemistry, IGF1R expression levels were determined in tissue microarrays of 187 cartilage tumours and ten paraffin embedded cell lines. Results: Mediators of IGF1R signalling are heterogeneously expressed and phosphorylated IRS1 was detected in 67 % of the tested chondrosarcoma cell lines, suggesting that IGF1R signalling is active in a subset of chondrosarcoma cell lines. In the cell lines with phosphorylated IRS1, inhibition of IGF1R signalling decreased phosphorylated Akt levels and increased IGF1R expression, but it did not influence MAPK or S6 activity. In line with these findings, treatment with IGF1R/IR inhibitors did not impact proliferation or migration in any of the chondrosarcoma cell lines, even upon stimulation with IGF1. Although synergistic effects of IGF1R/IR inhibition with doxorubicin are described for other cancers, our results demonstrate that this was not the case for chondrosarcoma. In addition, we found minimal IGF1R expression in primary tumours in contrast to the high expression detected in chondrosarcoma cell lines, even if both were derived from the same tumour, suggesting that *in vitro* culturing upregulates IGF1R expression. Conclusions: The results from this study indicate that the IGF pathway is not essential for chondrosarcoma growth, migration or chemoresistance. Furthermore, IGF1R is only minimally expressed in chondrosarcoma primary tumours. Therefore, the IGF pathway is not expected to be an effective therapeutic target for chondrosarcoma of bone.

Background

Chondrosarcoma is the second most common primary bone malignancy in humans (1) and represents a heterogeneous collection of cartilage forming tumours with different outcomes depending on subtype and histological grade. Conventional central chondrosarcoma, arising centrally in the medulla of bone, accounts for ~85 % of the cases and can be histologically divided into 3 grades (1). Sixty-one percent of these tumours are classified as atypical cartilage tumour (ACT) (previously known as grade I), for which first line treatment is curettage with local adjuvant treatment, resulting in a 5 year survival rate of 83 %. Grade II (36 %) and grade III (3 %) tumours are more prone to metastasize and have a combined 5 year survival rate of 53 % (1-3). These tumours are treated with *en bloc* resection. Dedifferentiated chondrosarcoma is a highly malignant variant with an overall survival rate of 7 ~ 24 % (4). Mesenchymal chondrosarcoma is a rare aggressive subtype, in which distant metastasis can be identified even after 20 years (5). It has a 10 year survival rate between 44 and 54 % (6, 7). Chondrosarcoma patients with unresectable disease, due to tumour location, tumour size or extensive metastatic disease, have a 5 year survival of only 2 % (8). Although chondrosarcoma is known for its resistance to chemo- and radiotherapy, it was recently described that patients with inoperable disease treated with doxorubicin-based chemotherapy have a 3 year survival rate of 26 % versus 8 % in patients who did not receive systemic treatment (8) and chemotherapy sensitivity differed between the chondrosarcoma subtypes (9). However, it is clear that overall efficacy of chemotherapy is limited. So far, the discovered genetic alterations and pathways involved in chondrosarcoma (reviewed in (10) and (11)) have not resulted in new treatment regimes. Therefore, further unravelling of critical signalling pathways in chondrosarcoma is essential to identify new therapeutic targets.

One pathway which has been implicated in chondrosarcoma proliferation is the IGF pathway. The IGF pathway has two closely related ligands: IGF1 and IGF2 (12). When a ligand binds to the IGF1 receptor (IGF1R), this receptor forms homodimers or hybrid receptors with the insulin receptor (IR). The resulting autophosphorylation of the receptor recruits the insulin receptor substrate (IRS) to the membrane causing subsequent downstream activation

of the PI3K/Akt/mTOR pathway and the Ras/Raf/MEK signalling pathway, which are known to be driver pathways in cancer (12). IGF2R functions to decrease the availability of IGF2 to IGF1R (12).

IGF1R can be the upstream receptor that is responsible for the well known activation of the PI3K/Akt/mTOR pathway, the Src-pathway and the Ras/Raf/MEK pathway in (a subset of) chondrosarcoma cell lines and primary cultures (13-17). In a heterogeneous group of sarcoma patients, a combination of an IGF1R antibody and mTOR inhibitor has been shown to have clinical activity but the level of IGF1R expression was not predictive for response (18). Takigawa *et al*. demonstrated that cells of a clonal human chondrosarcoma-derived chondrocyte cell line produce IGF ligands and express IGF1R and IGF2R (19). Seong *et al*. and Matsumari *et al*. described that IGF1 increases cell proliferation in a Swarm-rat chondrosarcoma model (20, 21). Interestingly, Ho. *et al* described that IGF binding protein 3 (IGFBP3), which binds the IGF ligands thereby inhibiting their interaction with the IGF receptors, decreases with increasing histological grade of chondrosarcoma (22). In addition, Wu *et al*. demonstrated that IGF1 induced migration of chondrosarcoma cell lines which could be blocked by an IGF1R antibody (23). Recently, functional profiling of receptor tyrosine kinases in chondrosarcomas revealed active IGF1R signalling in one out of five chondrosarcoma cell lines (13).

These above mentioned studies illustrate the need for a better understanding of the role of IGF1R signalling in chondrosarcoma to determine if it is a convincing target for therapy. Because chondrosarcoma is a very heterogenous disease, it is possible that the IGF1R directed therapy is only effective in a subset of patients. Hence, we used our large chondrosarcoma cell line panel, including three grade 2 and three grade 3 conventional chondrosarcomas, three dedifferentiated chondrosarcomas and one mesenchymal chondrosarcoma cell line. We analyzed expression levels of IGF1R and other important mediators of IGF1R signalling and determined the effect of IGF1R inhibitors. Our results indicate that the IGF pathway is not important for chondrosarcoma growth as IGF1R inhibition did not demonstrably impact chondrosarcoma cell line proliferation, migration and chemoresistance. In addition, IGF1R expression is low/absent in chondrosarcoma primary tumours in contrast to chondrosarcoma

cell lines. This illustrates that there is limited preclinical rationale for using IGF1R inhibitors for the treatment of chondrosarcoma of bone.

Methods

Compounds

The IGF1R inhibitors OSI-906, NVP-ADW742 and GSK1838705A were purchased from Selleck Chemicals LCC and dissolved in DMSO in a concentration of 10mM. The IGF1R inhibitors were tested in concentrations up to 1 μM as it was demonstrated previously that higher concentrations lead to an aspecific toxic response (24). Recombinant human IGF1 (PeproTech) was used in a concentration of 50 ng/ml (25). Doxorubicin was obtained from the in-house hospital pharmacy in a 0.9 % NaCl solution, and used in a concentration range of 1-100 nM.

Cell culture

The conventional chondrosarcoma cell lines JJ012 (26), SW1353 (ATCC), CH2879 (27), OUMS27 (28), L835 (29) and CH3573 (30), as well as the dedifferentiated chondrosarcoma cell lines L3252B (29), NDCS1 (31), and L2975 (29) were cultured in RPMI 1640 (Gibco, Invitrogen) supplemented with 1 % Glutamax (Gibco 35050, Invitrogen), 1 % penicillin/streptomycin (PS) (100U/mL) (Gibco, Invitrogen) and 10 % (JJ012, SW1353, CH2879, NDCS1, L2975) or 20 % (L835, L3252B, OUMS27, CH3573) heat-inactivated Fetal Bovine Serum (FBS) (F7524, Sigma-Aldrich). MCS170 (Mesenchymal chondrosarcoma) and TC-32 (Ewing Sarcoma, (32)) were cultured in IMDM (Gibco, Invitrogen) with 1 % PS with respectively 15 % and 10 % FBS. The cells were grown at 37°C with 5 % CO_2 in a humidified incubator. Mycoplasma tests were performed regularly. Identity of cell lines was confirmed using STR profiling with the CELL ID™ system (Promega Benelux BV).

qRT-PCR

RNA isolation and cDNA synthesis was performed as described (33). To determine the expression levels of IGF1, IGF1R, IGF2, IGF2R, IGFBP3 and IR, a standard quantitative reverse transcriptase PCR (qRT-PCR) was

performed as described previously (34). Primers were designed using primer3 software (http://bioinfo.ut.ee/primer3/). To correct for the amount of cDNA input, gene expression levels were normalized using the expression levels of CYPa and CPSF6 (35, 36). ΔΔCq values below 0.01 were considered negative. All qRT-PCRs were optimized on control tissue.

Immunoprecipitation and western blotting

Western blotting was performed as described previously (14). Per sample, 20μg of protein was loaded on SDS- PAGE gels. Rabbit antibodies against IGF1R (#3018), IR (#3025),IRS1 (#2382) and Phospho-S6 Ribosomal Protein (Ser235/236) (2 F9) (#4856) all diluted 1:1000, were obtained from Cell Signaling. Phospho-Akt (Ser473) (#9271), diluted 1:2000 was also obtained from Cell Signaling. Rabbit polyclonal antibody against phospho-IRS1 (Y612, 1:1000) was purchased from Biosource, Invitrogen. Monoclonal Anti-MAP Kinase, Activated (Diphosphorylated ERK-1&2) was obtained from Sigma (M8159) and diluted 1:2000. A mouse monoclonal antibody against α-tubulin (1:3000)(Abcam) was used as a loading control. Secondary antibodies were horseradish peroxidase (HRP) conjugated polyclonal goat-anti-rabbit IgG for components for IGF1R, IR, IRS1, phospho-IRS1, pAkt and pS6, and HRP conjugated polyclonal goat-anti-mouse for α-tubulin and diphos. ERK-1&2 (both 1:3000, BD Transduction Laboratories). Immunoprecipitation (IP) for IRS1 was performed according to the manufacturer's instruction. In short, cells where harvested at ±80 % confluence using the Cell lysis buffer (Cell Signaling) to which the PhosSTOP (REF 04906837001) and the Protease Inhibitor Cocktail Tablets (REF 11697498001) were added (Roche). The IRS1 antibody (1:50) was added to 200μl lysate at 1mg/ml and rotated over night at 4 ºC, followed by 30 min incubation with 40μl protein A magnetic beads (Cell Signaling). After washing using a magnetic separation rack, the pellet was suspended in 3x SDS sample buffer (containing 2- Mercaptoethanol). The sample was loaded on an SDS-PAGE gel and western blotting was performed as described above. TC-32 was used to optimize the protocol and was included as a positive control.

Proliferation assay

In all cell viability experiments, the cell lines were plated in triplicate at a

density of 3000 to 10000 cells per well depending on the growth rate. For the positive control (Ewing sarcoma cell line TC-32) the 96 well plates were coated with gelatine. After the cells were allowed to adhere overnight, the IGF1R inhibitors were added in their corresponding concentrations. In addition, we determined the effect of OSI-906 when IGF1 (50 ng/ml) was added to the medium. For the combination treatment of doxorubicin and OSI-906, both inhibitors were added at the same time. Because JJ012 and SW1353 are relatively more sensitive to doxorubicin, JJ012 and SW1353 were treated with 0, 1 nM, 10 nM or 100 nM while CH2879 and OUMS27 were treated with 0, 10 nM, 50 nM and 100 nM doxorubicin. These concentrations of doxorubicin were combined with DMSO, 0.1 μM, 0.5 μM or 1 μM OSI-906. After 72 h of incubation, cell viability was measured using the WST-1 reagent (Roche) (single treatment with OSI-906) or PrestoBlue Cell Viability Reagent (Promega Benelux BV) (single treatment with NVP-ADW742 and GSK1838705A and combinations with IGF1 and doxorubicin) according to the manufacturer's instructions. Colorimetric values in the plates were subsequently measured using a Wallac 1420 VICTOR2 (Perkin Elmer). Data were analysed in Graphpad Prism 5.0 (www.graphpad.com). The results shown are representative results from at least three independent experiments.

Migration assay

The real-time cell analyser xCELLigence system (Roche) based on cell-electrode subtract impedance detection technology (37) was used to study the effect of IGF1R/IR inhibition on migration as previously described (16). In short, cell lines were added at a density of 80.000 per well in the upper chamber of the Cell Invasion and Migration (CIM) plates in serum-free RPMI medium containing 0, 100 nM or 1 mM OSI-906. The lower chambers were filled with RPMI medium supplemented with 20 % FBS. The software calculated the Cell index, which was set at 1.0 migration at the last measurement.

Immunohistochemistry on tissue microarrays

The specificities of two IGF1R antibodies (#3018 and #3027, Cell Signaling) were compared by western blot (as described above) and immunohistochemistry on colon tissue (as described in (38)). The most specific antibody was selected

to determine the IGF1R expression in 5 enchondromas, 7 osteochondromas, 71 central conventional chondrosarcomas, 34 peripheral conventional chondrosarcomas, 32 dedifferentiated chondrosarcomas, 18 mesenchymal chondrosarcomas and 20 clear cell chondrosarcomas by using previously constructed and described tissue microarrays (TMAs) (16, 39). Slides were scored by an experienced pathologist (AHGC) as either positive or negative.

Results

IGF pathway members are expressed in a subset of chondrosarcoma cell lines

Using qRT-PCR analyses, we demonstrate that all cell lines express the receptors IGF1R, IGF2R and IR (Figure 1A). However, expression levels are highly variable as L835, OUMS27 and NDCS1 have a relatively high expression of the three receptors as compared to the other cell lines. For the IGF1R and the IR, we correlated the mRNA expression levels to levels of protein expression (Figure 1B). mRNA expression of the ligand IGF1 is restricted to four out of ten chondrosarcoma cell lines, with the highest expression in L835. Strikingly, IGF2 expression in OUMS27 is very high, comparable to the expression levels in a human placenta (data not shown). IGFBP3 mRNA expression is detected in 8 out of 10 chondrosarcoma cell lines. In addition, western blot analyses revealed protein expression of IRS1 in all cell lines, although expression levels are again variable amongst the different cell lines (Figure 1B).

IGF1R signalling is active in a subset of chondrosarcoma cell lines

To determine IGF pathway activity, the phosphorylation status of IRS1 was determined in three chondrosarcoma cell lines. Immunoprecipitation for IRS1 followed by western blot analyses with a phospo-IRS1 antibody revealed the presence of phosphorylated IRS1 in JJ012 and SW1353, but not in CH2879 (Figure 1C). This demonstrates that IGF1R signalling is active in a subset of chondrosarcoma cell lines. Furthermore, only in the cell lines in which phosphorylated IRS1 was detected, phosphorylated Akt levels were decreased and IGF1R levels were increased by OSI-906 (dual IGF1R and IR inhibitor) treatment (Figure 1D). However, phosphorylated S6, located downstream of Akt, and diphosphorylated ERK-1&2 were

unaffected, suggesting that activity of the downstream targets is not dependent on IGF1R signalling.

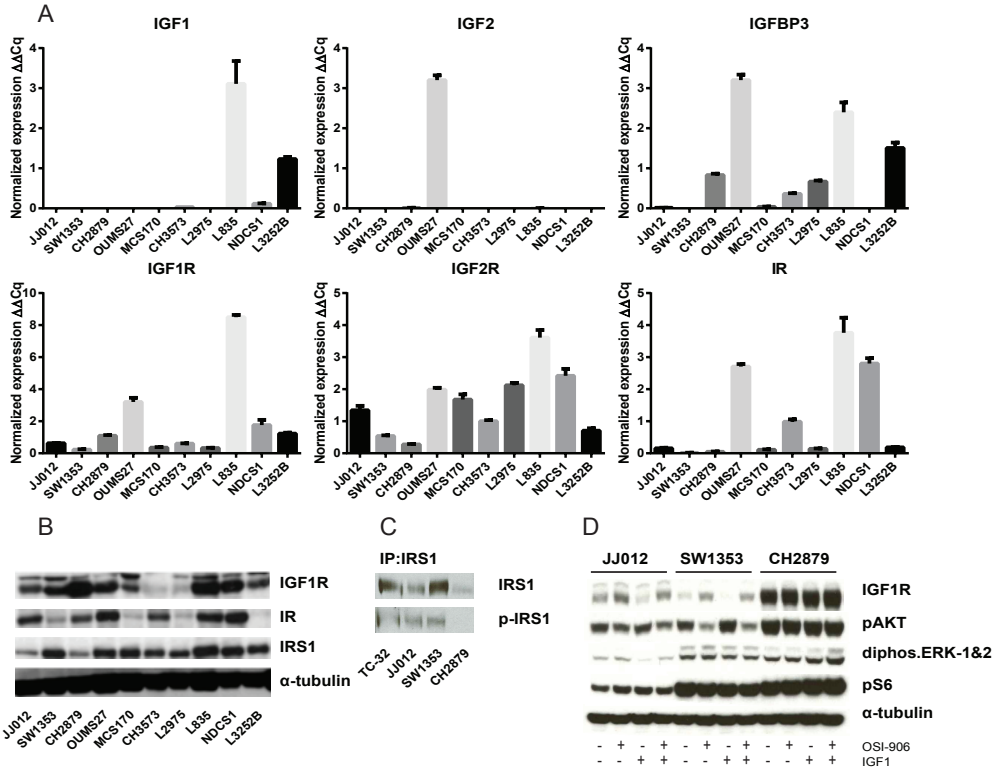

Figure 1. A-B: qRT-PCR and western blot analyses, respectively, reveal heterogeneous expression of IGF pathway members in chondrosarcoma cell lines. **C:** Immunoprecipitation with IRS1 followed by a western blot for phospho-IRS1 reveals pathway activity in two out of three chondrosarcoma cell lines tested. **D:** Evaluation of IGF1R downstream targets reveals an effect of OSI-906 on IGF1R and pAkt but not on pS6 and disphosphorylated ERK-1&2. Cell lines were treated for 72 hours with DMSO, 1 μM OSI-906 and/or 50 ng/ml IGF1.

Viability and migration of chondrosarcoma cell lines is not affected by IGF1R inhibition

Treating our full chondrosarcoma cell line panel for 72 h with concentrations from 0.01 to 1000 nM of OSI-906 revealed that chondrosarcoma cell viability was not affected by inhibition of the IGF pathway, whereas the positive control cell line TC-32 (Ewing sarcoma) showed dose-dependent decrease of cell viability (Figure 2A). Furthermore, addition of IGF1 to the medium did not increase cell proliferation nor sensitivity to OSI-906 (Figure 2B) in

three chondrosarcoma cell lines tested. In addition, four chondrosarcoma cell lines and the Ewing sarcoma cell line were treated with two other IGF1R/IR inhibitors (NVP-ADW742 and GSK1838705A) to determine if alternative targeting showed similar effects on cell viability (Figure 2C and 2D). Indeed, the results were highly comparable, demonstrating that the IGF pathway is not essential for chondrosarcoma cell viability.

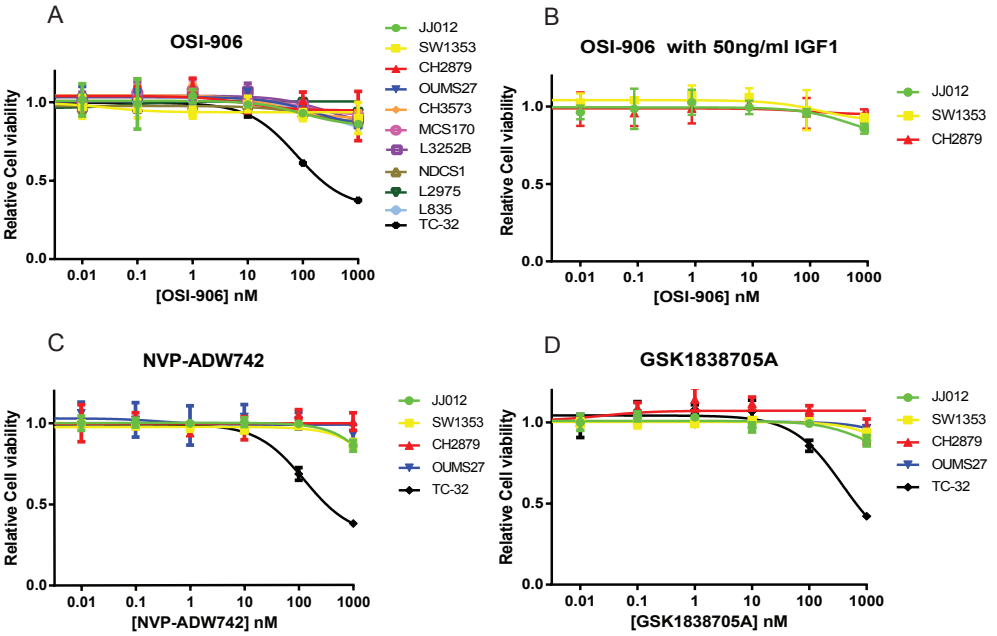

Figure 2. Relative cell viability after 72 hours of treatment with IGF1R/IR inhibitors. **A-B:** OSI-906 does not inhibit chondrosarcoma cell viability, even in the presence of IGF1 **C-D:** IGF1R inhibitors NVP-ADW742 and GSK1838705A do not inhibit chondrosarcoma cell viability.

By adding OSI-906 to the upper chamber of the CIM plates (xCELLigence), we demonstrated that IGF1R signalling was not essential for the migration of JJ012, SW1353, CH2879 and OUMS27 (Figure 3). To exclude the possibility that the absence of an effect of OSI-906 on chondrosarcoma cell migration was caused by an insufficient treatment duration, the cell lines were treated with 1 µM OSI-906 for 72 h before the onset of the experiment in one experimental condition. However, even after this pretreatment, chondrosarcoma cell line migration was not influenced by IGF1R/IR inhibition, illustrating that the IGF pathway does not play a role in chondrosarcoma cell migration.

The IGF pathway is not involved in chondrosarcoma chemoresistance

Because IGF1R signalling has been implicated in chemoresistance (40), four chondrosarcoma cell lines were treated with a combination of OSI-906 and doxorubicin. Although doxorubicin inhibited cell viability in a dose dependent manner, IGF1R/IR inhibition did not increase this cytotoxicity in any of the cell lines (Figure 4). This indicates that the IGF pathway is not involved in chondrosarcoma chemoresistance.

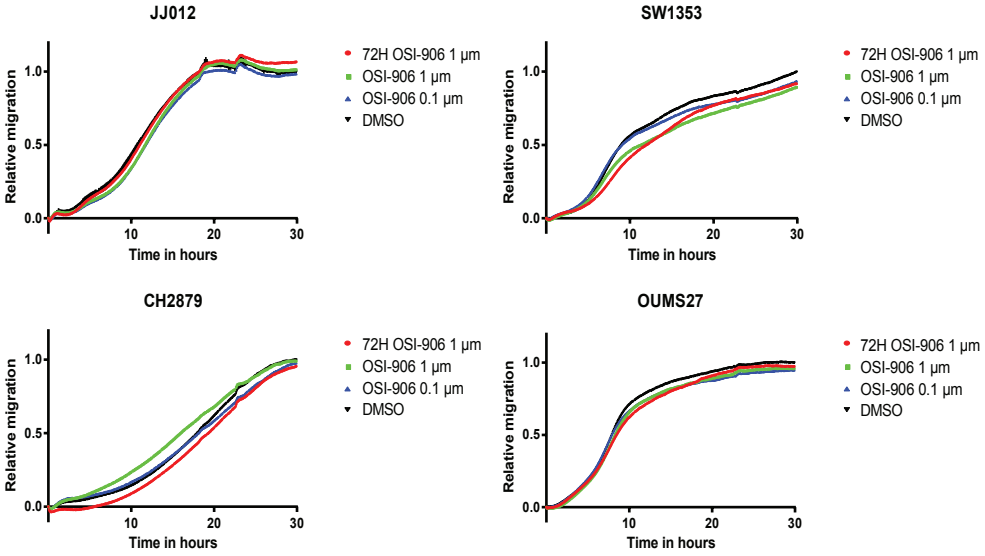

Figure 3. OSI-906 does not inhibit migration of four chondrosarcoma cell lines. 72H OSI 1 µM: cells were treated for 72 hours with OSI-906 before experimental onset.

IGF1R is not strongly expressed in uncultured cartilage tumours

To elucidate the discrepancy between the observed protein expression of IGF1R in cell lines and the absence of an effect of IGF pathway inhibition, we assessed IGF1R expression in clinical tumour samples versus cell lines that were formalin-fixed and paraffin-embedded (FFPE). IGF1R antibody #3018 was selected for these immunohistochemical stainings as both the positive immunohistochemical control and the control western blot demonstrated its high specificity compared to the IGF1R antibody #3027. Immunohistochemistry confirmed the western blot evidence of IGF1R expression (Figure 1B), with membranous expression of IGF1R shown in ten cell lines, and IGF1R expression levels were comparable in western blot and

immunohistochemistry evaluations (Figure 5A-B). In contrast, the primary tumours were either completely negative (66 %) or showed very weak staining (34 %) for IGF1R (Figure 5C, Table 1). To exclude the possibility that the discrepancy between the primary tumours and the cell lines was due to tissue handing procedures, we included a colon tissue sample that was decalcified by 20 % formic acid for 2 days which stained positive thereby excluding an effect of the decalcification procedure. To further study the difference in IGF1R expression between primary tumours and cell lines, we stained the primary tumours corresponding to the cell lines L835 (29), CH2879 (27), L3252B (29) and L2975 (29). Strikingly, the primary tumours were either completely negative (L835, CH2879) or showed weak staining (L3252B, L2975) for IGF1R (Figure 5D, Figure 6). This suggests that chondrosarcoma cells upregulate IGF1R upon prolonged culturing.

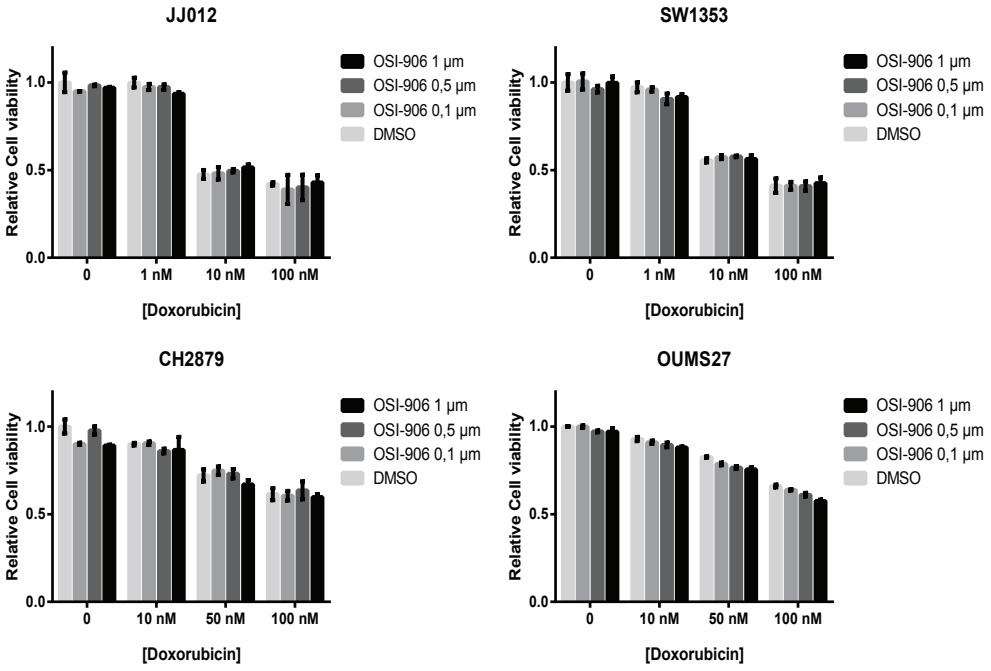

Figure 4. Relative cell viability after 72 hours of combination treatment with OSI-906 and Doxorubicin. OSI-906 does not sensitize the cells to doxorubicin.

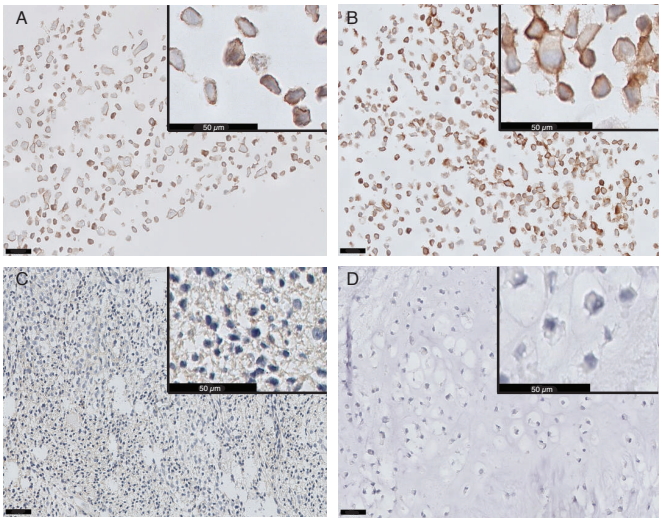

Figure 5. IGF1R expression is high in chondrosarcoma cell lines but low in primary tumours. **A:** IGF1R expression in chondrosarcoma cell line JJ012. **B and D:** IGF1R expression in L835 cell line and primary tumour, respectively. **C:** a conventional chondrosarcoma sample that was classified as weak IGF1R staining. Black bars represent 50 µm.

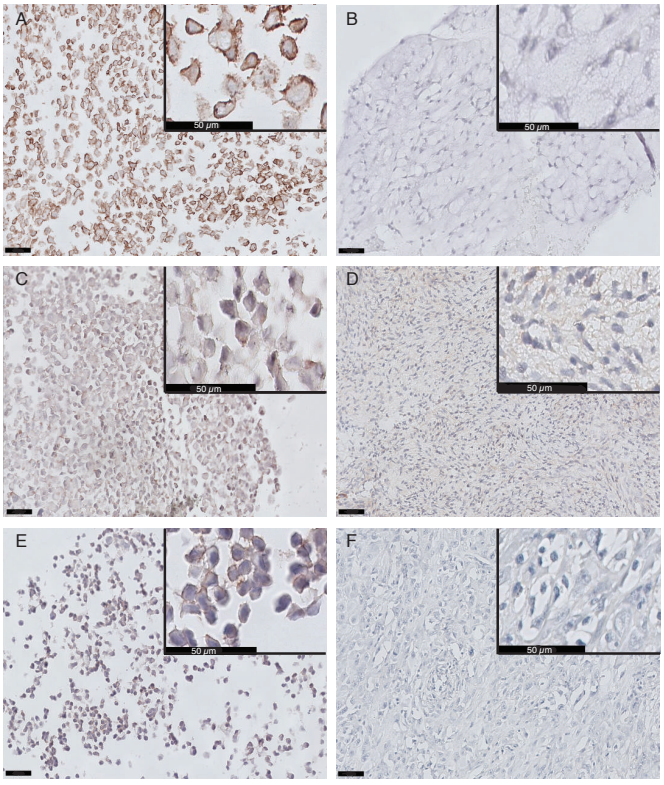

Figure 6. IGF1R expression in CH2879 (A-B), L3252B (C-D) and L2975 (E-F) cell lines and primary tumours, respectively. Black bars represent 50 µm.

Table 1. Immunohistochemistry demonstrates only weak IGF1R expression in uncultured cartilage tumours.

Diagnose	Weak IGF1R positivity
Enchondroma	3/5 (60%)
Osteochondroma	2/7 (29%)
Central Conventional chondrosarcoma	32/71 (45%)
Grade I	8/28 (29%)
Grade II	15/29 (51%)
Grade III	9/14 (64%)
Peripheral Conventional chondrosarcoma	14/34 (41%)
Grade I	8/21 (38%)
Grade II	5/10 (50%)
Grade III	1/3 (33%)
Dedifferentiated chondrosarcoma	11/32 (34%)
Mesenchymal chondrosarcoma	0/18 (0%)
Clear cell chondrosarcoma	1/20 (5%)
Total	63/187 (34%)

Discussion

The aim of this study was to investigate whether the IGF pathway is a suitable target for therapy in chondrosarcoma. Heterogeneous expression of IGF1R, IR, IGF2R, IGF1, IGF2, IRS1 and IGFBP3 was seen, both at the mRNA and protein levels, in chondrosarcoma cell lines. This indicates that essential IGF pathway components are present in cultured chondrosarcoma cells. Furthermore, detection of phosphorylated IRS1 in two out of three chondrosarcoma cell lines demonstrates that the IGF pathway is active in a subset of chondrosarcoma cell lines. In the cell lines with phosphorylated IRS1, IGF pathway inhibition decreased phosphorylated Akt levels and increased IGF1R expression; the latter suggests activation of a feedback loop, which is further supported by the downregulation of IGF1R expression by

IGF1 treatment. However, this did not influence the amount of phosphorylated S6, which is located further downstream in the PI3K/Akt/mTOR pathway. Furthermore, the activated MAPK levels were not affected by IGF pathway stimulation or inhibition, demonstrating that activity of the downstream targets is not dependent on IGF1R signalling.

In line with these findings, we demonstrate that despite activity of the pathway, IGF1R signalling is not essential for chondrosarcoma cell survival. Treatment with three different IGF1R/IR inhibitors does not have an effect on chondrosarcoma cell viability, irrespective of apparent pathway activity and stimulation with IGF1. Chondrosarcoma cell line OUMS27 was previously shown to be sensitive to IGF1R/IR inhibition by Zhang *et al*. (13). It is difficult to explain the discrepancy with the current study, as OSI-906 is a derivate of the IGF1R inhibitor used by Zang *et al*. with similar target potency (41). Moreover, we performed these assays at multiple cell densities, passage numbers and IGF1R/IR inhibitors (data not shown).

IGF1R signalling is involved in resistance to cytotoxic drugs in certain cancers (40). Since chondrosarcoma is resistant to chemotherapy, we explored a possible role of the IGF1R/IR pathway in chemoresistance. Doxorubicin reduced cell viability in a dose dependent manner; however, OSI-906 did not further inhibit cell viability in this cell line model. These results do not support a key role of the IGF pathway in chondrosarcoma cell survival and chemoresistance.

Our study could not confirm a role for the IGF pathway in chondrosarcoma cell migration. In contrast to the study from Wu *et al*., showing that IGF1 induced chondrosarcoma migration was inhibited by an IGF1R antibody (23), we chose not to pretreat the chondrosarcoma cells with IGF1 and not to use medium supplemented with IGF1 only as chemoattractant, thereby better mimicking the *in vivo* situation. These experimental differences might explain the difference in our findings.

Strikingly, we detected high expression of the IGF1R in chondrosarcoma cell lines compared to primary tumours. Moreover, we show that each of four

patients with matched cell lines and primary tumours had strong membranous IGF1R expression in the cultured cells compared to absent or very weak expression in the corresponding primary tumour. The finding that cell lines are insensitive to IGF1R inhibition despite their high IGF1R expression is in line with the results from the study by Schwartz *et al*, which described absence of a correlation between IGF1R expression levels and responsiveness to an IGF1R targeting antibody (18). This series included 38 chondrosarcomas of which 53 % had immunohistochemical staining with an IGF1R antibody (18). Therefore, we did not anticipate to find weak (34 %) or no expression (66 %) in our cartilage tumour series. The discrepancy between our results and the study from Schwartz *et al*. can likely be explained by use of another antibody. Lack of reproducibility is a well described phenomenon in preclinical studies with antibodies (42, 43). Our study further suggests that IGF1R expression is lower in clear cell chondrosarcoma and mesenchymal chondrosarcoma compared to the other cartilage tumours. However, as the staining is very weak in the samples scored positive and IGF1R expression levels do not correlate with responsiveness to IGF1R targeting antibodies, we do not think this difference in IGF1R expression has clinical significance. Furthermore, we did not see a difference in sensitivity to IGF1R inhibition between the mesenchymal, dedifferentiated and conventional chondrosarcoma cell lines included in this study.

Increased activity of the IGF pathway is implicated in several other cancers (12) including other bone tumours (44). In Ewing sarcoma, IGF binding protein 3 (IGFBP3) is downregulated by the EWSR1-FLI1 fusion gene (45), activating the IGF pathway (46). Recently, aberrant expression of IGF pathway members was described in osteosarcomas and OSI-906, a dual inhibitor of the IGF1R and the IR, inhibited proliferation in 3 out of 4 osteosarcoma cell lines with IC_{50} values within the therapeutic range (24).

Clinical trials to test the safety and efficacy of IGF1R antibodies, sometimes in combination with an mTOR inhibitor, have been performed in sarcoma patients (47), but only two trials enrolled chondrosarcoma patients (18, 44). In the study described by Olmos *et al*. one myxoid chondrosarcoma was included, which showed a small decrease in tumour size upon IGF1R inhibition

(44). It is unclear whether this was an extraskeletal myxoid chondrosarcoma or a chondrosarcoma of bone. In addition, 1 of 17 chondrosarcoma patients showed partial response to Cixutumumab (IGF1R antibody) and Temsirolimus (mTOR inhibitor), as described by Schwarz *et al*. (18). In future studies, dual inhibitors of both the IGF1R and the IR are preferably chosen because it has been shown in osteoblasts (48) and Ewing sarcoma cells (49) that cells can circumvent inhibition of IGF1R by increasing IR signalling.

Conclusions

In summary, the results of this study demonstrate that although chondrosarcoma cell lines have high IGF1R expression and activation of downstream targets, inhibition of IGF1R/IR signalling does not affect chondrosarcoma proliferation, migration and chemoresistance. Therefore, we conclude that there is no convincing preclinical rationale for using IGF1R/IR inhibitors in the treatment of chondrosarcoma.

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Chapter 6

NAD synthesis pathway interference is a viable therapeutic strategy for chondrosarcoma

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Abstract

Nicotinamide phosphoribosyltransferase (NAMPT) and nicotinic acid phosphoribosyltransferase (NAPRT) are rate-limiting enzymes in the nicotinamide adenine dinucleotide (NAD^+) synthesis pathway. Chondrosarcoma is a malignant cartilage forming bone tumor, in which mutations altering isocitrate dehydrogenase-1 and -2 (IDH1 and IDH2) activity have been identified as potential driver mutations. Vulnerability for NAD^+ depletion has been reported for *IDH1/2* mutant cells. Here, the potency of NAMPT inhibitors as a treatment of chondrosarcoma was explored. Eleven chondrosarcoma cell lines were treated with NAMPT inhibitors, in which the effect on cell viability, colony formation and 3D collagen invasion was assessed. The expression level of NAMPT and NAPRT transcripts in chondrosarcoma cells was determined by qRT-PCR. Methylation of the NAPRT promoter was evaluated using a previously published dataset of genome-wide methylation. In addition, a methylation dataset was used to determine methylation of the NAPRT promoter in twenty *IDH1/2*-mutated cartilage tumors. Chondrosarcoma cells showed a dose-dependent decrease in cell viability, 3D collagen invasion and colony formation upon treatment with NAMPT inhibitors, in which nearly half of the cell lines demonstrated absolute IC_{50}s in the low nanomolar range. Increasing IC_{50}s correlated to increasing NAPRT expression levels and decreasing NAPRT promoter methylation. No correlation between *IDH1/2* mutation status and sensitivity for NAMPT inhibitors was observed. Strikingly, higher methylation of the NAPRT promoter was observed in high-grade versus low-grade chondrosarcomas. In conclusion, this study identified NAMPT as a potential target for treatment of chondrosarcoma.

Introduction

Chondrosarcoma represents a heterogeneous group of cartilage forming tumors. It is the second most common primary bone malignancy in humans (1) with different outcomes depending on subtype and histologic grade. The far most prevalent subtype (72% of the cases) is conventional central chondrosarcoma, in which the tumor arises centrally in the medulla of the

bone. This subtype can be histologically subdivided into atypical cartilaginous tumor (ACT), grade II and grade III chondrosarcomas. ACT (previously known as grade I) accounts for 61% of the cases. The first line treatment is curettage with local adjuvant treatment, resulting in a 5 year survival rate of 83%. Grade II (36%) and grade III (3%) have a worse 5 year survival (combined 53%) due to the higher occurrence of metastases (1-3). These tumors are treated by *en bloc* resection. Two other subtypes with a worse prognosis are dedifferentiated chondrosarcoma, a highly malignant variant (4), and mesenchymal chondrosarcoma, a rare aggressive subtype in which distant metastasis can be identified even after 20 years (5-7). Chondrosarcoma patients with unresectable disease, due to tumor location, tumor size or extensive metastatic disease, have a 5 year survival of only 2% as the overall efficacy of chemotherapy is limited (8, 9).

Gain of function mutations in the *isocitrate dehydrogenase 1* and *-2* (*IDH1* and *-2*) genes have been identified as potential driver mutations of chondrosarcoma because of their high prevalence (38%-70% depending on the subtype) (10, 11). IDH1 and IDH2 are key enzymes in cell metabolism as they convert isocitrate to α-ketogluterate (α-KG) in respectively the cytoplasm and the mitochondria. The mutant enzyme acquires the activity to convert α-KG to D-2-hydroxyglutarate (*D*-2-HG). This leads to increased levels of this oncometabolite in cartilage tumors harboring an *IDH1/2* mutation (12) which competitively inhibits the α-KG dependent enzymes by the high structural similarities (13). Although AGI-5198, a specific small molecule inhibitor of the activity of mutant IDH1, was able to decrease *D*-2-HG levels in *IDH1* mutant chondrosarcoma cell lines, this did not influence the tumorigenic properties of these cells (14, 15) which is in line with findings in *IDH2* mutant leukemia (16, 17) and *IDH1* mutant glioma models (15, 18). This suggests that although the *IDH1* or *-2* mutations are an early event in tumorigenesis, at later stages other processes involved in chondrosarcoma progression render these cells independent of the mutant IDH1/2 enzymes, which is in line with findings in other *IDH1/2*-mutated cancers (15, 16). To identify metabolic targets for *IDH1/2*-mutated glioma, Tateishi and colleagues performed a systematic metabolic profiling on glioma cells after short- and long-term mutant IDH1 inhibition (15). This study revealed a vulnerability to nicotinamide adenine

dinucleotide (NAD^+) depletion in *IDH1/2* mutant cells, demonstrated by an increased sensitivity for nicotinamide phosphoribosyltransferase (NAMPT) inhibitors. This increased sensitivity could be explained by decreased expression levels of nicotinic acid phosphoribosyltransferase (NAPRT) in *IDH1/2*-mutated tumors, potentially caused by an increased methylation of the NAPRT promoter (15). NAMPT and NAPRT are rate limiting enzymes of respectively the primary salvage pathway and the Preiss-Handler pathway, which are involved in NAD^+ synthesis (Figure 1). Tumor cells depend on these pathways for their rapid NAD turnover, as they lack expression of key enzymes in the *de novo* synthesis of NAD from tryptophan (19-21). Therefore, interfering with NAD^+ biosynthesis holds great promise as a therapeutic strategy for cancer, which is why we further explored this pathway in chondrosarcoma. We used our large chondrosarcoma cell line panel (n =11) to determine sensitivity to NAMPT inhibitors. In contrary to glioma cell lines, chondrosarcoma cell lines harboring an endogenous *IDH1* or *IDH2* mutation can grow as a monolayer culture. We determined expression levels of NAMPT and NAPRT, and methylation of the NAPRT promoter in cell lines and in primary tumors. Our results indicate that NAMPT is a promising therapeutic target in chondrosarcoma.

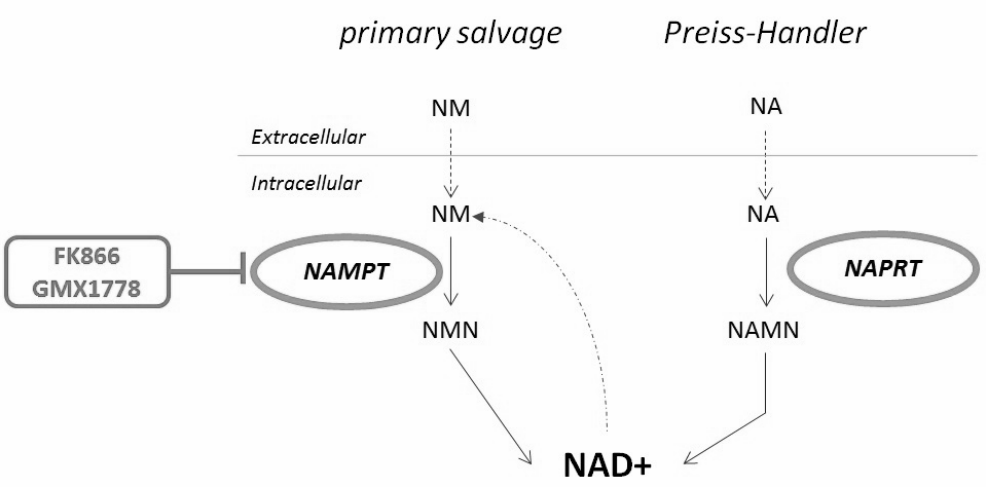


Figure 1. Schematic representation of nicotinamide adenine dinucleotide $(NAD)^+$ biosynthesis. NAMPT and NAPRT are the rate-limiting enzymes of the primary salvage pathway and the Preiss-Handler pathway, respectively. NAD^+ is recycled in the primary salvage pathway. NM, nicotinamide; NMN, nicotinamide mononucleotide; NA, nicotinic acid (vitamin B3); NAMN, nicotinic acid mononucleotide.

Materials and Methods

Compounds

NAD^+ and the NAMPT inhibitors FK866 (also known as APO866) and GMX1778 (also known as CHS-828) were purchased from Sigma-Aldrich. FK866 and GMX1778 both likely function as substrate mimetics (19). FK866 and GMX1778 were dissolved in DMSO in a concentration of 20 and 36.16 mmol/L, respectively, and stored at -20 °C. NAD^+ was dissolved in culture medium at a concentration of 10 mmol/L, stored at -20 °C, and used in a concentration of 10, 50, and 100 nmol/L. AGI-5198 (Cayman Chemical) was dissolved in DMSO in a concentration of 10 mmol/L, stored at -20 °C and used in a concentration of 1 and 10 μmol/L.

Cell culture

The conventional chondrosarcoma cell lines JJ012 (22), SW1353 (ATCC), CH2879 (23), OUMS27 (24), L835 (25) and CH3573 (26), the dedifferentiated chondrosarcoma cell lines L3252B (25), NDCS-1 (27), and L2975 (25), as well as the chondrocyte cell line LBPVA (28) and the cell line HT1080 (ATCC) were cultured in RPMI1640 (Gibco, Invitrogen) supplemented with 10% (JJ012, SW1353, CH2879, NDCS-1, L2975, HT1080) or 20% (L835, L3252B, OUMS27, CH3573, LBPVA) heat-inactivated Fetal Bovine Serum (FBS) (F7524, Sigma-Aldrich). HT1080 was originally reported to be derived from a fibrosarcoma of bone. As this is a diagnosis of exclusion and this cell line is now known to harbor an *IDH1* mutation, this tumor cell line is most likely derived from a dedifferentiated chondrosarcoma (14). MCS170 (Mesenchymal chondrosarcoma) (29) was cultured in IMDM (Gibco, Invitrogen) with 15% FBS. The lowest passage number possible was thawed (passage number between 11 and 42), the authenticity confirmed by STR profiling with the GenePrint10 (Promega Benelux BV) and tested for mycoplasma using MycoAlert (Lonza, Switzerland) before the start of the experiments. The cells were grown at 37°C with 5% CO_2 in a humidified incubator. Cell lines were never cultured for more than 3 months, and were tested for mycoplasma every 4 weeks (using RT-PCR).

qRT-PCR

RNA isolation and cDNA synthesis was performed as described previously (30). A standard quantitative reverse transcriptase PCR (qRT-PCR) was performed (31) to determine the expression levels of NAMPT and NAPRT. Primers were designed using primer3 software (http://bioinfo.ut.ee/primer3/). To correct for the amount of cDNA input, gene expression levels were normalized using the expression levels of CYPa, CPSF6 and GPR108 (32, 33). Data were normalized using the ΔΔCq method using Bio-Rad CFX Manager (Bio-Rad).

Proliferation assay

The cell lines were plated in triplicate at a density of 3,000 to 20,000 cells per well depending on the growth rate. After the cells adhered overnight, the compounds were added in their corresponding concentrations. To determine the effect of AGI-5198 on NAMPT inhibitor sensitivity, cells were pretreated for 72 hours with 1 or 10 μmol/L AGI-5198, after which the cells were counted and the same number of cells were plated for the different pretreatment conditions. Treatment with AGI-5198 was continued when the NAMPT inhibitors were added. To validate that NAD^+ could reverse the effect of the NAMPT inhibitors, NAD^+ was added in a concentration of 10, 50, or 100 nmol/L at the same time as the NAMPT inhibitors. After 72 hours of incubation, cell viability was measured using the PrestoBlue Cell Viability Reagent (Promega Benelux BV) according to the manufacturer's instructions. Colorimetric values in the plates were subsequently measured using a Wallac 1420 VICTOR2 (PerkinElmer). Data were analyzed in Graphpad Prism 5.0 (www.graphpad.com). The results shown are the results of three independent experiments. Absolute IC_{50}s for FK866 and GMX1778 were compared with NAPRT expression levels using Pearson correlation (IBM SPSS Statistics 20).

Cell counting

To confirm that a decrease in cell viability corresponds to an absolute decrease in cell number, the cell lines JJ012, SW1353 and CH2879 were plated in black 96-well μ-Clear Plates (Greiner) in a fully independent experiment. After the cells adhered overnight, the corresponding concentrations GMX1778

were added to the wells in duplicates. After 72 hours of incubation, the PrestoBlue Cell Viability Reagent was added as described above. After measuring the Colorimetric values, cells were washed with PBS, fixed in 4% paraformaldehyde for 30 minutes and stained with Hoechst 33342 (Thermo Fisher Scientific). The cells were counted using Cellomics (Thermo Fisher Scientific) according to manufacturer's instructions. Results were normalized to mocktreated wells.

3D outgrowth/invasion assay

The invasion assay, based on invasion of cells in a 3D extracellular matrix scaffold, was performed as described previously (34). In short, trypsinized monolayer cultured cells were suspended in PBS containing 2% polyvinylpyrrolidone (PVP; Sigma–Aldrich), after which they were printed into 70 μL solidified collagen gels in glass-bottom 96 well plates (Greiner) using injection robotics. Three droplets were injected per well, forming three collagen-embedded tumor spheroids per well. Subsequently, 130 μL medium containing compounds at indicated concentrations was added to each well. To assess outgrowth and invasion of the spheroids, images were taken 1 hour postinjection and 3 days postinjection using the Motic Motical 3 CMOS 3.0MP Color Digital Camera and the corresponding Motic Images Plus 2.0 ML Software.

Colony formation assay

The colony formation assay was performed according to the "plating before treatment" method as described by Franken and colleagues (35). After counting, 1,000 NDCS-1 cells and 1,500 JJ012 cells were seeded per well. The next day, FK866 and GMX1778 were added in the indicated concentrations. The 6-well plates were analyzed at day 10 after staining with 0.05% crystal violet – 6% glyceraldehyde. Colonies were quantified by manual counting, followed by normalization to the negative control. Quantification is done for at least three separate experiments.

Methylation analysis

Methylation of the NAPRT promoter in chondrosarcoma cell lines was determined using a previously described genome-wide methylation dataset

(14). In addition, we used a previously conducted but unpublished methylation array of *IDH1/2* mt cartilage tumors, for which genomic DNA of four enchondromas and five ACTs, nine grade II chondrosarcomas and four grade III chondrosarcomas was bisulfite-converted using the EZ DNA Methylation Gold Kit (Zymo Research) and used for microarray-based DNA methylation analysis, performed at ServiceXS using the HumanMethylation450 BeadChip array (Illumina). The bisulfite-converted DNA was processed and hybridized to the arrays according to the manufacturer's instructions. We performed data analysis in R version 3.2.3. 'methylumi' (36) was used to load data from the raw data files and perform data quality checks. One grade II sample was excluded from the analysis based on an inflated average detection *P* value. Samples were normalized using the BMIQ procedure from the 'wateRmelon' package (37). We selected probes around the NAPRT gene from base pair position 144658390 until 144668845 on chromosome 8. A heatmap from this region including all samples was generated from the β-values using Gene-E (Broad Institute). We compared the level of methylation of the promoter region of NAPRT (from 144659831 to 144660631 (15)) between low-grade (enchondroma and ACT) and high-grade (grade II and grade III) tumors. A *t* test was performed to compare the groups. In addition, we compared methylation of the CpG island of the NAPRT promoter region with NAPRT mRNA expression in chondrosarcoma cell lines and in a subset (n=13) of these cartilage tumors using the Spearman correlation (IBM SPSS Statistics 20).

Results

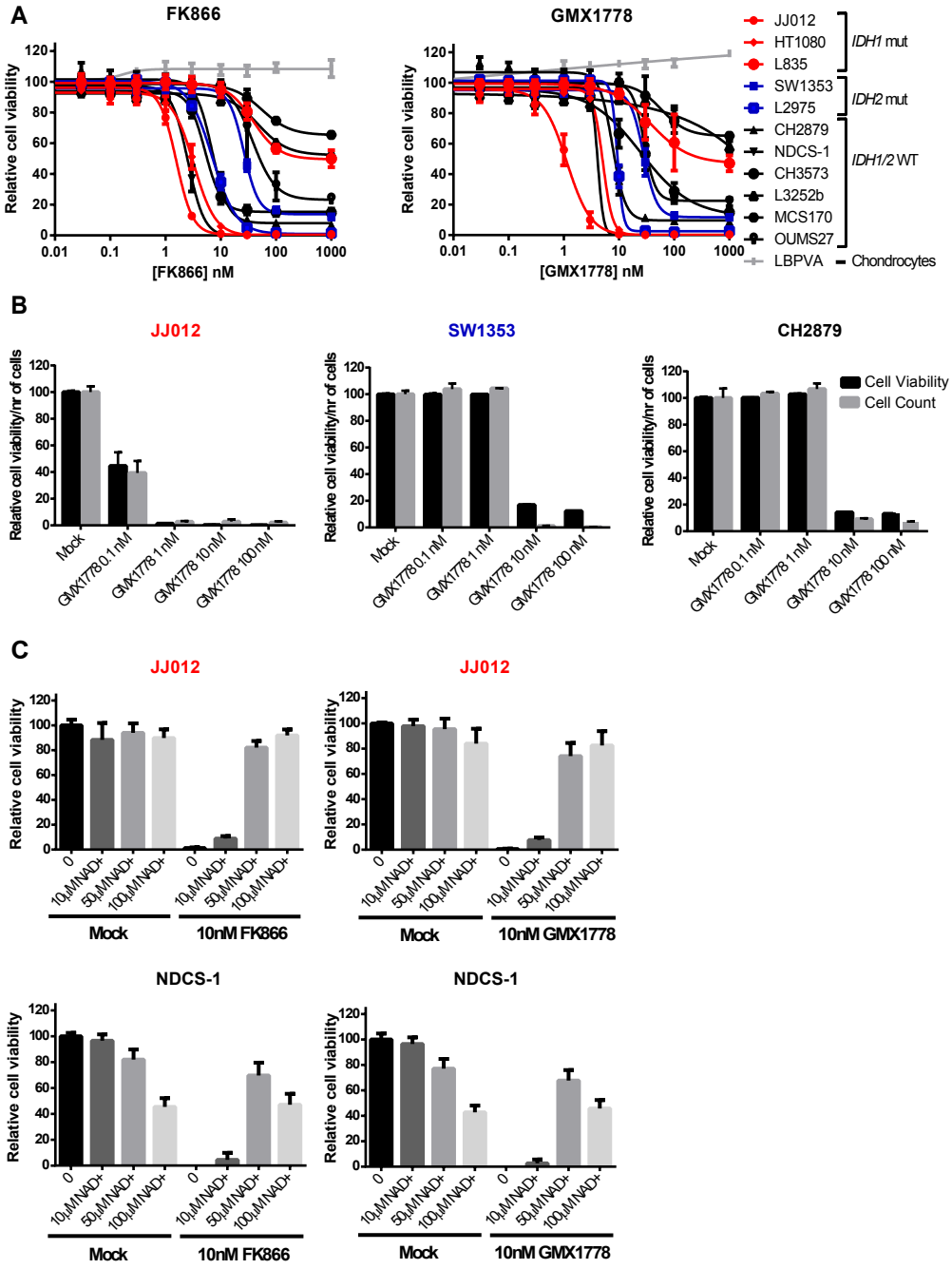
NAD^+ depletion inhibits chondrosarcoma cell viability, invasion and colony formation

To explore whether NAMPT could be a therapeutic target for chondrosarcoma, we treated 11 chondrosarcoma cell lines with FK866 and GMX1778. In contrast to the chondrocyte cell line LBPVA, all chondrosarcoma cell lines showed dose-dependent decreases in cell viability and 8 out of 11 chondrosarcoma cell lines showed a more than 75% reduction in cell viability upon treatment with 1 μmol/L NAMPT inhibitor (Figure 2A). Sensitivities for FK866 and GMX1778 were highly comparable within cell lines (Figure 2A). Comparing cell counts with cell viabilities confirmed that a decrease in

cell viability was caused by an absolute decrease in cell number (Figure 2B). Cell viability could be rescued by coincubation with NAD^+ in two cell lines tested, demonstrating that the treatment with FK866 and GMX1778 indeed caused on target inhibition (Figure 2C). Interestingly, high concentrations of NAD^+ also reduced cell viability in NDCS-1, suggesting that NAD^+ levels are tightly regulated in these cells. Absolute IC_{50} values varied between the different chondrosarcoma cell lines: six cell lines had absolute IC_{50} values below 10 nmol/L and three cell lines had absolute IC_{50} values above 100 nmol/L (Table 1). In addition to determining the effect of NAMPT inhibition on cell viability, we studied its effect on JJ012 spheroid outgrowth and invasion using a 3D collagen scaffold model (Figure 2D). Upon treatment with FK866 or GMX1778, a clear decrease in invasive outgrowth was observed. In addition to cell viability and spheroid outgrowth and invasion, these compounds inhibited colony formation of JJ012 and NDCS-1 cells already at a concentration of 0.5 nmol/L (Figure 2E and F). This demonstrates that chondrosarcoma cell lines depend on NAMPT for their NAD^+ generation and that NAD^+ is essential for their tumorigenic properties.

Table 1. Absolute IC_{50} values for the NAMPT inhibitors FK866 and GMX1778 of the chondrosarcoma cell lines.

Cell Line	IC_{50} FK866 (nmol/L)	IC_{50} GMX1778 (nmol/L)
JJ012	1.54	1.09
HT1080	3.04	5.02
L835	497.15	358.18
SW1353	28.08	29.11
L2975	7.17	9.44
CH2879	7.51	8.39
NDCS-1	2.49	4.09
CH3573	5.67	30.68
L3252b	>1000	>1000
MCS170	>1000	>1000
OUMS27	50.70	32.94


A
FK866
GMX1778
Relative cell viability
[FK866] nM
[GMX1778] nM
JJ012
HT1080
L835
SW1353
L2975
CH2879
NDCS-1
CH3573
L3252b
MCS170
OUMS27
LBPVA
Chondrocytes
IDH1 mut
IDH2 mut
IDH1/2 WT
B
JJ012
SW1353
CH2879
Relative cell viability/nr of cells
Cell Viability
Cell Count
Mock
GMX1778 0.1 nM
GMX1778 1 nM
GMX1778 10 nM
GMX1778 100 nM
C
JJ012
JJ012
NDCS-1
NDCS-1
Relative cell viability
0
10 µM NAD+
50 µM NAD+
100 µM NAD+
Mock
10nM FK866
10nM GMX1778

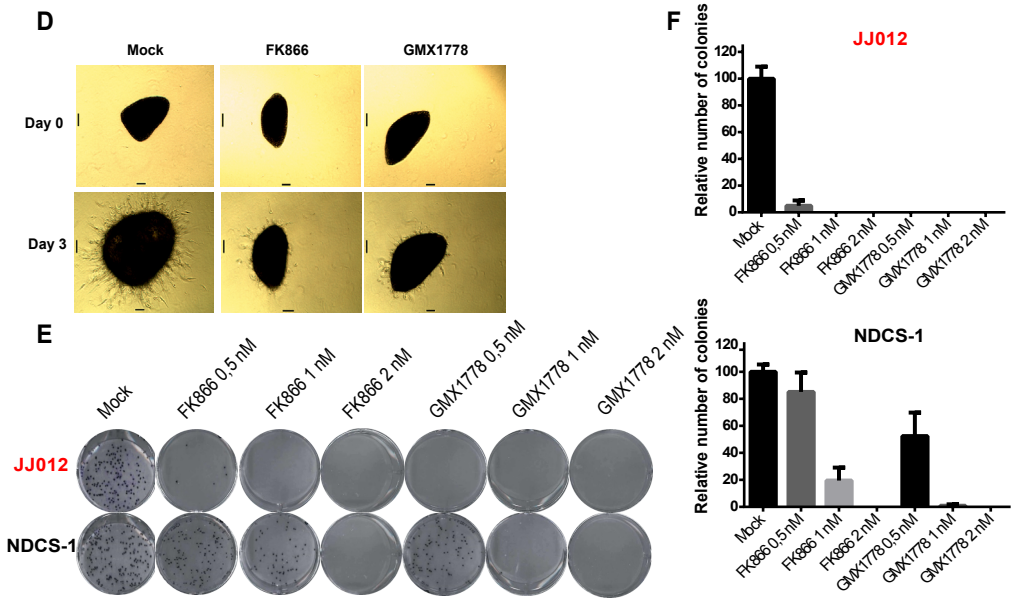


Figure 2. Chondrosarcoma cell lines are sensitive for NAD^+ depletion. **A:** Eleven chondrosarcoma cell lines and a chondrocyte cell line were treated for 72 hours with two NAMPT inhibitors, FK866 and GMX1778, after which cell viability was assessed by a PrestoBlue assay. All cell lines showed a dose-dependent decrease in cell viability except the LBPVA control and in 8 of 11 cell lines this reduction was more than 75%. **B:** Cell count results (using Cellomics) were very similar to cell viability results (using PrestoBlue). **C:** JJ012 (*IDH1* mt) and NDCS-1 (*IDH* WT) were cotreated with NAMPT inhibitors and 10, 50, or 100 µmol/L NAD^+. NAD^+ abolished the effect of the NAMPT inhibitors, demonstrating that the effect of NAMPT inhibitors is caused by an NAD^+ depletion. **D:** 3D outgrowth and invasion of JJ012 cells in a collagen scaffold-embedded spheroid model. JJ012 spheroid outgrowth and invasion was inhibited by treating the cells with NAMPT inhibitors. A total of 130µl medium containing 10 nmol/L compounds was added on top of the 70µl collagen gel. **E:** Representative wells from JJ012 and NDCS-1 colony formation during treatment with FK866 and GMX1778. **F:** Quantification of at least three different colony formation assays.

NAPRT methylation correlates with sensitivity for NAMPT inhibitors

We hypothesized that the variation in sensitivity to NAMPT inhibitors between cell lines could be attributed to expression of NAMPT or NAPRT, the rate limiting enzymes of pathways involved in NAD^+ synthesis. We performed qRT-PCR analyses in chondrosarcoma cell lines to determine NAMPT and NAPRT expression levels. Expression levels of NAMPT and NAPRT are highly variable between chondrosarcoma cell lines (Figure 3A). NAPRT has the highest expression in CH2879, L3252b, L835, and OUMS27, of which three out of four have IC_{50} values above 30 nmol/L for the NAMPT inhibitors. Plotting IC_{50}s for FK866 and GMX1778 versus NAPRT expression of 10 chondrosarcoma cell lines revealed a correlation between low IC_{50} values and

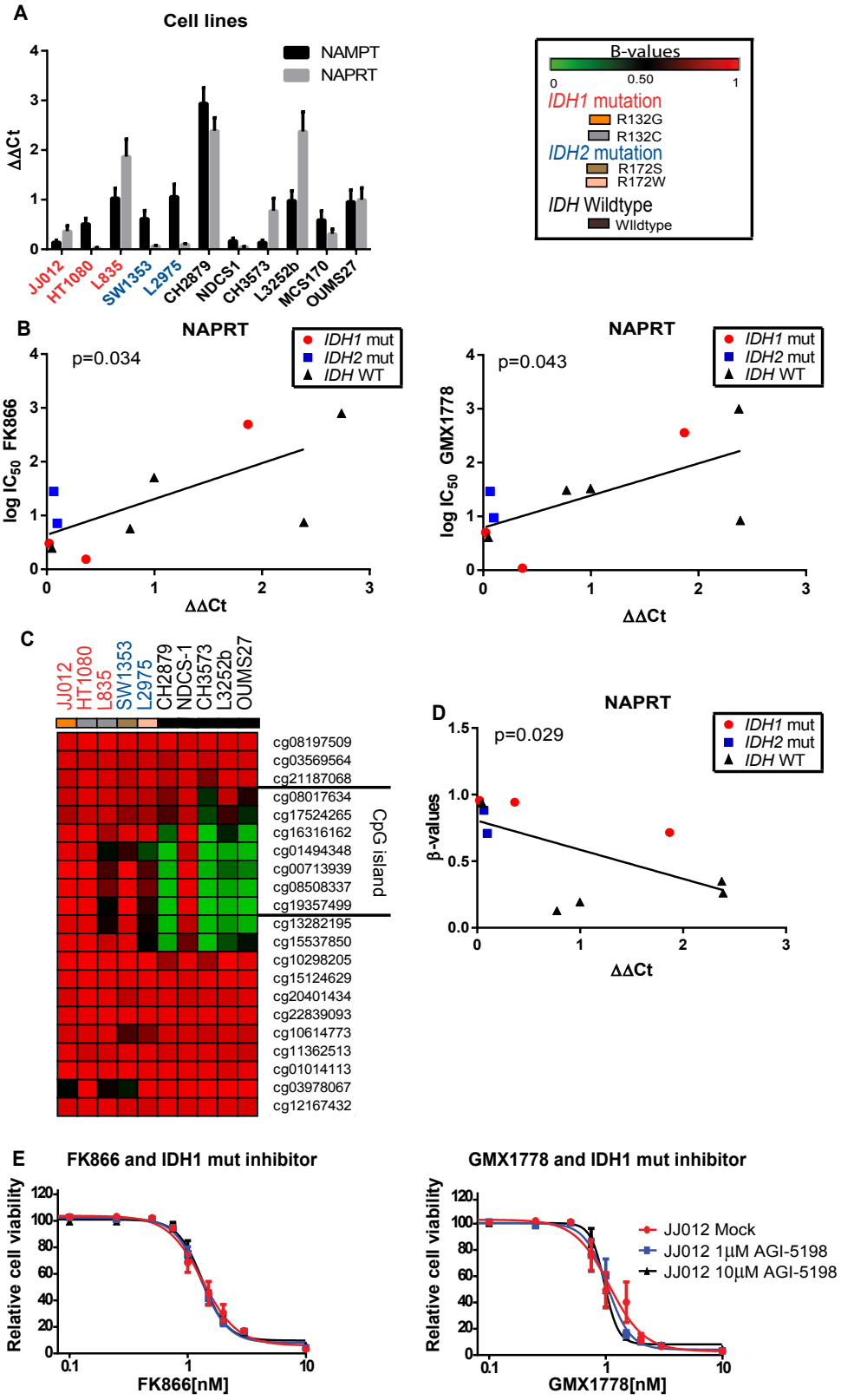

A
Cell lines
NAMPT
NAPRT
ΔΔCt
JJ012
HT1080
L835
SW1353
L2975
CH2879
NDCS1
CH3573
L3252b
MCS170
OUMS27
B-values
0
0.50
1
IDH1 mutation
R132G
R132C
IDH2 mutation
R172S
R172W
IDH Wildtype
WIldtype
B
NAPRT
p=0.034
IDH1 mut
IDH2 mut
IDH WT
log IC50 FK866
ΔΔCt
NAPRT
p=0.043
log IC50 GMX1778
ΔΔCt
C
JJ012
HT1080
L835
SW1353
L2975
CH2879
NDCS-1
CH3573
L3252b
OUMS27
cg08197509
cg03569564
cg21187068
cg08017634
cg17524265
cg16316162
cg01494348
cg00713939
cg08508337
cg19357499
cg13282195
cg15537850
cg10298205
cg15124629
cg20401434
cg22839093
cg10614773
cg11362513
cg01014113
cg03978067
cg12167432
CpG island
D
NAPRT
p=0.029
β-values
ΔΔCt
E
FK866 and IDH1 mut inhibitor
Relative cell viability
FK866[nM]
GMX1778 and IDH1 mut inhibitor
Relative cell viability
GMX1778[nM]
JJ012 Mock
JJ012 1μM AGI-5198
JJ012 10μM AGI-5198

Figure 3. NAPRT expression correlates to sensitivity for NAMPT inhibitors and to methylation of the CpG island of the NAPRT promoter. **A:** NAMPT and NAPRT are heterogeneously expressed in the different chondrosarcoma cell lines. Expression levels were determined using qRT-PCR analyses. **B:** Log IC_{50}s for the NAMPT inhibitors FK866 and GMX1778 significantly correlated to NAPRT expression levels. **C:** NAPRT methylation in a previously published methylation array of 10 chondrosarcoma cell lines. β-Values are visualized using Gene-E (Broad Institute), where red represent high methylation of the NAPRT promoter and green represents low methylation of the NAPRT promoter. **D:** NAPRT expression significantly correlates to methylation of the CpG island of the NAPRT promoter in chondrosarcoma cell lines. **E:** Pretreating JJ012 cells for 72 hours with AGI-5198, followed by a 72-hour combined treatment of AGI-5198 and the corresponding NAMPT inhibitor did not influence the dose-dependent decrease in cell viability. Cell viability was determined by a PrestoBlue assay.

low NAPRT expression levels ($P = 0.034$ and $P = 0.043$, respectively) (Figure 3B). As it has been suggested that low expression of NAPRT is mediated by methylation of the NAPRT promoter (15, 38), we assessed the methylation of the NAPRT promoter in 10 chondrosarcoma cell lines (Figure 3C) using a previously published genome wide methylation array dataset (14). The analysis showed that high CpG island methylation was associated with low NAPRT expression levels (Figure 3D) ($P = 0.029$). JJ012, HT1080, SW1353 and NDCS-1 have high β values, demonstrating high methylation of the NAPRT promoter. L835 and L2975 have medium β values, whereas the *IDH* WT cell lines CH2879, CH3573, L3252b and OUMS27 have low β values. This suggested a potential correlation between *IDH1/2* mutation status and sensitivity for NAMPT inhibitors in chondrosarcoma cell lines. However, (pre)treating JJ012 with AGI-5198, a specific IDH1 mutant inhibitor, did not affect the sensitivity of JJ012 to NAMPT inhibitors (Figure 2E). Furthermore, using previously published datasets and mRNAs of four chondrosarcoma cell lines treated for 10 and 20 passages with AGI-5198 and one *IDH* WT cell line for 10 passages with *D*-2-HG (14), we demonstrated that the mutant IDH1 enzyme did not influence the methylation of the NAPRT promoter nor NAMPT and NAPRT expression levels (data not shown).

The NAPRT promoter is hypermethylated in high-grade chondrosarcomas

To extend our study to primary tumors, we determined methylation of the NAPRT promoter in an available dataset of 20 *IDH1/2*-mutated cartilage tumors. Interestingly, we found significantly higher methylation of the CpG island of the NAPRT promoter in the high-grade (grade II and III)

chondrosarcomas compared with the low-grade (ACT and enchondroma) cartilage tumors *(P* = 0.002213: Figure 4A). To compare methylation to expression in this set of tumors, mRNA of 13 of these 20 tumors was collected, in which we could not identify a significant correlation between NAPRT CpG island promoter methylation and decreased NAPRT expression levels (*P* = 0.271: Figure 4B). In addition, we determined NAMPT and NAPRT expression levels in an independent cohort of 32 cartilage tumors, in which also 9 *IDH* WT tumors were also included (Figure 4C and D). Similar to the cell lines, expression of NAMPT and NAPRT was variable in the primary tumors. Expression levels seemed slightly lower in the high-grade tumors as compared with low-grade, although the difference was not statistically significant (*P* = 0.255, *t* test).

Discussion

The results of this study demonstrate that chondrosarcoma cell lines are vulnerable to NAD^+ depletion. Five of 11 chondrosarcoma cell lines have IC_{50} values below 10 nmol/L for the two tested NAMPT inhibitors, suggesting that these cell lines depend on the primary salvage pathway for NAD^+ synthesis. Strikingly, the chondrocyte cell line LBPVA was unaffected by NAMPT inhibition, further demonstrating the therapeutic potential of NAMPT inhibitors for chondrosarcoma patients.

To identify a possible biomarker for distinguishing sensitivity to NAMPT inhibition, we assessed methylation and expression levels of NAPRT, the rate limiting enzyme of the Preiss-Handler pathway for NAD^+ synthesis. Comparing IC_{50} values with NAPRT expression levels demonstrated that low NAPRT expression is significantly correlated to increased sensitivity for NAMPT inhibitors. Therefore, NAPRT expression partly explains the difference in NAMPT inhibitor sensitivity between the different cell lines. Strikingly, we observed higher methylation of the CpG island of the NAPRT promoter in high-grade chondrosarcomas versus low-grade cartilage tumors, suggesting that NAMPT can be a promising target especially in these clinically challenging patients.

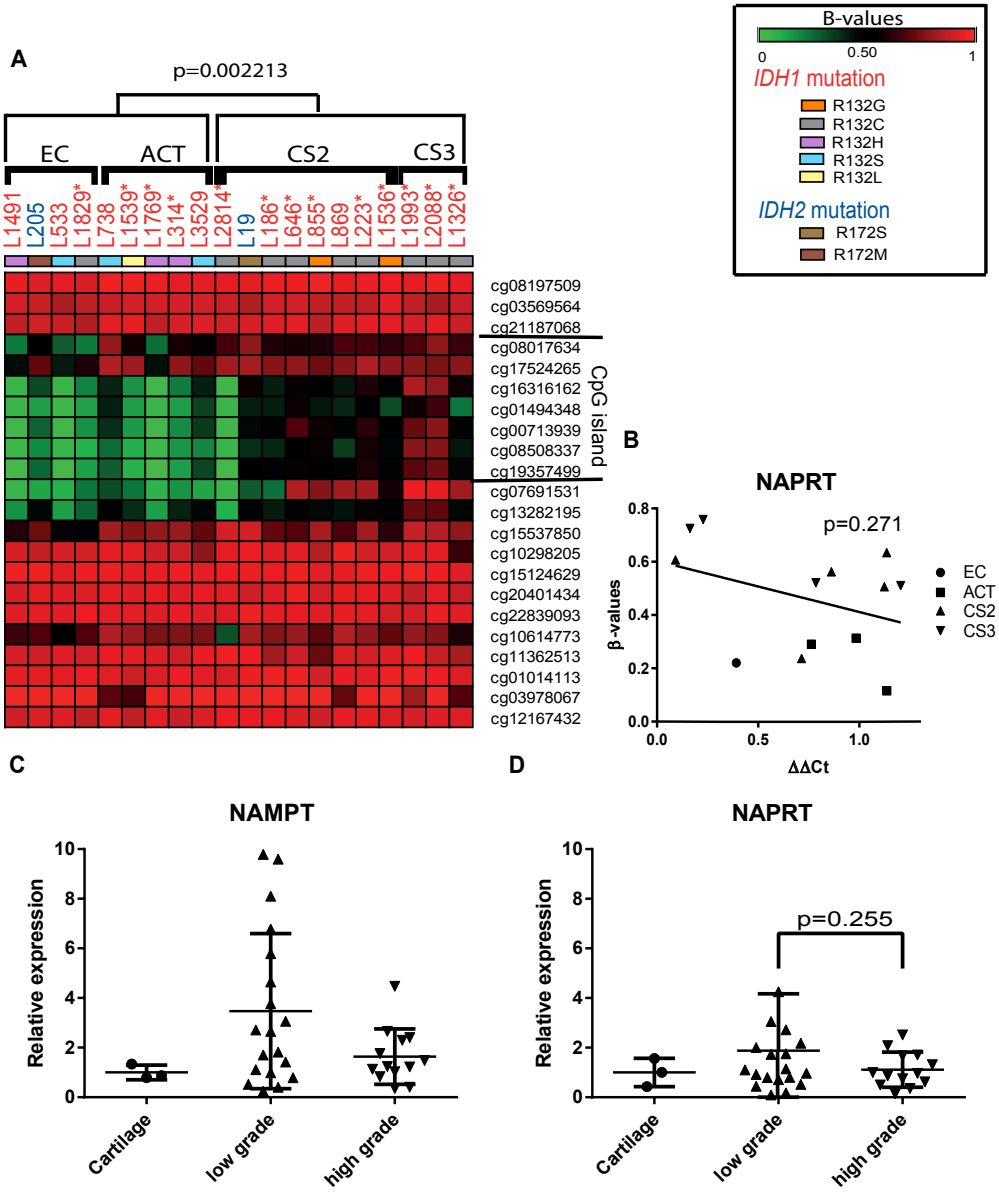


Figure 4. A: The CpG island of the NAPRT promoter is hypermethylated in high-grade compared with low-grade *IDH1/2* mutant cartilage tumors. β-Values are visualized using Gene-E (Broad Institute), where red represent high methylation of the NAPRT promoter and green represents low methylation of the NAPRT promoter. mRNA was available of cell lines marked with a *. **B:** Methylation of the CpG island of the NAPRT promoter weakly correlated to NAPRT expression in cartilage tumors. **C:** qRT-PCR analyses of NAMPT expression in an independent cohort of 32 cartilage tumors. **D:** NAPRT expression in a cohort of 32 cartilage tumors shows a nonsignificant trend for higher expression in low-grade versus high-grade cartilage tumors. Expression was normalized to cartilage. EC, enchondroma; ACT, atypical cartilaginous tumor; CS2, chondrosarcoma grade II; CS3, chondrosarcoma grade III.

We did not observe a correlation between the presence of a mutant IDH1/2 enzyme and sensitivity for NAMPT inhibitors in chondrosarcoma. Furthermore, inhibition of the IDH1 mutant enzyme by AGI-5198 did not influence sensitivity for NAMPT inhibitors nor methylation of the NAPRT promoter. This is in line with previous studies from our group, where we demonstrated that *IDH1/2* mutations do not affect IHC levels of 5-hmC, 5mC, and trimethylation of H3K4, -9, and -27 (39), and prolonged inhibition of the IDH1 mutant enzyme does not affect global gene expression, CpG island methylation nor histone H3K4, -9, and -27 trimethylation in chondrosarcoma cell lines (14). Our observations are in contrast to the conclusion from Tateishi and colleagues, who suggest that mutant IDH1 downregulates NAPRT expression, making *IDH1* mutant cell lines more sensitive to NAMPT inhibitors (15). However, only two chondrosarcoma cell lines were included in that study, which both harbor an *IDH1 or IDH2* mutation and are sensitive to NAMPT inhibition. No cotreatment with a mutant IDH1 inhibitor and a NAMPT inhibitor was performed, and NAPRT expression was not assessed in primary tumors; the link between mutant *IDH1* and NAPRT expression was demonstrated by the introduction of an *IDH1* mutation in *IDH* WT glioma cells. Therefore, experimental differences and a different tumor type may explain the discrepancy between experimental results.

Phase I clinical trials to test the safety of NAMPT inhibition have been performed with FK866, GMX1778, and its prodrug GMX1777 (19). However, further evaluation was discontinued due to dose-limiting toxicities (19). To increase the therapeutic index of NAMPT inhibitors, co-administration with nicotinic acid (NA) has been proposed (19). NA can be used to synthesise NAD^+ in NAPRT-proficient cells, thereby decreasing the toxicity without interfering with its efficacy in the treatment of NAPRT-deficient tumors. Indeed, it was shown that the effect of NAMPT inhibitors on tumorigenic properties of HT1080 and SW1353 chondrosarcoma cell lines and HT1080 xenografts was not affected by co-administration of NA (40), suggesting that this could be a suitable approach to decrease dose-limiting toxicities in chondrosarcoma patients.

Collectively, this study demonstrates that NAMPT inhibitors hold potential

therapeutic promise for chondrosarcoma patients, especially for those with high histologic grade as these, due to hypermethylation of the NAPRT promoter, are dependent on the primary salvage pathway for their NAD^+ synthesis.

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Chapter 7

Targeting glutaminolysis in chondrosarcoma in context of the *IDH1/2* mutation

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Abstract

Chondrosarcoma is a malignant cartilage-forming bone tumour in which mutations in *IDH1* and *IDH2* frequently occur. Previous studies suggest an increased dependency on glutaminolysis in *IDH1/2* mutant cells, which resulted in clinical trials with the drugs CB-839, metformin and chloroquine. In this study, the preclinical rationale for using these drugs as a treatment for chondrosarcoma was evaluated. Expression of glutaminase was determined in 120 cartilage tumours by immunohistochemistry. Ten chondrosarcoma cell lines were treated with the metabolic compounds CB-849, metformin, phenformin (lipophilic analogue of metformin) and chloroquine. A difference in glutaminase expression levels between the different tumour grades (p = 0.001, one-way ANOVA) was identified, with the highest expression observed in high-grade chondrosarcomas. Treatment with CB-839, metformin, phenformin or chloroquine revealed that chondrosarcoma cell lines are sensitive to glutaminolysis inhibition. Metformin and phenformin decreased mTOR activity in chondrosarcoma cells, and metformin decreased LC3B-II levels, which is counteracted by chloroquine. In conclusion, targeting glutaminolysis with CB-839, metformin, phenformin or chloroquine is a potential therapeutic strategy for a subset of high-grade chondrosarcomas, irrespective of the presence or absence of an *IDH1/2* mutation.

Introduction

Chondrosarcoma is the second most common primary bone malignancy in humans. It represents a heterogeneous collection of cartilage-forming tumours, which can be divided in several subtypes and histological grades (1). The most common subtype is conventional chondrosarcoma (85%), which arises centrally in the medulla of the bone. Conventional chondrosarcoma is histologically graded to determine treatment strategy and the patient's prognosis. The atypical cartilaginous tumour (ACT, previously known as chondrosarcoma grade 1), accounts for 61% of cases. First-line treatment consists of curettage with local adjuvant treatment, resulting in a 5-year survival rate of 95%. Grade II (36%) and grade III (3%) chondrosarcomas have a worse 5-year survival of 86% and 58%, respectively, due to the occurrence of metastases (1-3). These tumours

are treated with *en bloc* resection. Dedifferentiated chondrosarcoma is a highly malignant subtype with an overall survival rate of 7–24% (4). Mesenchymal chondrosarcoma has a 10-year survival rate between 44 and 54% (5, 6). It is a rare aggressive subtype in which distant metastasis can be identified even after 20 years (5-7). Chondrosarcoma patients with inoperable disease, due to tumour location, tumour size or extensive metastatic disease benefit from a doxorubicin-based chemotherapeutic regimen, which increases the 3-year survival from 8 to 26% (8). As the overall efficacy of chemotherapy is limited, new treatment options are needed, which can be identified by further unravelling the essential driver genes and pathways of these tumours.

Potential driver mutations of central conventional and dedifferentiated chondrosarcoma are gain of function mutations in *isocitrate dehydrogenase 1* and *2 (IDH1* and *IDH2)*, which have been identified in 38–70% of the cases (9, 10). Its occurrence in the benign precursors lesions (enchondromas), of which 52–87% harbour an *IDH1/2* mutation (11, 12), further demonstrates that *IDH1/2* mutations are an early event in chondrosarcoma genesis. IDH1 and IDH2 are essential enzymes in cell metabolism, as they convert isocitrate to α-ketoglutarate (α-KG) in respectively the cytoplasm and the mitochondria. The mutant enzyme acquires the activity to convert α-KG to *D*-2-hydroxyglutarate (*D*-2-HG), an oncometabolite that competitively inhibits the α-KG dependent enzymes by the high structural similarities (13). Processes involved in chondrosarcoma progression make these cells independent of the mutant IDH enzymes, as treatment with AGI-5198, a specific IDH1 mutant inhibitor, did not influence the tumourigenic properties of these cells (14). Therefore, we propose to exploit the metabolic vulnerability caused by the *IDH1/2* mutations as therapeutic strategy for chondrosarcoma.

IDH1/2 mutant cells need α-KG for the production of *D*-2-HG, which can be generated via glycolysis and glutaminolysis. It has been suggested that *IDH1/2* mutated tumours depend on glutaminolysis for their α-KG supply (15-17), which led to two clinical trials that were recently started in *IDH1/2* mutated solid tumours, including chondrosarcomas. The first one is a phase I trial with the drug CB-839 (NCT02071862 clinicaltrials.gov), an inhibitor of glutaminase (Figure 1). The second one is a phase IB/II trial with the drugs

metformin and chloroquine (NCT02496741 clinicaltrails.gov), after which the feasibility of phenformin may be explored as an alternative to metformin in case of lack of effect of metformin (18). Metformin is a first-in-line drug used for the treatment of type II diabetes mellitus that inhibits gluconeogenesis in the liver. It has several effects on cellular proteins, among which it (1) activates adenosine monophosphate activated protein kinase (AMPK), thereby inhibiting the mammalian target of rapamycin (mTOR) (19); (2) inhibits complex 1 of the electron transport chain (20); and (3) indirectly inhibits glutaminase, the enzyme that converts glutamine to glutamate, via c-Myc; (Figure 1) (21, 22). Phenformin is a lipophilic analogue of metformin with similar working mechanisms, but in contrast to metformin it does not depend on solute carrier (SLC) 22A1-3 transport to get into cells (20, 23); The anti-malaria drug chloroquine, in addition to its well-known anti-autophagy potency, is able to inhibit glutamate dehydrogenase, an enzyme converting glutamate to α-KG (Figure 1)(24, 25).

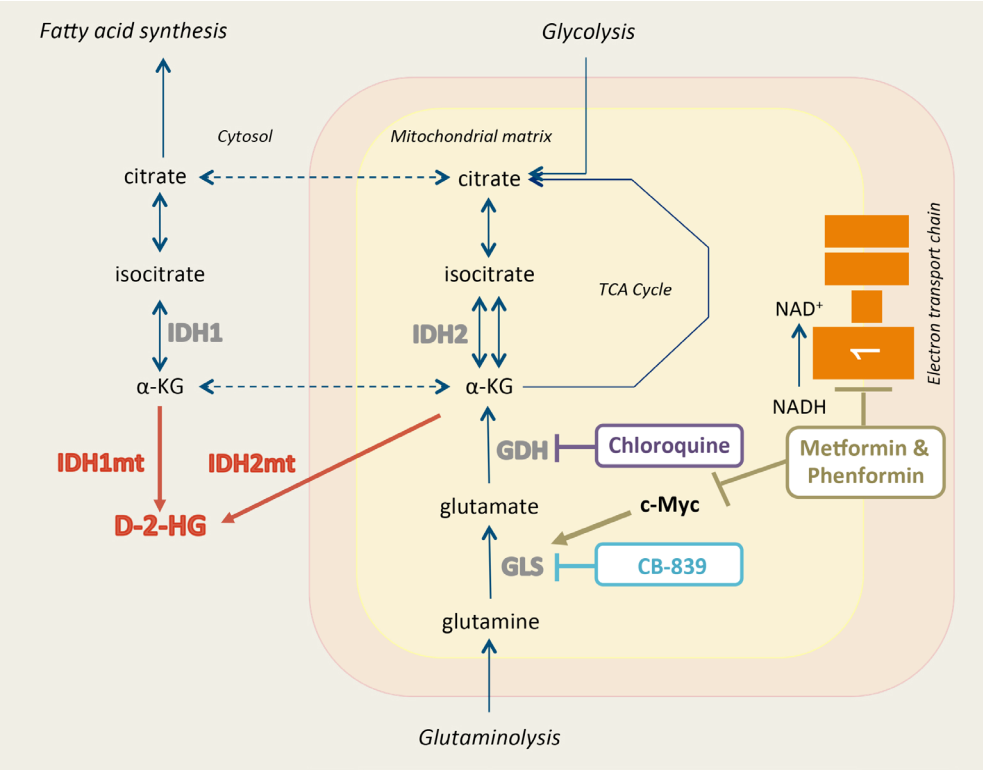

Figure 1. Schematic representation of glutamine metabolism and the compounds used in this study. IDH isocitrate dehydrogenase, IDHmt mutated IDH, *D*-2-HG *D*-2-hydroxyglutarate, α-KG α-ketoglutarate, GLS glutaminase, GDH glutamate dehydrogenase, NAD Nicotinamide adenine dinucleotide, TCA tricarboxylic acid

In this study, we evaluate whether there is preclinical rationale to target glutaminolysis as a treatment for chondrosarcoma by determining the expression levels of glutaminase in chondrosarcoma primary tumours and by evaluating the effect of metformin, phenformin, chloroquine and CB-839 on chondrosarcoma cells.

Material and methods

Immunohistochemistry on tissue microarrays

Glutaminase monoclonal antibody (AB156776, Abcam) (1:400) was used for immunohistochemical stainings on previously generated and published formalin-fixed, paraffin-embedded tissue microarrays (26) as described (27). Hundred and twenty cartilage tumours could be scored, consisting of 12 benign (enchondromas or osteochondromas), 56 ACTs, 36 grade II and 16 grade III tumours. Two independently operating observers used the following scoring procedure: intensity score (0 negative, 1 weak, 2 moderate, 3 strong) + percentage score (0 = 0%, 1 = 1–24%, 2 = 25–49%, 3 = 50–74% and 4 = 75–100%). Discrepancies were discussed to reach consensus. Of the central cartilage lesions that could be scored, the *IDH* mutation status was known of 54 tumours, of which 33 harboured an *IDH1* or *IDH2* mutation and 21 were wildtype.

Statistical analysis

Statistical analysis on immunohistochemistry data was performed using Statistical Package for the Social Sciences 23 (SPSS Statistics, IBM). One-way ANOVA with the Fisher's least significant difference (LSD) post-hoc analysis was used to compare glutaminase expression levels between different tumour grades. The difference in glutaminase protein expression between high-grade cartilage tumours (grade II and grade III cartilage tumours) and low-grade cartilage tumours (enchondromas, osteochondromas and ACT) was determined using independent-samples *T* test. Results were considered significant at the $\alpha = 0.05$ level.

Cell culture

Five *IDH1 or IDH2* mutated (JJ012 (28), SW1353 (ATCC #HTB-94), L2975 (29), L835 (29) and HT1080 (30)) and five *IDH1/2* wildtype (CH2879 (31), MCS170 (32), CH3573 (33), NDCS1 (34) and L3252b (29)) chondrosarcoma cell lines were analysed. Five of these originate from conventional chondrosarcoma (JJ012, SW1353, L835, CH2879 and CH3573), three from dedifferentiated chondrosarcoma (L2975, NDCS1 and L3252b) and one from mesenchymal chondrosarcoma (MCS170). HT1080 was originally reported as a fibrosarcoma of bone. As this is a diagnosis of exclusion and this cell line is now known to harbour an *IDH1* mutation, this tumour most probably reflects a dedifferentiated chondrosarcoma (14). Cells were cultured at 5% CO2 and 37°C in a humidified incubator using RPMI 1640 (Gibco) with 10% (JJ012, SW1353, L2975, HT1080, CH2879 and NDCS1) or 20% (L835, CH3573 and L3252b) heat inactivated foetal bovine serum (FBS) (F7524, Sigma Aldrich). MCS170 was cultured in IMDM (Gibco) with 15% FBS. The authenticity of the cells was confirmed by STR profiling with the GenePrint10 (Promega Benelux BV) and cells were tested for mycoplasma using MycoAlert (Lonza, Switzerland) before the start of the experiments. Cell lines were never cultured for more than three months, and were tested for mycoplasma every 4 weeks (using RT-PCR).

qRT-PCR

RNA isolation of chondrosarcoma cells and the anonymised controls (growth plate and articular cartilage) was performed using TRIzol (Ambion biosystems, Invitrogen) followed by a standard RNA isolation protocol and cDNA synthesis (35). Product size and sequence were validated using Qiaxcel (Qiagen) and Sanger sequencing (Applied Biosystems 48- or 96-cappilary 3730 system, Leiden genome technology centre), respectively. Standard qRT-PCR analyses were performed as described previously (36) to determine glutaminase, SLC22A1, SLC22A2 and SLC22A3 expression levels. GPR108, CYPa and CPSF6 were used as housekeeping genes for normalization (37, 38). Data were normalised using the delta-delta Cq method using Bio-Rad CFX Manager (Bio-Rad).

Cell viability assay

Cells were counted with the Muse Cell Analyzer (Millipore BV) using the Muse calibration kit (Millipore BV) according to manufacturer's instruction. Plating was done in densities optimised for each cell line and condition *i.e.,* 3000–15,000 cells per well for 72 h, 200–400 cells per well for one week incubation in triplicates. CB-839 (s7655, Selleckchem), metformin hydrochloride (215169110, Bioconnect), phenformin hydrochloride (219590, Santa Cruz Biotechnology) and chloroquine diphosphate salt (c6628, Sigma Aldrich) were added after the cells adhered overnight. The metabolic drugs were incubated for 72 h or 1 week after which cell viability was measured using the PrestoBlue Cell Viability Reagent (Promega Benelux BV) according to the manufacturer's instructions. Colorimetric values in the plates were subsequently measured using a Wallac 1420 VICTOR2 (Perkin Elmer). Data were analysed in Graphpad Prism 5.0 (www.graphpad.com). For the combination of metformin, phenformin and chloroquine with AGI-5198 (14624, Life technologies), cells were pretreated for 72 h with AGI-5198 (1 and 10 μM) or DMSO. For the analyses in which the effect of FBS on CB-839 sensitivity was evaluated, the medium with or without FBS and the corresponding concentrations of the metabolic compounds were added at the same time, so after the cells were allowed to adhere overnight.

Cell count assay

As the PrestoBlue assay measures mitochondrial activity, we confirmed that the effects of metformin, phenformin and chloroquine on cell viability were caused by an absolute decrease in cell number by fixing the cells in 4% paraformaldehyde for 15 min, followed by nuclear staining using Hoechst 33342 (Fischer Scientific). The plates were imaged using a BD Pathway 855 imager (Becton Dickinson), after which the images were processed using an Image-Pro Analyser 7.0 algorithm. Hoechst area was used as a read out to quantify the amount of cells in each well.

Analysis of apoptosis

For analysis of apoptosis, the caspase-glo 3/7 assay (Promega) was used according to manufacturer's instructions. Cells were seeded in white walled

96-wells plates (Corning BV Life Sciences) in densities which resulted in 70% confluence after 24 h as described previously (39). HT1080, JJ012, SW1353, NDCS1 and CH2879 cells were treated with their IC_{75} of metformin, phenformin, chloroquine and CB-839 (based on 72 h dose response curves). The concentration of compounds used was 10 mM metformin, 100 μM phenformin, 50 μM chloroquine and 6 μM CB-839 if IC_{75}, were above these concentrations. CH2879 cells treated with 1 μM doxorubicin (obtained from the in-house hospital pharmacy) and 50 μM ABT-737 (Catalog No. S1002, Selleckchem) were used as positive control. For the negative control doxorubicin and ABT-737 were combined with Z-vad-FMK (550377, BD Biosciences). After 24h the caspase-glo substrate was added 1:1 followed by incubation of 60 min at room temperature. Wells were analysed using Wallac 1420 VICTOR2. The experiment was performed two times in duplicate. Data was corrected for plane RPMI control and normalised to untreated control for each cell line. Viability was measured on a simultaneously treated plate after 24 h.

Western blot analysis

HT1080, JJ012, SW1353, NDCS1 and CH2879 cells are treated with their half maximal inhibitory concentration (IC_{50}) values (based on dose response curves of 72h) of metformin, phenformin, chloroquine or CB-839 and lysed after 72h. A maximum concentration of compounds of 10 mM metformin, 100 μM phenformin and 6 μM CB-839 was used if the IC_{50} was above these concentrations. Western blotting was performed for LC3B (1:1000, clone D11, #3868, Cell Signaling Technology) and phospho-S6 (1:1000, 2F9, #4856, Cell Signaling Technology). As a loading control, α-tubulin (1:10,000, clone DM1A, Sigma-Aldrich Chemie) expression was used. Cells were lysed using hot-SDS buffer (1% SDS, 10 mM Tris/EDTA with complete inhibitor and phosSTOP). For each sample, 10 μg protein was loaded on TGX Stain-Free™ FastCast™ 12% Acrylamide Gels (Bio-Rad). Proteins were transferred to a Polyvinylidene difluoride (PVDF) membrane using Trans-Blot® Turbo™ Transfer System (Bio-Rad) and Trans-Blot® Turbo™ RTA Transfer kit PVDF (Bio-Rad) and detected using enhanced chemo luminescence (Pierce ECL Western Blotting Substrate Fisher Scientific), followed by exposure of 30 s to 5 min and development of the film (ECL hyperfilm, Amersham, GE Healthcare).

Cell line metabolic profiling

A Seahorse XFe 96 analyser (Seahorse Bioscience, Agilent) was used to measure the oxygen consumption rate (OCR) and the extracellular acidification rate (ECAR) in chondrosarcoma cell lines JJ012, SW1353 and CH2879 after metformin treatment. Thirty hours prior to the assay, cells were plated in optimised densities being 15,000, 13,000 and 30,000 for JJ012, SW1353 and CH2879, respectively. After 6 h cells were treated with 5 mM metformin for 24 h. Before the measurement, cells were incubated for 1 h in glucose-free RPMI-1640 supplemented with 5% FBS. During the assay, sequential injections of 10 mM glucose (Sigma-Aldrich), 2.0 μM oligomycin A, 2 μM carbonyl cyanide-4-(trifluoromethoxy)phenylhydrazone (FCCP) and 0.5 μM 1:1 rotenone : antimycin A (Cayman Chemicals) established the metabolic profile of all cell lines. Data was normalised to cell numbers measured in each individual well, determined using a Cellomics HCS fluorescent microscope (Thermoe Fisher) after fixation and Hoechst staining. Data represented as the average ±SD of triplicate measurements for metformin treated cells and 5–7 replicates for controls.

Results

Glutaminase is a potential therapeutic target in a subset of chondrosarcomas

By immunohistochemistry, a difference in glutaminase expression levels between the different tumour grades was identified (ANOVA, $p = 0.001$), and the post-hoc analysis revealed that specifically the grade II tumours and the grade III tumours had higher expression levels compared to the ACTs (both $p = 0.001$) (Figure 2A-E). As no difference between central and peripheral cartilage tumours was observed, these were combined in the analyses. Grouping the high-grade cartilage tumours (grade II and grade III chondrosarcomas) and the low-grade cartilage tumours (ACTs and enchondromas/osteochondromas) further demonstrated the significant difference in glutaminase expression levels between high-grade and low-grade cartilage tumours ($p < 0.0001$, independent-samples T test) (Figure 2A). No difference in glutaminase expression between *IDH1/2* mutant and *IDH1/2* wildtype central cartilage tumours was observed (Figure 2B). Therefore, glutaminase is higher expressed in high-grade compared to low-grade cartilage tumours but does not correlate to *IDH1/2* mutation status.

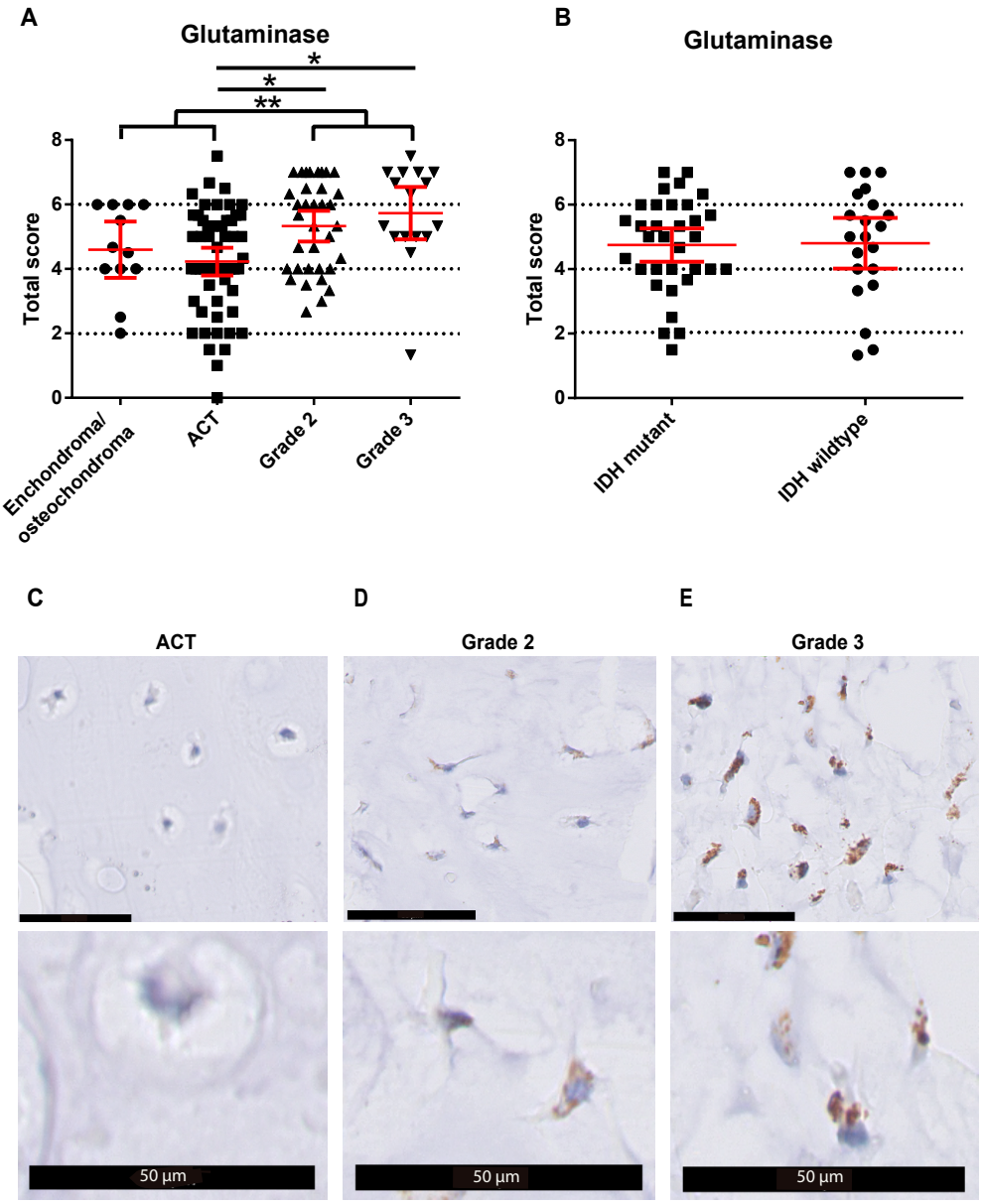

Figure 2. Glutaminase expression correlates to tumour grade but not to *IDH1/2* mutation status. **A:** Total score (intensity + percentage) of glutaminase expression. * $p = 0.001$ by one-way ANOVA with the LSD post-hoc analysis. ** $p < 0.0001$ by independent-samples *T* test, grouping the high-grade and the low-grade cartilage tumours. **B:** No difference between *IDH1/2* mutant and *IDH1/2* wildtype central tumours was observed. **C:** ACT without expression of GLS, scored as percentage 0, intensity 0. **D:** Grade II chondrosarcoma with medium expression of GLS, scored as percentage 2, intensity 2. **E:** Grade III chondrosarcoma with high expression of GLS, percentage 4 intensity 3. Black bars represent 50 µm.

Using qRT-PCR analyses, we demonstrate that all cell lines have higher expression levels of glutaminase compared to the controls (growth plate and cartilage), although expression levels are variable (Figure 3A). Inhibition of glutaminase using CB-839 in ten chondrosarcoma cell lines revealed that HT1080 (*IDH1*^{R132C}), SW1353 (*IDH2*^{R172S}) and, to a lesser extent, JJ012 (*IDH1*^{R132G}), were very sensitive for glutaminase inhibition, with IC_{50} values below 5 µM (Figure 3B, Table 1). L2975 (*IDH2*^{R172W}), NDCS1 (*IDH1/2*^{WT}) and CH3573 (*IDH1/2*^{WT}) had IC_{50} values of 10.2, 13.5 and 17.5 µM, while the remaining four cell lines (one *IDH1*^{R132C}, three *IDH1/2*^{WT}) had IC_{50} values above 50 µM. Interestingly, absence of FBS, increased the sensitivity to CB-839 especially in the *IDH1/2* mutant cell lines, while there was no clear difference in the cell lines with wildtype *IDH1/2* (Figure 3C). In conclusion, these experiments demonstrate that a subset of chondrosarcoma cell lines is dependent on glutaminase- mediated glutaminolysis to maintain cell viability.

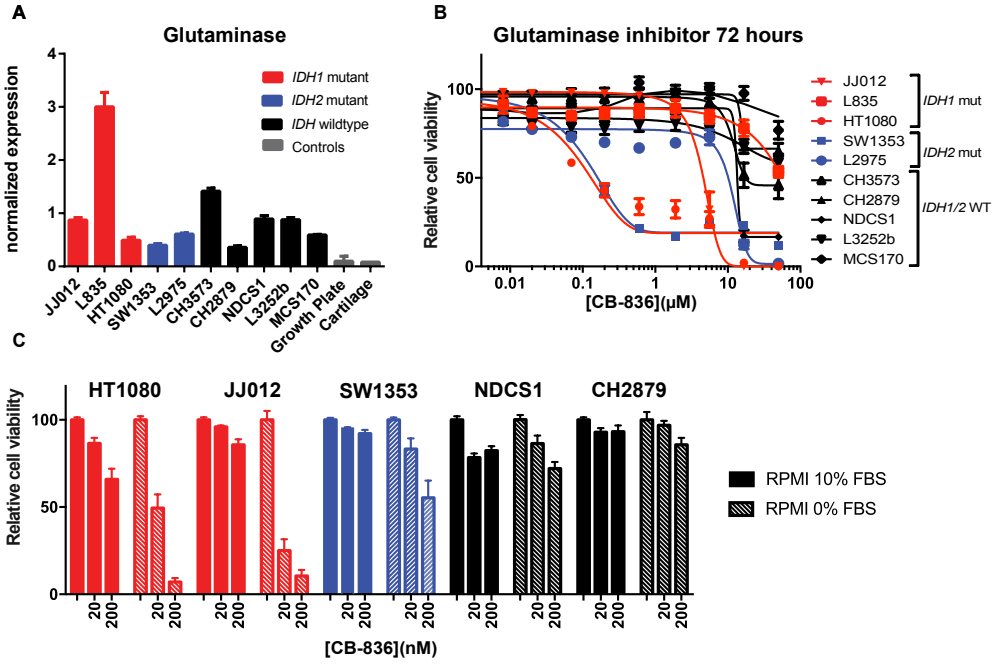

Figure 3. The glutaminase inhibitor CB-839 inhibits chondrosarcoma cell viability. **A:** Expression levels of glutaminase in ten chondrosarcoma cell lines and two controls. Glutaminase is higher expressed in chondrosarcoma cell lines compared to growth plate and articular cartilage. **B:** Ten chondrosarcoma cell lines were treated for 72 h with CB-839, a glutaminase inhibitor. Sensitivity differed between the different cell lines. **C:** Indicated cell lines were treated with 0, 20 or 200 nM CB-839 in the presence or absence of FBS. In the absence of FBS, all cell lines are more sensitive for inhibition with CB-839, especially the *IDH1/2* mutant cell lines.

Table 1. Absolute IC_{50} values of CB-839, metformin, phenformin and chloroquine upon 72 h of treatment, as determined by PrestoBlue Cell Viability.

Compound/ Cell line	CB-839 (μM)	Metformin (mM)	Phenformin (μM)	Chloroquine (μM)	*IDH1/2* status	Reference
HT1080	0.1	1.20	17.1	19.5	*IDH1* p.Arg132Cys	Rasheed *et al.* 1974
JJ012	4.6	19.0	504.1	27.1	*IDH1* p.Arg132Gly	Scully *et al.* 2000
L835	>50	>20	870.3	31.1	*IDH1* p.Arg132Cys	Van Oosterwijk *et al.* 2012
SW1353	0.2	8.26	74.5	24.6	*IDH2* p.Arg172Ser	ATCC
L2975	10.2	10.6	116.6	30.5	*IDH2* p.Arg172Trp	Van Oosterwijk *et al.* 2012
CH2879	>50	10.9	193.7	57.9	WT	Gil Benso *et al.* 2003
NDCS1	13.5	18.1	187.9	16.7	WT	Kudo *et al.* 2007
CH3573	17.5	>20	343.4	34.0	WT	Calabuig-Farinas *et al.* 2012
L3252b	>50	16.7	>1000	39.4	WT	Van Oosterwijk *et al.* 2012
MCS170	>50	10.5	294.7	14.4	WT	De Jong *et al.* 2016

Metformin, phenformin and chloroquine inhibit chondrosarcoma cell viability

Treating the chondrosarcoma cell line panel for 72 h with metformin, phenformin or chloroquine demonstrated that sensitivity for these compounds differed between the different chondrosarcoma cell lines (Figure 4A). With an IC_{50} of 1.20 mM and 17.1 μM after 72 h of treatment, HT1080 cells have a higher sensitivity for respectively metformin and phenformin compared to the other cell lines (Table 1). Treating the chondrosarcoma cell lines for 7 days increased the effect of metformin, phenformin and chloroquine on cell viability (Figure 4B). Hoechst quantification confirmed that the effects on cell viability were caused by an absolute decrease in cell amount (data not shown). No difference between *IDH1/2* mutant and *IDH1/2* wildtype chondrosarcoma cell lines in sensitivity for metformin, phenformin and chloroquine was observed. To further demonstrate that the IDH1 mutant enzyme does not influence sensitivity to these compounds, the inhibitors were combined with AGI-5198, a specific inhibitor of the mutant IDH1 enzyme, in *IDH1* mutant JJ012 and HT1080 cells. Cell viability (Figure 4C) was unaffected by cotreatment with AGI-5198. These results demonstrate that chondrosarcoma cell lines can be targeted by metformin, phenformin and chloroquine and further demonstrates the dependency of chondrosarcoma cell lines on glutaminolysis independent of the presence of the IDH1/2 mutant enzyme.

Cellular effects of glutaminolysis inhibition

To investigate the effect on apoptosis, caspase-glo 3/7 assays were performed. Chloroquine slightly increased caspase 3/7 activity in three out of five cell lines tested (Figure 5A). While the other compounds did impact cell viability after 24 h (Figure 5B), no effect on caspase 3/7 activity was observed. Next, we evaluated the effect of the four metabolic compounds on phosphorylated S6 protein levels, as this is an indicator of mTOR activity. As shown in Figure 5C, metformin and phenformin decreased phosphorylated S6 levels in four out of five and three out of five cell lines respectively. However, metformin and phenformin did not affect phosphorylated S6 levels in HT1080 cells, the cell line that is most sensitive to metformin. Interestingly, metformin decreased LC3B-II levels in four out of five cell lines, which indicates an increase in

autophagy (40). As expected, chloroquine greatly increased LC3B-II levels. CB-839 did not affect phosphorylated S6 or LC3B protein levels. Thus, while the induction of apoptosis is very limited, metformin and phenformin decreased mTOR activity in chondrosarcoma cells, and metformin decreased autophagy, an effect that is counteracted by chloroquine.

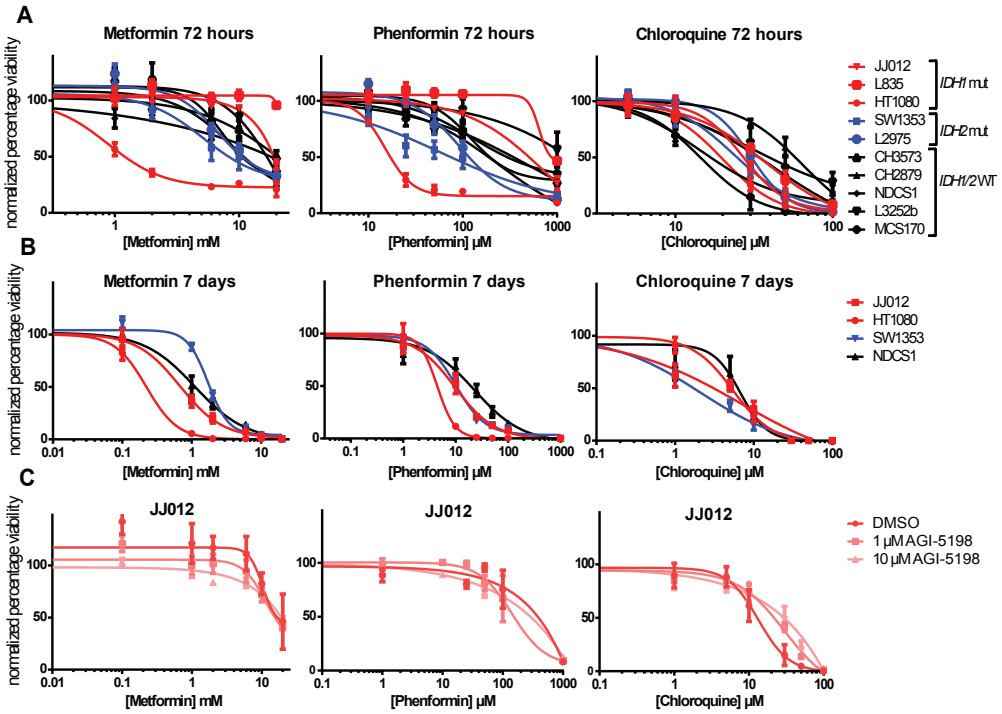

Figure 4. Chondrosarcoma cell lines are sensitive for metformin, phenformin and chloroquine, irrespective of the *IDH1/2* mutation. **A:** Chondrosarcoma cell lines were treated for 72 h with corresponding compounds. Cell viability was measured using the PrestoBlue assay. **B:** Four chondrosarcoma cell lines were treated for 7 days with the corresponding inhibitors. **C:** *IDH1* mutant JJ012 cells were pretreated for 72 h with 10 µM AGI-5198, 1 µM AGI-5198 or DMSO, after which they were treated with a combination of AGI-5198 and the corresponding compounds for 72 h. No effect of AGI-5198 was observed.

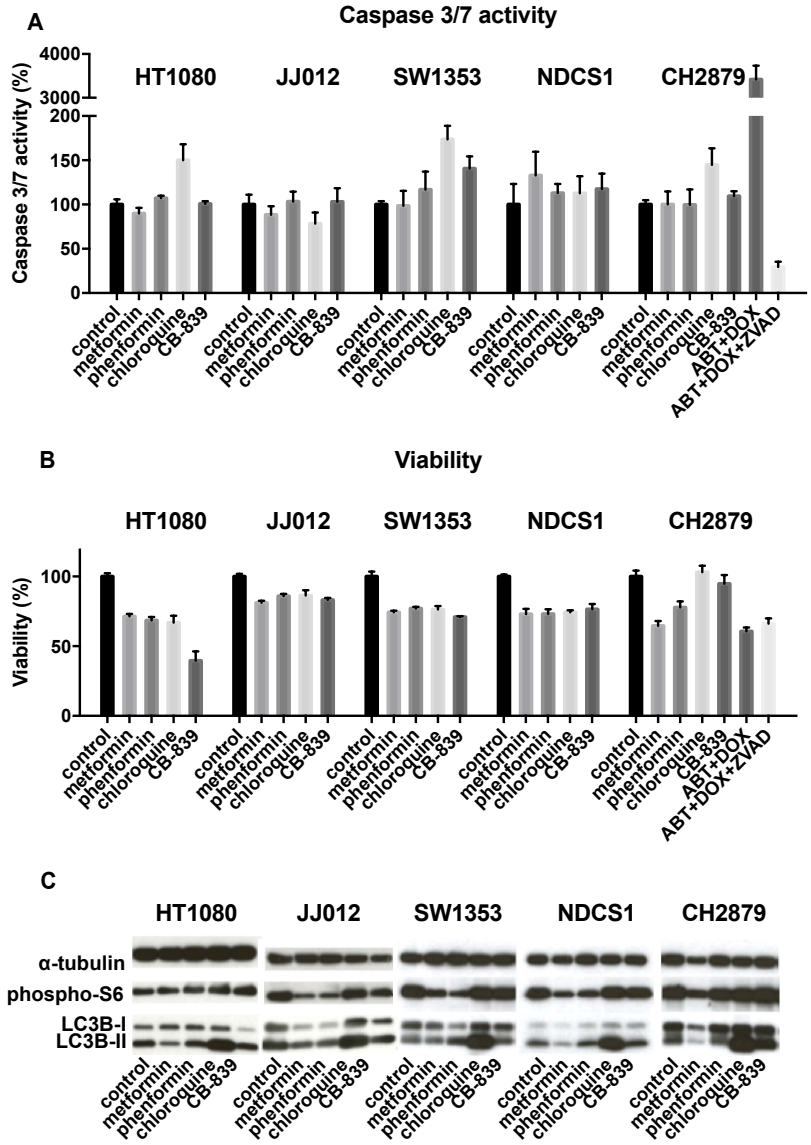

Figure 5. The different compounds provoke different intracellular responses. **A:** Caspase 3/7 activity of HT1080, JJ012, SW1353, NDCS1 and CH2879 cells after 24 h treatment with the metformin, phenformin, chloroquine and CB-839, as determined by caspase-glo 7/3 assays. Only chloroquine slightly increases Caspase 3/7 activity in 3/5 cell lines. CH2879 cells treated with ABT-737 and doxorubicin were used as positive control. For the negative control these compounds were combined with Z-vad-FMK. **B:** Simultaneously to the measurement of caspase 3/7 activity, cell viability was measured using a Presto-Blue assay. **C:** Western blot to evaluate the effect of metformin, phenformin, chloroquine and CB-839 on phosphorylated S6 and LC3B levels in five chondrosarcoma cell lines. Cell lines were treated for 72 h with their corresponding IC_{50} values. Metformin and phenformin decreased levels of phosphorylated S6 in 4/5 and 3/5 cell lines, respectively, and decreased levels of LC3B in 4/5 and 1/5 cell lines, respectively. Chloroquine increased LC3B in all cell lines.

Metformin is sufficiently transported into chondrosarcoma cells to completely inhibit mitochondrial respiration

Next, expression levels of SLC22A1–3 were determined by qRT-PCR analyses, as these transporters are essential for the cellular uptake of metformin and might explain the variability in sensitivity for metformin. Although expression is variable, all cell lines express SLC22A1 (Figure 6A). Interestingly, the two *IDH2* mutated cell lines had the highest expression of SLC22A1. SLC22A2 is only expressed by L2975 (Figure 6B) and SLC22A3 is only expressed by SW1353, MCS170 and CH2879 (Figure 6C). This demonstrates that all cell lines express transporters for the cellular uptake of metformin, but expression levels differ.

To evaluate the effect of metformin on chondrosarcoma cell metabolism, seahorse experiments with three chondrosarcoma cell lines (one *IDH1* mutated, one *IDH2* mutated and one *IDH* wildtype) were performed. Strikingly, metformin completely inhibited mitochondrial respiration in all cell lines tested independent of *IDH1/2* mutation status or SLC22A1 levels (Figure 6D). The small increase in OCR observed after the addition of FCCP and increased ECAR levels after glucose injection demonstrate that the metformin treated cells are still viable. Interestingly, metformin treated cells showed higher levels of glycolysis when glucose is present compared to the controls. However, oligomycin A injection increased glycolytic levels in controls but not in treated cells, indicating a maximum in glycolytic energy production is achieved in treated cells after glucose addition (Figure 6E). The small difference in glycolytic activity between treated and untreated cells cannot compensate for the observed loss in ATP production through mitochondrial respiration, suggesting that chondrosarcoma cells utilize other pathways for energy production.

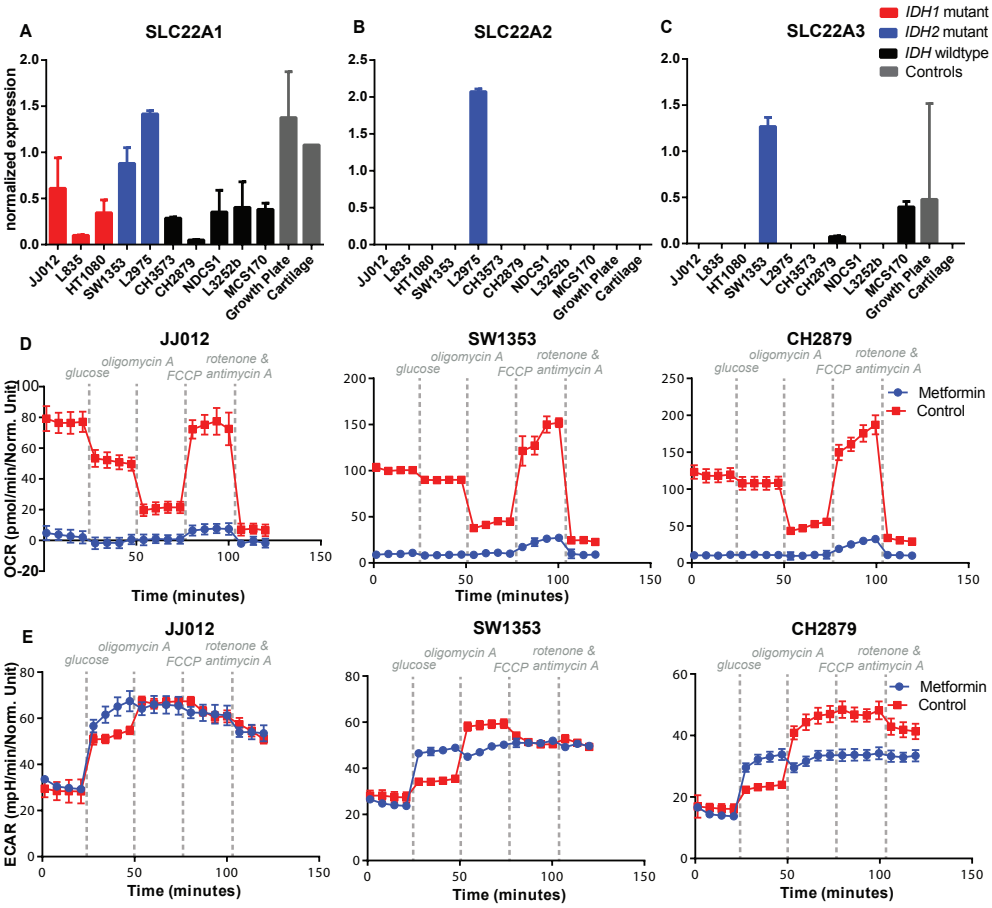

Figure 6. Chondrosarcoma cells sufficiently express SLC22A1 for metformin to completely inhibit mitochondrial respiration. **A:** All chondrosarcoma cell lines express SLC22A1. **B:** SLC22A2 is only expressed by L2975. **C:** Three out of ten chondrosarcoma cell lines express SLC22A3. **D-E:** The impact of 24 h treatment with 5 mM metformin on the Oxygen Consumption Rate (OCR)(D) and extracellular acidification rate (ECAR) (E) of JJ012, SW1353 and CH2879 was measured by Seahorse experiments. Metformin completely blocks mitochondrial respiration, which is only accompanied by a small increase in glycolysis.

Discussion

In this study, we demonstrated a difference in glutaminase expression levels between the different chondrosarcoma grades, with the highest expression observed in high-grade tumours. We therefore examined whether glutaminolysis could be exploited as a therapeutic target for high-grade chondrosarcoma. Based on current ongoing clinical trials targeting glutaminolysis, to which chondrosarcoma patients can be enrolled, we interfered with glutaminolysis in chondrosacoma cell lines at different levels (Figure 1).

First, we inhibited glutaminase using the glutaminase inhibitor CB-839, and indeed, six out of ten chondrosarcoma cell lines showed IC_{50} values below 20 μM. CB-839 did not induce apoptosis, autophagy or mTOR activity, suggesting that it likely impacts cell viability via other mechanisms.

Second, we used the widely used anti-diabetic drug metformin, which, among other effects, indirectly inhibits glutaminase via c-Myc; (Figure 1), (21, 22) inhibits complex 1 of the electron transport chain (20), and inhibits mTOR signalling(19). Indeed, a subset of chondrosarcoma cell lines was sensitive to metformin, especially when treated for a longer time period. mTOR signalling was previously shown to be important in chondrosarcoma (41) and we confirmed that metformin decreased mTOR activity in all but one chondrosarcoma cell lines tested, which is in line with findings in other studies (19). Interestingly, mTOR activity was not inhibited in HT1080 which is the most sensitive for metformin treatment, suggesting that mTOR inhibition alone cannot explain the impact of metformin on chondrosarcoma cell viability. Furthermore, while apoptosis was absent, metformin seemed to induce autophagy in the majority of cell lines tested, which can likely be linked to the effect of metformin on AMPK (42). In this study, we further demonstrate that metformin completely blocks mitochondrial respiration in chondrosarcoma cells, likely caused by its effect on complex I of the electron transport chain. However, blocking complex I of the electron transfer chain (and therefore oxidative glutaminolysis) alone was not sufficient to inhibit cell viability, as an even higher concentration of metformin only had a small impact on cell viability within the 24-h time frame of the mitochondrial

respiration experiments (Figure 5B). The small difference in glycolysis observed upon treatment with metformin is likely insufficient to compensate for the total los of ATP production through oxidative means, suggesting that chondrosarcoma cells are not dependent on oxidative metabolism and require other sources, in addition to the increased glycolysis, to supply the necessary energy. In prostate cancer cell lines, it was demonstrated that metformin treatment increased the dependency on reductive glutaminolysis (43). Further identifying these pathways might provide interesting targets for combination treatment with metformin.

Third, as an alternative to metformin, we used its lipophilic analogue phenformin. In contrast to metformin, phenformin does not need SLC22A1-3 transport to get into cells. As expected, the effect of phenformin on cell viability, mTOR activity, and apoptosis was very similar to metformin. Contrary to metformin, phenformin is not used in the clinic due to an increased risk of lactic acidosis. Moreover, we show that all chondrosarcoma cell lines sufficiently express the SLC22A1 transporter that is necessary for metformin uptake, suggesting that there is limited rationale to move to phenformin trials for chondrosarcomas when the metformin trial demonstrates limited efficacy.

Fourth, we evaluated the anti-malaria drug chloroquine, which, in addition to inhibiting glutamate dehydrogenase, is a well-known inhibitor of autophagy and thereby inhibits many other metabolic and signal transduction pathways (44, 45). The chondrosarcoma cell lines were sensitive to chloroquine. Also, we confirmed that chloroquine inhibited autophagy in all chondrosarcoma cell lines tested. Moreover, a slight induction of apoptosis was seen in three out of five chondrosarcoma cell lines.

Thus, we used four different drugs to evaluate whether the increased dependence on glutaminolysis could be therapeutically exploited using repurposing of existing drugs, and confirmed that a subset of chondrosarcoma cell lines is indeed sensitive to glutaminolysis inhibition. We could not identify a correlation between levels of glutaminase expression and sensitivity for any of these metabolic compounds in the panel of ten chondrosarcoma cell lines. Of note, there was also no correlation of the *IDH1/2* mutation status

of chondrosarcoma cells with sensitivity to these compounds, or with the expression levels of glutaminase in primary tumours. We could therefore not confirm the prevailing hypothesis that *IDH1/2* mutant chondrosarcoma cells rely on glutaminolysis to generate sufficient α-KG for *D*-2-HG production (15-17), as it seems that also high-grade chondrosarcomas that are wildtype for *IDH1/2* depend on glutaminolysis. To explain this dependency of *IDH1/2* wildtype chondrosarcoma on glutaminolysis, it is tempting to speculate that the hypoxic microenvironment, which is a characteristic of chondrosarcoma (46), is equally important or even overrates the effect of the *IDH1/2* mutation in chondrosarcoma. HIF-1α activates pyruvate dehydrogenase kinase, which inactivates pyruvate dehydrogenase, thereby inhibiting the influx of pyruvate into the TCA cycle (47). In this context, TCA cycle anaplerosis is required for the synthesis of fatty acids, which is then primarily mediated via glutaminolysis (48). Therefore, the hypoxic microenvironment of chondrosarcomas potentially explains why chondrosarcomas depend on glutaminolysis irrespective of the presence or absence of an *IDH1/2* mutation

Our results indicate that there is limited preclinical rationale to select chondrosarcoma patients for treatment with these compounds based on their *IDH1/2* mutation status. This is in contrast to the studies by Cuyas *et al*. (16), and Molenaar *et al*. (49), where, respectively, an increased sensitivity for metformin was identified in an engineered *IDH1* mutant breast cancer cell line, and in an engineered *IDH1* mutant colorectal cancer cell line compared to their wildtype parental cells. The differences in tumour types and the fact that these cell lines harbour an engineered instead of an endogenous *IDH1/2* mutation likely explain the differences in experimental findings.

In conclusion, our results demonstrate that glutaminase is higher expressed in high-grade compared to low-grade chondrosarcomas. High-grade chondrosarcomas are dependent on glutaminolysis which is independent of *IDH1/2* mutation status. This dependence can be therapeutically exploited by repurposing existing drugs that inhibit glutaminolysis, including CB-839, metformin, phenformin and chloroquine.

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Chapter 8

Summary and future perspectives

Osteosarcoma and chondrosarcoma are the first and second most common primary bone cancers, respectively. The aim of this thesis was to explore therapeutic strategies by unravelling cellular pathways that are essential for chondrosarcoma and osteosarcoma cell survival, taking the insulin-like growth factor pathway as a starting point. **Chapter 1** gives an introduction to these two sarcoma subtypes and to the cellular pathways that have been studied in this thesis.

Potential targets for treatment of osteosarcoma

The large heterogeneity in osteosarcoma patients is reflected by the heterogeneity in osteosarcoma cell lines, which provide adequate models to study mechanisms of osteosarcoma genesis, cell biology, and drug responsiveness (1). It cannot have escaped the notice of researchers working in the field of osteosarcoma that the number of publications on *in vitro* studies of this relatively rare disease has increased considerably in the past five years. On the one hand, this is desirable, as such studies may lead to the identification of new treatment options, which are urgently required for a deadly disease occurring in young patients and for which no improvement of survival is observed since the eighties. On the other hand, an increase in quantity of low quality studies does more harm than good, as it is a waste of time and resources. **Chapter 2** systematically identified osteosarcoma *in vitro* studies performed between 1996 and 2015, and demonstrates an almost exponential increase in the number of these studies in the last few years. Unfortunately, the majority of these studies have limited scientific value as they use questionable study designs. While in osteosarcoma *in vitro* studies the use of multiple cell lines is essential to represent the heterogeneity in patients, many studies were performed with only one or two cell lines, i.e. U-2 OS or MG-63. Furthermore, approximately 1/3 of all drugs described in the past three years could be classified as traditional medicine, for which the evidence of specific intracellular targets is lacking. The huge increase in osteosarcoma *in vitro* studies can be attributed by publications from Chinese institutes. This can be explained by the fast growing economy of China, combined with the evaluation system of Chinese medical doctors (2). In China, the Science Citation Index is used as the main indicator for medical career evaluation,

leading to a huge amount of pressure to publish articles each year. 53% of the publications in 2015 were published by Chinese institutes. The increase in publications from China is not restricted to osteosarcoma research. However, the easiness to grow osteosarcoma cell lines and their extremely high growth rate that boosts their drug responsiveness can probably explain why the amount of reports on osteosarcoma is relatively high compared to other research areas. Therefore, chapter 2 sketches the current situation in the osteosarcoma field, and emphasizes the general idea that there is a need to change the evaluation system of medical research (3).

In **chapter 3**, genome-wide gene expression data was analysed to identify new possibilities for targeted treatment of osteosarcoma. A difference in mRNA expression levels of genes involved in insulin-like growth factor receptor 1 (IGF1R) signalling between osteosarcoma and osteosarcoma progenitors was identified. In osteosarcoma cell lines and pre-treatment biopsies, *IGFBP4* and *GAS6* showed the highest significant downregulation, which are upstream inhibitors of IGF1R signalling (4). We therefore hypothesized that we could inhibit osteosarcoma growth by inhibiting this pathway. Osteosarcoma cell lines were treated with OSI-906, a dual inhibitor of the IGF1R and the insulin receptor (IR), as signalling via the IR can take over in case of IGF1R blockage. OSI-906 inhibited osteosarcoma cell viability in three out of four osteosarcoma cell lines tested. Furthermore, OSI-906 resulted in a decreased phosphorylation of insulin-receptor substrate 1, a direct downstream target of the IGF1R, thereby validating on target inhibition. Therefore, chapter 3 identified the IGF pathway as a potential target for treatment of osteosarcoma.

In the gene set analysis performed in chapter 3, nineteen osteosarcoma cell lines were included. This broad panel of osteosarcoma cell lines optimally reflects the heterogeneity observed in osteosarcoma patients. The potency of targeting the IGF pathway in osteosarcoma sarcoma was subsequently explored in four osteosarcoma cell lines, which might question the degree in which the heterogeneity of osteosarcoma patients is captured in these experiments. However, these four cell lines demonstrated different potential to undergo osteo- chondro- and adipogenic differentiation *in vitro*, and showed different potency to induce tumour growth *in vivo* (1). Therefore, although

only four cell lines were used, the captured heterogeneity was considered sufficient for this study.

In **chapter 4**, a commentary on a study by Yang *et al*. (5) is given. Similar to our investigations described in chapter 3, the study by Yang *et al*. also compared gene expression between osteosarcoma (biopsies and cell lines), and osteosarcoma progenitor cells. Interestingly, they also identified downregulation of genes upstream of the IGF1R, and thereby pointed toward a role for the IGF pathway in osteosarcoma genesis. In the first section of our commentary, we describe where we are now. While preclinical models have shown promising results, evidence for the efficacy in large-scale randomized controlled trials is lacking, resulting in pharmaceutical companies discontinuing the production of all IGF pathway-targeting agents. In the second section, it is described where we need to go. Biomarkers should be identified, and trials with compounds that target both the IGF1R and the IR should be performed. The last section, we describe how we should get there. Biomarkers that will be identified in a clinical trial with OSI-906 in Ewing sarcoma (NCT02546544, clinicaltrials.gov) should be tested in other tumour types including osteosarcoma. As this trial incorporates a strong translational research program, it sets an example of how future trials with IGF1R-IR inhibitors in osteosarcoma should be performed.

Currently, no clinical trials with IGF1R inhibitors are open for osteosarcoma patients. The clinical trials that have been performed with IGF1R inhibitors in osteosarcoma patients are summarized in Table 1. As mentioned above, an important limitation of these trials is that they all involved IGF1R monoclonal antibodies. By targeting the IGF1R, the endocrine feedback loop is disrupted resulting in a strong increase in GH and IGF1 levels (6). These increased IGF1 levels can still activate the IGF pathway by stimulating IGF1 receptors that are not blocked by the treatment and by signalling via the IR, thereby stimulating tumour growth. The increased GH and IGF1 levels upon IGF1R directed therapy can potentially explain the difference in efficacy of IGF1R inhibitors in xenografts and human patients, as most antibodies are specific for human IGF1R binding and therefore do not disrupt the endocrine feedback loop in rodents (7).

Table 1. Overview of clinical trials and results of IGF inhibitors in osteosarcoma and chondrosarcoma patients. All inhibitors are monoclonal antibodies to the IGF1R.

Inhibitor	**Osteosarcoma patients**	**Efficacy**	**Phase**	**Reference**
AVE1642	N=3 bone sarcomas, refractory disease	1 osteosarcoma unconfirmed partial response	I	(8)
Figitumumab	N=11, patients with no other treatment options	No responders	I	(9)
Figitumumab and Everolimus (mTOR)	N=3, no curative treatment options	1 prolonged stable disease	I	(10)
Cixutumumab	N=3, patients with relapsed/refractory tumours	No responders	I	(11)
Cixutumumab	N=11, elapsed refractory solid tumours	No responders	II	(12)
Cixutumumab and temsirolimus (mTOR)	N=24, received previous treatments	3 partial responses	II	(13)
RG1507	N=3, relapsed or refractory solid tumours	2 stable disease for >52 weeks	I	(14)
R1507	N=38, recurrent or refractory osteosarcoma	2 partial responses 10 stable disease	II	(6)
Cixutumumab and temsirolimus (mTOR)	N=11, relapsed or refractory osteosarcoma	No responders	II	(15)
Robatumumab	Group 1: N=68 relapsed, resectable recurrences < 6 months after prior treatment Group 2: N=35, relapsed unresectable metastasis	Group 1: 3 partial responders, 17 stable disease Group 2: 6 stable disease	II	(16)
Inhibitor	**Chondrosarcoma patients**	**Efficacy**	**Phase**	**Reference**
Cixutumumab and temsirolimus (mTOR)	N=17, received previous treatments	1 partial response	II	(13)
Figitumumab	N=1, myxoid chondrosarcoma* that received previous treatments	Small decrease in tumour size	I	(17)
Figitumumab and docetaxel	N=2, received previous treatments	1 stable disease after 6 months	Ib	(18)
BIIB022	N=1, relapsed or refractory solid tumour	Not clear, results presented for all sarcomas combined	I	(19)

* unclear whether this was an extraskeletal myxoid chondrosarcoma or a chondrosarcoma of bone

A recent whole genome sequencing study by Behjati *et al*. reported alterations in IGF receptor genes in 7% of the osteosarcoma cases, and additional driver mutation in pathways downstream of IGF1R in an additional 20% of the cases (20). Therefore, 27% of the tumours had perturbed IGF1R signalling due to somatic mutations. In addition, 14% of the osteosarcoma cases had 15 copies or more of the IGF1R gene. These findings suggest that a substantial subgroup of osteosarcoma patients may give a significant response to IGF-directed therapy and that we might be able to select patients based on their genotype in the future. Due to driver mutations downstream of the IGF1R, resistance to IGF1R-IR inhibitors might occur. Therefore, these inhibitors should potentially be combined with agents that target the IGF pathway at different levels.

Potential targets for treatment of chondrosarcoma

In **chapter 5** it is reported whether, in addition to osteosarcoma, the IGF pathway is a potential target for chondrosarcoma therapy. Mediators of IGF1R signalling were heterogeneously expressed in chondrosarcoma cell lines, and phosphorylated IRS1 was identified in 2 out of 3 cell lines tested. Although these results indicated that the IGF1R pathway was active in a subset of chondrosarcoma cell lines, treating chondrosarcoma cell lines with three different IGF1R inhibitors did not influence chondrosarcoma cell line viability, migration, or chemoresistance. To elucidate the discrepancy between the absence of an effect of IGF1R inhibition and pathway activity in chondrosarcoma cell lines, IGF1R expression level was assessed in chondrosarcoma cell lines and primary tumours using immunohistochemistry. In contrast to chondrosarcoma cell lines, we found no expression (66%) or weak expression (34%) in the primary tumours. Moreover, determining expression in four patients with matched cell lines and primary tumours suggested that chondrosarcoma cells had upregulated IGF1R upon culturing, thereby questioning the validity of using monolayer-cultured cells as a model to study IGF pathway activity. In conclusion, chapter 5 demonstrates that the IGF pathway is not expected to be an effective therapeutic target for chondrosarcoma.

Chapter 5 illustrates the need to develop more representative *in vitro* models for chondrosarcoma. In the last decade, models with cancer cell culture in a three-

dimensional (3D) environment have been developed. Our group has been using a spheroid model in which chondrosarcoma cells are injected into a collagen gel (21). Lhuissier *et al*. recently published a model in which chondrosarcoma cells were embedded in an alginate hydrogel (22). 3D *in vitro* models contain several *in vivo* features such as drug penetration, drug resistance, cell-cell interaction, a hypoxic gradient and the deposition of extracellular matrix (23). Therefore, 3D models should be the focus of future research.

As can be seen in Table 1, a few clinical trials with IGF1R inhibitors included chondrosarcoma patients, which in line with our preclinical study, do not support using IGF1R inhibitors in the clinic of chondrosarcoma. The largest is the study by Schwartz *et al*., which included 17 chondrosarcoma patients that did not respond to previous treatments. This reflects that despite the huge difference in aetiology between different sarcomas, clinical trials often combine patients from different subtypes. This can be explained by the rareness of these cancers. However, these trials do not fully evaluate the potency of a drug for a specific sarcoma subtype, potentially leading to incorrect conclusions. Large international research consortia can solve this problem, as by these consortia, enough patients of a particular sarcoma subtype can be included in a clinical study. Therefore, international collaborations should be facilitated even more in the future (24).

As chapter 5 demonstrates that IGF1R signalling is not a potential target for treatment of chondrosarcoma, other therapeutic strategies are needed. Therefore, in chapters 6 and 7, a different approach was taken to identify potential targets for chondrosarcoma therapy, exploiting the genetic properties of this tumour type. About 50% of the conventional chondrosarcomas carry an *IDH1* or *IDH2* hotspot mutation. It is acknowledged that the oncogenic activity of these mutations lies in the aberrant production of the oncometabolite D-2-hydroxyglutarate. However, as IDH1 and IDH2 are key enzymes in cell metabolism, these mutations potentially may lead to metabolic vulnerabilities that can be targeted.

Chapter 6 investigated whether nicotinamide adenine dinucleotide (NAD+) depletion can be used to target *IDH1/2* mutant chondrosarcoma cells, based

on a study from Tateishi *et al*. (25) who identified this vulnerability in *IDH1/2* mutant glioma cells. Nicotinamide phosphoribosyltransferase (NAMPT) and nicotinic acid phosphoribosyltransferase (NAPRT) are rate-limiting enzymes in the NAD+ synthesis pathway. Treating eleven chondrosarcoma cell lines with two NAMPT inhibitors revealed that chondrosarcoma cell lines showed a dose-dependent decrease in cell viability, 3D collagen invasion and colony formation upon treatment with NAMPT inhibitors. Nearly half of the cell lines demonstrated IC_{50}s in the low nM range. qRT-PCR analyses demonstrated that increasing IC_{50}s for NAMPT inhibitors correlated to increasing NAPRT expression levels, and datasets of genome-wide methylation arrays revealed that the increasing NAPRT expression levels were correlated to decreasing NAPRT methylation. Strikingly, higher methylation of the NAPRT promoter was observed in high-grade versus low-grade tumours. In contrast to our initial hypothesis, we did not observe a correlation between the *IDH1/2* mutation status and sensitivity to NAMPT inhibitors, nor could we find a difference in NAPRT methylation between *IDH1/2* mutant and wildtype primary tumours. Therefore, this study identified NAMPT as a potential target for treatment of chondrosarcoma, especially for those of high histological grade, irrespective of the *IDH1/2* mutation status.

The phase I clinical trials that tested the safety of NAMPT inhibition (also including FK866 and GMX1778) were discontinued due to dose-limiting toxicities (26). Co-administration with nicotinic acid (NA) has been proposed to increase the therapeutic index of NAMPT inhibitors, as NA can be used to synthesise NAD+ in NAPRT-proficient cells. In chondrosarcoma xenograft, it was shown that the inhibitory effect of NAMPT inhibitors was not affected by co-administration of NA (27), suggesting that this could be a suitable approach to decrease dose-limiting toxicities in chondrosarcoma patients. Therefore, NAMPT inhibitors (in combination with NA) should be evaluated in chondrosarcoma mouse models. In addition, a Phase I clinical trial with KPT-9274, a dual inhibitor of PAK4 and NAMPT, is currently recruiting patients with advanced solid malignancies (including sarcomas) and non-Hodgkin's lymphoma (NCT02702492, clinicaltrials.gov). Interestingly, NAPRT1 and *IDH1* tumour status will be determined prior to enrolment. Hopefully, this study will recruit (sufficient) chondrosarcoma

patients to be able to indicate whether NAMPT inhibitors can be used as a treatment of chondrosarcoma.

As multiple studies point towards an increased dependency on glutaminolysis in *IDH1/2* mutant glioma cells, **chapter 7** evaluated if there was preclinical rationale for targeting glutaminolysis in chondrosarcoma. By immunohistochemistry, it was demonstrated that increasing glutaminase expression levels correlated to increasing tumour grades, and qRT-PCR analyses of chondrosarcoma cell lines revealed a higher expression of glutaminase in chondrosarcoma cell lines compared to the controls (growth plate and cartilage). Treating chondrosarcoma cell lines with the glutaminase inhibitor CB-839 revealed that a subset of chondrosarcoma cell lines is dependent on glutaminase-mediated glutaminolysis to maintain cell viability. As the safety of CB-839 for patient treatment is still under investigation, the effects of the widely used anti-diabetic drug metformin, its lipophilic analogue phenformin, and the anti-malaria drug chloroquine were evaluated *in vitro*, as these drugs also inhibit glutaminolysis. These four metabolic compounds inhibited chondrosarcoma cell viability in a subset of chondrosarcoma cell lines tested. To further investigate the cellular mechanism by which these four metabolic drugs inhibit chondrosarcoma cell viability, cell apoptosis, mTOR activity and LC3B-II levels were determined. While the induction of apoptosis was limited, metformin and phenformin decreased mTOR activity in chondrosarcoma cells, and metformin decreased autophagy, which is counteracted by chloroquine. The mechanisms by which CB-839 decreased cell viability remain to be identified. In conclusion, chapter 7 suggests that targeting glutaminolysis with CB-839, metformin, phenformin or chloroquine is a potential therapeutic strategy for a subset of high-grade chondrosarcomas.

Similar to chapter 6, we could not confirm the prevailing hypothesis that *IDH1/2* mutant chondrosarcoma cells are more dependent on glutaminolysis as compared to *IDH1/2* wildtype cells, since there was also no correlation of the *IDH1/2* mutation status of chondrosarcoma cells with sensitivity to these compounds, or with the expression levels of glutaminase in primary tumours. Two clinical trials that target glutaminolysis are currently recruiting patients, which select patients based on the *IDH1/2* mutation status. These

so called “basket trials” recruit patients of a variety of tumour types at the same time, based on the presence of a specific mutation, thereby increasing the number of eligible patients. The first one is a trial with the glutaminase inhibitor CB-839 (NCT02071862, clinical trials.gov), and the second one is a trial that combined metformin and chloroquine (NCT02496741, clinicaltrials.gov). These trials already started before the results of our preclinical studies became available. Chapter 7 demonstrates that in contrast to our initial hypothesis based on findings in gliomas, there is limited preclinical rationale to only include chondrosarcoma patients that harbour an *IDH1/2* mutation for these trials. This is in line with previous studies from our group, which demonstrated that in contrast to gliomas, *IDH1/2* mutation status does not correlate to chondrosarcoma prognosis, *IDH1/2* mutations do not affect immunohistochemical levels of 5-hmC, 5mC and trimethylation of H3K4, -9, and -27, and prolonged inhibition of the IDH1 mutant enzyme does not affect global gene expression, CpG island methylation nor histone H3K4, -9, and -27 trimethylation in chondrosarcoma cell lines. Therefore, together with other studies from our group, Chapter 6 and Chapter 7 suggest that *IDH1/2* mutant chondrosarcomas do not require a different therapeutic approach than *IDH1/2* wildtype chondrosarcomas. This can have two potential explanations. First, it might be that although the *IDH1/2* mutation is involved in the initiation of a chondrosarcoma, it does not play a role in chondrosarcoma progression. This is supported by the lack of an *in vitro* effect of IDH1 mutant inhibitors on the tumorigenic properties of chondrosarcoma cells (28). Second, it might be that chondrosarcomas without an *IDH1/2* mutation have other aberrant intracellular routes that have the same effect as an *IDH1/2* mutation on chondrosarcoma cell metabolism. A key regulator of cell metabolism is mTOR, a kinase that integrates input from many upstream pathways and passes the signal to multiple target proteins. Currently, one clinical trial that recruits *IDH1/2* mutant and wildtype chondrosarcoma patients, run in the LUMC, uses the compound sirolimus (mTOR inhibitor) in combination with cyclophosphamide (NCT02821507, clinicaltrials.gov). As this trial incorporates a translational research program, it will help unravelling the intracellular biology of these tumours, which might provide useful insights regarding which of these potential explanations is accurate.

Chondrosarcoma and osteosarcoma model systems

In this thesis, mainly monolayer-cultured cells were used to study the drug responsiveness of osteosarcoma and chondrosarcoma cells. Testing the potency of a drug in this model system is a first step in developing new treatment options. However, monolayer-cultured cells are being criticized for two reasons. First, cell lines have repeatedly been discovered as being misidentified, which can be caused by cross-contaminations or mislabelling of samples. The international cell line authentication committee created a database of these cell lines, now containing 488 cell lines. It is estimated that 0.8% of the total literature on cells involved the use of these misidentified cell lines (29). Although techniques have been introduced for cell line authentication, misidentification of cell lines remains a substantial problem. Second, cell lines are grown without a reflective three-dimensional structure and microenvironment. Therefore, as discussed above, new *in vitro* models have been developed that better reflect the original tumours. These 3D models will become more and more important in future research.

In addition to *in vitro* models, *in vivo* models can be used the study the potency of new osteosarcoma and chondrosarcoma therapies. Ideally, this is the second step in the development of new therapies. Four groups of *in vivo* models can be distinguished:

- Spontaneous models, such as canine osteosarcoma.
- Induced models, such as the induction of murine osteosarcoma by radiation.
- PDX models, i.e. patient derived xenografts, in which osteosarcoma or chondrosarcoma cells from a patient are transplanted in mice or zebrafish.
- Transgenic mice, in which the (conditional) knock-out of a certain gene results in the development of a particular tumour, such as the osterix-cre-mediated deletion of p53 and pRb in murine osteosarcoma models.

As these models all have advantages and disadvantages, the best *in vivo* model depends on the research question (30). When subsequently *in vitro* and *in vivo* models demonstrate the potency of a drug, clinical trials can be initiated as a final step in the development of new therapies.

Summary

In conclusion, this thesis explored potential new therapeutic strategies by identifying cellular pathways that are essential for chondrosarcoma and osteosarcoma cell survival. Although clinical trials with IGF1R inhibitors have disappointing results in osteosarcoma, this thesis strengthens the view that the IGF pathway can be an effective target for osteosarcoma therapy if an appropriate selection of patients is treated with IGF1R/IR dual inhibitors. When optimized clinical trials targeting the IGF pathway will be performed in the future, chondrosarcoma patients should not be recruited, as there is limited preclinical rationale for the efficacy of IGF1R targeting agents in chondrosarcoma. We identified two promising pathways that can be used to target chondrosarcoma; the NAD+ synthesis pathway and glutaminolysis. Our results suggest that chondrosarcoma patients should be included in future studies with drugs that interfere in these pathways, regardless of their *IDH1/2* mutation status.

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Chapter 9

Nederlandse samenvatting
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Nederlandse samenvatting

Het osteosarcoom en het chondrosarcoom zijn de meest voorkomende kwaadaardige primaire bottumoren. Osteosarcomen komen met name op jonge leeftijd voor, terwijl chondrosarcomen het meest voorkomen tussen de 30 en de 60 jaar. Chondrosarcomen kunnen alleen curatief behandeld worden als deze nog niet uitgezaaid zijn en de volledige tumor door middel van chirurgie verwijderd kan worden. Sinds de introductie van chemotherapie in de jaren ‘80 is de 5-jaars overleving van osteosarcoom patiënten gestagneerd op 65–70%. Het doel van de studies beschreven in dit proefschrift is het identificeren van signaaltransductieroutes die aangrijpingspunten zouden kunnen zijn voor nieuwe doelgerichte therapieën voor de behandeling van osteosarcomen en chondrosarcomen. **Hoofdstuk 1** introduceert het osteosarcoom en het chondrosarcoom, en signaaltransductieroutes die mogelijk een aangrijpingspunt voor doelgerichte therapieën zouden kunnen zijn.

Doelgerichte therapieën voor de behandeling van osteosarcomen

In de laatste vijf jaar is er een enorme toename geweest in het aantal wetenschappelijke publicaties over *in vitro* studies naar het osteosarcoom. Aan de ene kant is dit gewenst, omdat deze studies mogelijk kunnen leiden tot het identificeren van nieuwe therapieën voor de behandeling van deze dodelijke ziekte. Aan de andere kant heeft een verhoging van het aantal studies met lage kwaliteit voornamelijk negatieve consequenties omdat het een verspilling is van tijd en financiële middelen. In **hoofdstuk 2** is het aantal *in vitro* onderzoeken naar het osteosarcoom, gepubliceerd tussen 1996 en 2015, systematisch geanalyseerd. Deze analyse toont een bijna exponentiele toename van het aantal publicaties in de afgelopen jaren. Helaas heeft de meerderheid van deze studies weinig wetenschappelijke waarde omdat ze een bedenkelijke studieopzet hebben. Veel van deze studies hebben slechts gebruik gemaakt van één of twee cellijnen (U-2 OS of MG-63), terwijl het voor *in vitro* onderzoek naar osteosarcomen essentieel is om een grotere hoeveelheid cellijnen te gebruiken om de heterogeniteit van osteosarcoom patiënten na te kunnen bootsen. Daarnaast was van ongeveer een derde van de

onderzochte medicijnen niet bekend wat ze precies doen op cellulair niveau. De enorme toename van *in vitro* onderzoek naar het osteosarcoom betreft een stijging van het aantal publicaties van Chinese instituten. Deze toename kan verklaard worden door de snelgroeiende Chinese economie in combinatie met het evaluatiesysteem van Chinese artsen. In China wordt namelijk de Science Citation Index gebruikt als belangrijkste indicator voor de evaluatie van een medische carrière, waardoor er een enorme druk is om artikelen te publiceren. 53% van de *in vitro* onderzoeken naar het osteosarcoom in 2015 werd gepubliceerd door Chinese instituten. De toename in het aantal publicaties van Chinese instituten beperkt zich niet tot studies naar het osteosarcoom, maar de toename in dit veld is relatief hoog ten opzichte van andere onderzoeksvelden. Dit kan waarschijnlijk verklaard worden doordat osteosarcoom cellijnen erg makkelijk en snel groeien. Samenvattend schetst hoofdstuk 2 de huidige trend in het aantal *in vitro* studies naar het osteosarcoom. De exponentiele groei in het aantal *in vitro* studies naar het osteosarcoom onderschrijft dat het evaluatiesysteem voor medisch onderzoek moet veranderen.

Hoofdstuk 3 beschrijft de analyse van genexpressie in osteosarcoom cellen met als doel het vinden van nieuwe doelgerichte therapieën voor de behandeling van osteosarcomen. mRNA expressieniveau's werden vergeleken tussen osteosarcoom cellen en voorlopercellen van het osteosarcoom. De genen die betrokken zijn bij de insulinegroeifactor (IGF) signaaltransductieroute, die een rol speelt bij botgroei, bleken verschillend tot expressie te komen. De genen *IGFBP4* en *GAS6* waren het sterkst gedownreguleerd in de osteosarcoom cellen. Beide coderen voor negatieve regulatoren van de IGF1 receptor (IGF1R). Dit zou kunnen duiden op een verhoogde activiteit van de IGF signaaltransductieroute. Op basis van dit gegeven veronderstellen wij dat de groei van osteosarcomen onderdrukt zou kunnen worden door deze signaaltransductieroute te remmen. Osteosarcoom cellijnen werden daarom behandeld met OSI-906, een inhibitor van zowel de IGF1R als de insulinereceptor (IR). Het is essentieel om beide receptoren te remmen om resistentie tegen IGF1R remmers te voorkomen. OSI-906 remde de groei van drie van de vier geteste osteosarcoom cellijnen. Daarnaast resulteerde de behandelingen met OSI-906 in een verminderde fosforylering van het insulinereceptorsubstraat 1 (IRS1). Dit toont aan dat het

effect van OSI-906 op de cellen specifiek veroorzaakt wordt door het remmen van de IGF signaaltransductieroute. Derhalve identificeert de studie beschreven in hoofdstuk 3 de IGF signaaltransductieroute in osteosarcomen als mogelijk aangrijpingspunt voor therapie.

Hoofdstuk 4 betreft een beschouwing van de studie van Yang en collega's. Vergelijkbaar met onze studie die beschreven is in hoofdstuk 3, vergeleek deze studie ook genexpressiedata tussen osteosarcoom cellen en voorlopercellen van het osteosarcoom. In lijn met onze resultaten vond deze studie ook een downregulatie van negatieve regulatoren van de IGF signaaltransductieroute. In het eerste gedeelte van deze beschouwing wordt beschreven wat de huidige stand van zaken is met betrekking tot de mogelijk om de IGF signaaltransductieroute te remmen als behandeling van osteosarcomen. Preklinische modellen suggereren dat de IGF signaaltransductieroute een mogelijk aangrijpingspunt voor therapie zou kunnen zijn. Echter, de resultaten van klinische studies laten zien dat maar een kleine groep patiënten mogelijk baat heeft van behandeling met IGF1R remmers, en daarom zijn farmaceutische bedrijven gestopt met de productie van medicijnen die de IGF signaaltransductieroute remmen. In het tweede gedeelte wordt beschreven hoe er voor gezorgd kan worden dat in de toekomst remmers van de IGF signaaltransductieroute toch mogelijk gebruikt kunnen worden in de kliniek. Biomarkers dienen geïdentificeerd te worden die patiënten kunnen selecteren waarin het remmen van de IGF signaaltransductieroute wel effectief is. Daarnaast moeten studies gedaan worden met medicijnen die simultaan de IGF1R en de IR remmen; de klinische studies die tot nu toe uitgevoerd zijn, maakten gebruik van medicijnen die alleen de IGF1R remmen. In het laatste gedeelte van de beschouwing wordt beschreven hoe biomarkers geïdentificeerd kunnen worden, en hoe toekomstige klinische studies met IGF1R remmers verder geoptimaliseerd kunnen worden. Momenteel worden de data geanalyseerd van een klinische studie naar de werking van OSI-906 in patiënten met het Ewing-sarcoom. Biomarkers die geïdentificeerd worden in deze studie, die gecoördineerd wordt door prof. dr. A.B. Hassan van de Universiteit van Oxford, dienen getest te worden in patiënten met een osteosarcoom. De studie in patiënten met een Ewing-sarcoom is door zijn sterke translationele onderzoeksprogramma een voorbeeld voor toekomstige klinische studies in patiënten met een osteosarcoom.

Doelgerichte therapieën voor de behandeling van chondrosarcomen

In **hoofdstuk 5** wordt onderzocht of het remmen van de IGF signaaltransductieroute net als voor osteosarcomen ook een mogelijke doelgerichte therapie is voor de behandeling van chondrosarcomen. Eiwitten die deel uitmaken van de IGF signaaltransductieroute kwamen heterogeen tot expressie in chondrosarcoom cellijnen. Gefosforyleerd IRS1 werd gevonden in twee van de drie geteste cellijnen, wat een indicatie is voor activatie van de IGF signaaltransductieroute. Echter had het behandelen van chondrosarcoom cellijnen met drie verschillende remmers van deze signaaltransductieroute geen effect op chondrosarcoom proliferatie, migratie en chemoresistentie. Om uit te zoeken waarom, het remmen van deze signaaltransductie route geen effect had, ondanks dat de IGF signaaltransductie route wel actief is in een gedeelte van de cellijnen, werd expressie van de IGF1R in chondrosarcoom cellijnen en primaire tumoren bestudeerd. Door middel van immunohistochemie werd aangetoond dat alle cellijnen expressie hebben van de IGF1R, terwijl er geen (66%) of matige (34%) expressie van de IGF1R gevonden werd in primaire tumoren. Het vergelijken van de expressieniveau's van de IGF1R tussen cellijnen en primair tumor materiaal afkomstig uit dezelfde patiënt, suggereerde dat de expressie van de IGF1R tijdens de kweek *in vitro* verhoogd wordt. Dit trekt de representativiteit van chondrosarcoom cellijnen om de IGF signaaltransductie te bestuderen in twijfel. Concluderend laat hoofdstuk 5 zien dat inhibitie van de IGF signaaltransductieroute geen geschikte therapie is voor de behandeling van het chondrosarcoom.

Om andere doelgerichte therapieën voor de behandeling van chondrosarcomen te identificeren werd in hoofdstuk 6 en 7 een andere benadering gekozen, waarbij de genetische eigenschappen van deze tumor benut werden. Ongeveer de helft van de conventionele chondrosarcomen heeft een mutatie in het *isocitraatdehydrogenase-1* (*IDH1*) of *IDH2* gen. Deze mutaties leiden mogelijk tot specifieke veranderingen in het celmetabolisme, welke als aangrijpingspunt voor therapie zouden kunnen dienen. **Hoofdstuk 6** beschrijft een studie naar nicotinamideadeninedinucleotide (NAD^+)-depletie, wat reeds geïdentificeerd was als een mogelijk doelgerichte therapie voor de behandeling van *IDH1/2*

gemuteerde gliomen. Nicotinamidefosforibosyltransferase (NAMPT) en nicotinezuurfosforibosyltransferase (NAPRT) zijn de limiterende enzymen in the synthese van NAD^+. De behandeling van elf chondrosarcoom cellijnen met twee NAMPT-remmers resulteerde in een dosisafhankelijke afname van de proliferatie, invasie en kolonievorming. Bij bijna de helft van de cellijnen was de concentratie NAMPT-remmer die nodig is om proliferatie te halveren onder de 10 nanomolair. qRT-PCR-experimenten toonden aan dat er een negatieve correlatie is tussen NAPRT expressie en de gevoeligheid van de cellen voor NAMPT-remmers. Daarnaast correleerde lagere methylatie van de NAPRT-promotor met hogere NAPRT-expressie. Tevens werd er hogere methylatie van de NAPRT-promotor in hooggradige ten opzichte van laaggradige kraakbeentumoren gevonden. In tegenstelling tot onze initiële hypothese gaf de *IDH1/2*-mutatiestatus geen verschil in gevoeligheid voor NAMPT-inhibitie. Tevens was er geen verschil in NAPRT-methylatie tussen *IDH1/2* gemuteerde en ongemuteerde tumoren. Samenvattend toont hoofdstuk 6 aan dat medicijnen die NAMPT remmen een doelgerichte therapie voor de behandeling van het chondrosarcoom zouden kunnen zijn, in het bijzonder voor hooggradige tumoren, onafhankelijk van de *IDH1/2*-mutatiestatus.

Omdat meerdere studies hebben aangetoond dat *IDH1/2* gemuteerde gliomen afhankelijk zijn van glutaminolyse, beschrijft **hoofdstuk 7** een preklinische onderzoek naar het remmen van glutaminolyse als doelgerichte therapie voor de behandeling van het chondrosarcoom. Door middel van immunohistochemie werd aangetoond dat hogere glutaminase-expressie correleerde met een hogere tumorgraad, en qRT-PCR-analyses van chondrosarcoom cellijnen wezen uit dat glutaminase hoger tot expressie komt in chondosarcoom cellijnen dan in normaal controleweefsel (groeischijf en kraakbeen). Het behandelen van chondrosarcoom cellijnen met de glutaminaseremmer CB-839 toonde aan dat een gedeelte van de chondrosarcoom cellijnen inderdaad gevoelig is voor glutaminolyse inhibitie. Omdat de veiligheid van CB-839 voor mensen nog onderzocht wordt, werden ook de medicijnen metformine (antidiabetesmedicijn), fenformine (lipofiele analoog van metformine) en chloroquine (antimalariamedicijn) *in vitro* onderzocht omdat deze medicijnen net als CB-839 de glutaminolyse remmen. Deze vier medicijnen remden de groei van een gedeelte van de chondrosarcoom cellijnen. Om de cellulaire mechanismen van deze inhibitie te bestuderen,

werden apoptose, autofagie en mTOR-activiteit bestudeerd. De medicijnen die in deze studie gebruikt zijn leken geen apoptose te induceren, maar metformine en fenformine verminderde wel de activiteit van mTOR. Daarnaast verlaagde metformine autofagie, iets dat juist geïnduceerd werd door chloroquine. Hoe CB-839 de groei van chondrosarcomen remt moet in de toekomst verder onderzocht worden. Net als in hoofdstuk 6 konden we onze initiële hypothese dat *IDH1/2* gemuteerde chondrosarcomen meer afhankelijk zouden van glutaminolyse niet bevestigen, aangezien er geen correlatie was tussen de *IDH1/2*-mutatiestatus en de gevoeligheid voor glutaminolyse-remmende medicijnen of de expressie levels van glutaminase in primaire tumoren. Concluderend toont hoofdstuk 7 aan dat het remmen van de glutaminolyse een doelgerichte therapie voor de behandeling van chondrosarcomen zou kunnen zijn, onafhankelijk van de *IDH1/2*-mutatiestatus.

Samenvatting

In dit proefschrift zijn studies beschreven die nieuwe aangrijpingspunten voor therapieën verkennen doordat zij cellulaire signaaltransductieroutes identificeren die essentieel zijn voor de overleving van osteosarcoom- en chondrosarcoom cellen. Ondanks dat klinische studies met IGF signaaltransductieremmers tegenvallende resultaten geven, ondersteunt dit proefschrift de visie dat deze remmers wel degelijk gebruikt zouden kunnen worden als doelgerichte therapie voor behandeling van het osteosarcoom, mits de juiste patiënten geselecteerd worden en er gebruik wordt gemaakt van medicijnen die simultaan de IGF1R en de IR remmen. Als geoptimaliseerde klinische studies in de toekomst uitgevoerd worden, dienen er geen patiënten met een chondrosarcoom geïncludeerd te worden omdat hier geen preklinische rationale voor is. Wij hebben twee signaaltransductieroutes geïdentificeerd in chondrosarcomen die wel een mogelijk aangrijpingspunt zijn voor doelgerichte therapieën, namelijk NAD^+-synthese en glutaminolyse. Chondrosarcoom patiënten dienen geïncludeerd te worden in klinische studies met remmers van deze signaaltransductieroutes, ongeacht de aan- of afwezigheid van een *IDH1/2* mutatie.

Curriculum Vitae

Elisabeth Francisca Patricia Peterse was born on the 29th of January 1991 in Diessen, the Netherlands. She attended pre-university education at the Odulphuslyceum in Tilburg. After graduating in 2009, she started the Bachelor's program Biomedical Sciences at Leiden University. During her Bachelor's, Elisabeth participated in the Honours College Bèta and Life Science, and studied at the Karolinska Institutet in Stockholm, Sweden, as part of an Erasmus exchange program. In addition, she enrolled in the MSc/PhD-track and started extracurricular research under supervision of dr. A.M. Cleton-Jansen at the Department of Pathology, Leiden University Medical Center (LUMC). After receiving her Bachelor's degree in 2012, Elisabeth continued her studies by attending the Research Master program Biomedical Sciences at Leiden University. Her first Master's internship was at the Ludwig Institute for Cancer Research, University of Oxford, United Kingdom under the supervision of dr. G.L. Bond, where she analysed genetic variants that affect human cancer risk. Upon her return, Elisabeth enrolled in the Leiden Leadership Program from the Leiden University Honours Academy and continued her MSc/PhD research under the supervision of dr. A.M. Cleton-Jansen and prof. dr. J.V.M.G. Bovée as part of her second Master's internship. After receiving her Master of Science degree with distinction in 2014, she received funding from the MSc/PhD-track to continue her PhD. The results obtained during this PhD are described in this thesis. In June 2016, Elisabeth started her second PhD in the group of dr. I. Lansdorp-Vogelaar and prof. dr. H.J. de Koning at the Department of Public Health, Erasmus Medical Center, Rotterdam.

List of publications

- Peterse EFP, Niessen B, Addie RD, de Jong Y, Cleven AHG, Kruisselbrink AB, van den Akker BEWM, Molenaar RJ, Cleton-Jansen AM, Bovée JVMG (2018) Targeting glutaminolysis in chondrosarcoma in context of the IDH1/2 mutation. *British Journal of Cancer* **In Press**

- Peterse EFP, van den Akker BEWM, Niessen B, Oosting J, Suijker J, de Jong Y, Danen EHJ, Cleton-Jansen AM, Bovée JVMG (2017) NAD Synthesis Pathway Interference Is a Viable Therapeutic Strategy for Chondrosarcoma. *Molecular Cancer Research* **15**(12)**:** 1714-1721

- Peterse EFP, van Leeuwen TN, Cleton-Jansen AM (2017) A researcher's perspective on the quantity of osteosarcoma *in vitro* studies. *Journal of Bone Oncology* **7:** 29-31

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- Zeron-Medina J, Wang X, Repapi E, Campbell MR, Su D, Castro-Giner F, Davies B, Peterse EFP, Sacilotto N, Walker GJ, Terzian T, Tomlinson IP, Box NF, Meinshausen N, De Val S, Bell DA, Bond GL (2013) A polymorphic p53 response element in KIT ligand influences cancer risk and has undergone natural selection. *Cell* **155**(2)**:** 410-22

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