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A screening based approach to find new paths for targeted treatment in chondrosarcoma

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Yvonne de Jong

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Cover photo: Slap Savica, Slovenia, picture by Robin the Jong on the 2nd of November 2019

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A screening based approach to find new paths for targeted treatment in chondrosarcoma

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Voor mijn Lieve broertje Robin

‘Liefde is Sterker dan de Dood’

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Chapter 1

General introduction and Thesis outline

Chondrosarcoma

Chondrosarcoma is a malignant cartilage tumour representing 20% of malignant bone tumours [1]. It is most common in adults around the age of fifty and develops predominantly in the bones of the ribs, pelvis and bones of the extremities. The most frequently observed subtype is conventional chondrosarcoma, which represents 85% of all the chondrosarcomas, followed by rarer chondrosarcoma subtypes dedifferentiated chondrosarcoma (10%) [2], mesenchymal chondrosarcoma (2%) [3], clear cell chondrosarcoma (2%) [4] and periosteal chondrosarcoma (1%) [1] (see figure 1).

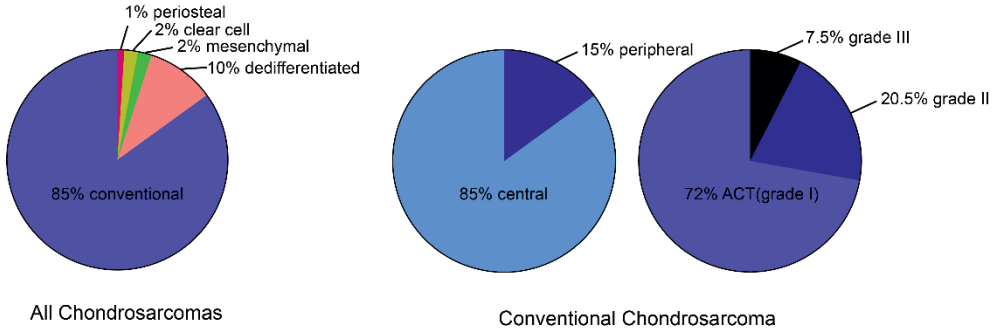


Figure 1. Chondrosarcoma subtypes and grades. Conventional chondrosarcomas are the most common subtype, followed by dedifferentiated chondrosarcomas. Of all conventional chondrosarcomas, central chondrosarcoma is most frequently observed.

Conventional Chondrosarcoma

Conventional chondrosarcoma can be found either as a central subtype, when it is located inside the medulla of the bone or as a peripheral subtype when it is located next to the bone. Central conventional chondrosarcoma accounts for 85% of all conventional chondrosarcomas, while peripheral chondrosarcoma is rarer and comprises 15% of all conventional chondrosarcomas. Conventional chondrosarcoma can be further subdivided into three histologically different grades; Atypical cartilaginous tumour /Grade I) (72%), Grade II (20.5%) and Grade III (7.5%) [5], which histologically show an increase in cellularity and a decrease in matrix deposition with increasing grade (see figure 2). Based on the latest WHO classification from 2020 the term Atypical cartilaginous tumour is given to

low grade cartilaginous lesions located in the long and short tubular bones, while the term Grade I is restricted for low grade cartilaginous lesions located in the flat bones. The histological appearance is similar, but lesions located at the flat bones represent with a poorer clinical outcome, and need more extensive surgery [6]. Histological grade is the most important predictor of survival and metastasis. Atypical cartilage tumours rarely metastasize and show a relatively good prognosis with a 10-year survival rate of 83-88%. Grade II chondrosarcomas show a 10 years survival of 62-64% and grade III chondrosarcomas show a very poor prognosis with a 10 years survival rate of 26-29% and a high risk of developing metastasis [1, 5, 7, 8]. Recurrence of a low-grade tumour into a higher histological grade occurs in ~13% of the cases, leading to a worse prognosis for these patients [7, 9].

Central chondrosarcomas can arise *de novo* or from a pre-existing enchondroma, which are benign cartilage lesions that can arise in the medulla of the bone. Characteristic mutations identified in 87% of enchondromas and 38-70% of central chondrosarcomas are mutations in *isocitrate dehydrogenase 1 (IDH1)* or *isocitrate dehydrogenase 2 (IDH2)* [10, 11]. These are enzymes catalysing the conversion of isocitrate to alpha-ketoglutarate in the Krebs Cycle. A mutation in one of these genes leads to a gain of function in which alpha-ketoglutarate is converted to the oncometabolite D2HG (See figure 3). This oncometabolite competes with alpha-ketoglutarate to bind to alpha ketoglutarate dependent enzymes resulting in inhibition of these enzymes. This group of enzymes is involved in different important processes such as methylation, collagen folding and oxygen sensing [12-14].

Peripheral chondrosarcomas always develop from a pre-existing benign osteochondroma. Osteochondromas are benign bone tumours developing adjacent to the bone, which can progress towards secondary peripheral ACT/CS1. Osteochondromas can either occur as sporadic or as hereditary disease in which case multiple osteochondromas develop. Alterations in *Exostosin glycosyltransferase 1 (EXT1)* or *Exostosin glycosyltransferase 2 (EXT2)* are involved in the development of osteochondromas. Heterozygous germline mutations in these genes have been identified in 70-95% of patients with multiple osteochondromas [15]. In addition, homozygous inactivation of *EXT1* is observed in 80% of sporadic osteochondromas [16-18]. Although homozygous inactivation is observed in osteochondromas, only a small percentage (~15%) of peripheral chondrosarcomas show homozygous inactivation of the *EXT* genes [18-22]. This leads to the

hypothesis that wildtype cells with functional EXT that are located in or near this EXT-null microenvironment are the precursors of secondary peripheral chondrosarcoma [23]. EXT1 and EXT2 are involved in biosynthesis of heparan sulfate chains of proteoglycans [24]. Proteoglycans are important for cell to cell contact and regulate growth plate organization. The absence of EXT1 or EXT2 in the developing bone will lead to the formation of an osteochondroma [25].

Rare chondrosarcoma subtypes

Dedifferentiated chondrosarcoma is a malignant high-grade chondrosarcoma subtype. Histologically it shows a mixed appearance of a usually low grade cartilaginous component and a high grade dedifferentiated component [2] (see figure 2). The 10 years survival is only 28%, and when the patient presents with metastasis at diagnosis this if further decreased to 10% [26]. Like in conventional central chondrosarcomas *IDH1* and *IDH2* mutations have been identified in 54% of the cases [10].

Mesenchymal chondrosarcoma is a high-grade tumour with a reported ten years survival rate between 27 and 67% [27, 28]. Its histology shows a mixture of differentiated cartilage combined with undifferentiated small round cells [3] (see figure 2). Genetically, mesenchymal chondrosarcoma is characterized by a fusion between *HEY1* (Hes-Related Family BHLH Transcription Factor with YRPW Motif 1) and *NCOA2* (Nuclear Receptor Coactivator 2). The function of HEY1 is as transcription factor downstream of Notch signalling. NCOA2 is a nuclear hormone receptor coactivator. Their role in development of mesenchymal chondrosarcoma still needs to be investigated.

Clear cell chondrosarcoma is a low-grade chondrosarcoma subtype histologically showing malignant cells with clear empty cytoplasm and hyaline cartilage matrix (see figure 2). It is mainly found in the epiphysis of the femoral or humoral head and the prognosis is relatively good with a mortality rate of approximately 15% [29]. No recurrent possible initiating mutations have been identified in this subtype.

Periosteal Chondrosarcoma originates from the periosteum and is a very rare chondrosarcoma subtype accounting for less than 1% of the cases [1]. Little is known about its development and progression however *IDH1* mutations have been identified in 15% of periosteal chondrosarcomas [30].

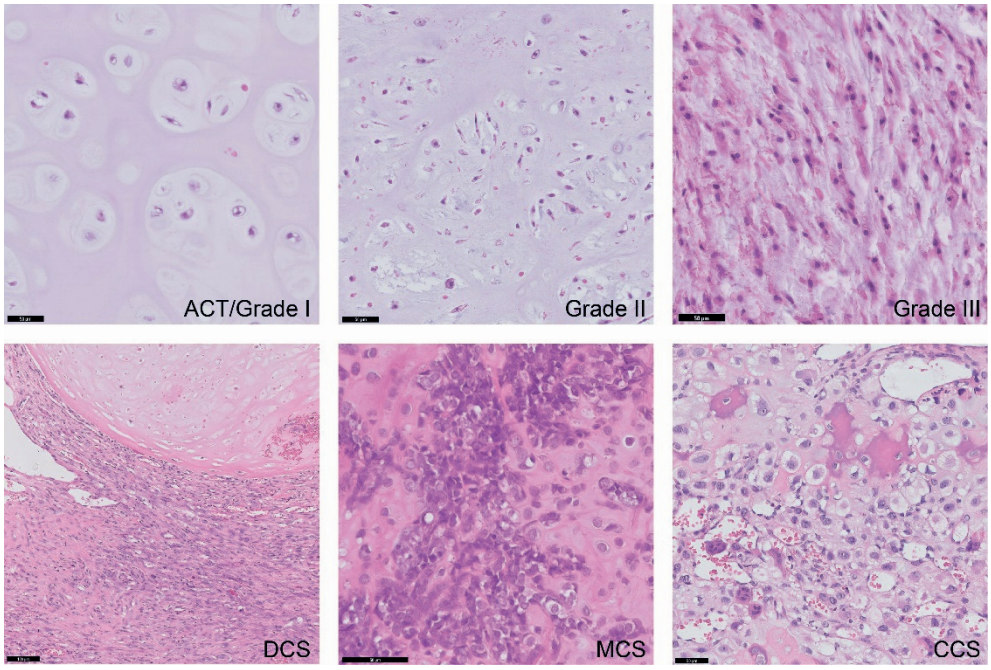


Figure 2. Histological appearance of conventional (ACT, Grade II and Grade III) and rare chondrosarcoma subtypes. (ACT: atypical cartilage tumour, DCS: dedifferentiated chondrosarcoma, MCS: mesenchymal chondrosarcoma, CCS: clear cell chondrosarcoma).

Current treatment options for patients with chondrosarcoma

Surgery

Chondrosarcomas are relatively resistant towards chemo- and radiotherapy, consequently the best treatment option for patients with chondrosarcoma is surgery. Complete surgical removal of the tumour provides the only chance for cure. Grade II and III chondrosarcoma is treated if possible, by wide, *en-bloc* excision of the tumour, while atypical cartilaginous tumour that is confined to the tubular bone, is removed by intralesional curettage often followed by adjuvant treatment. When the tumour is large or has grown into the surrounding soft tissue wide resection is always performed [8].

Radiotherapy

Chondrosarcomas are considered radioresistant, however after incomplete resection or when surgery is not feasible radiotherapy is given to

chondrosarcoma patients. This is challenging since high doses are needed to achieve an improvement especially when the tumour is located in the skull or spine [8]. Proton beam therapy has shown to be a much better option compared to conventional radiotherapy [31], especially for tumours close to vital organs. This type of radiotherapy can deliver the particles precisely to the tumour without damaging the surrounding tissue and since last year two centres in the Netherlands are able to offer treatment with proton radiotherapy.

Chemotherapy

Chemotherapy is not standard treatment for patients with chondrosarcoma, since there is no evidence whether this is beneficial for these patients. A small increase in median overall survival was observed in patients with unresectable chondrosarcoma when treated with conventional chemotherapy [32]. Mesenchymal chondrosarcoma has been described in several reports to be more sensitive to chemotherapy compared to other chondrosarcoma subtypes, and has shown a reduced risk of recurrence and a better overall survival after chemotherapy [27, 28, 33-38]. However in a meta-analysis this could not be confirmed [39].

Therapy resistance mechanisms

There are several possible explanations why chondrosarcoma cells are resistant to chemo- and radiotherapy; the tumour is slow growing and has a large amount of cartilage matrix, which may hamper access to the cells. This is however not the case for high grade chondrosarcomas, which proliferate faster and have myxoid instead of hyaline cartilaginous matrix, and they also show chemo- and radiotherapy resistance. A second possible mechanism is the expression of multi-drug resistance pumps, which has been shown on chondrosarcoma cell lines and patient tissues. However, despite the expression of these pumps doxorubicin was still able to accumulate in the nuclei of 3D chondrosarcoma cell pellets [40-42]. Another possible mechanism is the expression of anti-apoptotic Bcl-2 family members, which has been shown previously as a mechanism of chemoresistance in chondrosarcoma cell lines. Inhibition of Bcl-2 family members could sensitize conventional, dedifferentiated and, as shown in this thesis, mesenchymal chondrosarcoma cells towards doxorubicin and cisplatin chemotherapy [41, 43]. Furthermore, upregulation of other pro-

survival and down regulation of pro-cell death mechanisms are likely to play a role in therapy resistance.

Possible new therapeutic options

IDH1 and IDH2 mutations as therapeutic vulnerability

Mutations in *IDH1* or *IDH2* have been identified in 38-70% of central chondrosarcomas, 87% of enchondromas, 54% of dedifferentiated chondrosarcomas [10, 11] and 15% of periosteal chondrosarcomas [30]. Compounds specifically targeting mutant IDH1 or IDH2 have been developed by several pharmaceutical companies. Inhibiting mutant IDH1 using AGI-5198 decreased the production of D2HG in in vitro chondrosarcoma cell models, but did not lead to any reduction in viability, proliferation and migration, unless used at high, probably toxic dosages [44, 45]. This indicates that the mutation in *IDH1* or *IDH2* is an early event in tumorigenesis, but after progression chondrosarcomas are not dependent on it anymore. Although preclinical studies do not seem promising, clinical trials assessing the efficacy of IDH1 and IDH2 mutant inhibitors in chondrosarcoma patients are ongoing (NCT022737399, NCT02481154, NCT02073994, NCT03684811).

The oncometabolite D2HG competes with alpha-ketoglutarate to bind to alpha ketoglutarate dependent enzymes, which leads to inhibition of these enzymes [12-14] (Figure 3). Two groups of alpha ketoglutarate dependent enzymes that affect methylation are demethylating enzymes of the ten eleven translocation (TET) family and histone demethylases of the jumonji C domain containing (JMJD) family. TET enzymes convert 5-methylcytosine (5-mC) to 5-hydroxymethylcytosine (5-hmC). Inhibition of TETs will lead to an increase in methylation. Indeed *IDH1* and *IDH2* mutant chondrosarcoma cell lines and tissues do show a CpG island methylator phenotype (CIMP) [11, 45, 46], however surprisingly no differences in 5-mC and 5 hmC immunostaining were observed in a panel of ~100 chondrosarcomas between *IDH1* or *IDH2* mutant and wildtype [47]. Likewise, no difference in histone methylation marks H3K27me3, H3K9me3 and H3K27me3 was observed in the same cohort. Further studies should determine whether *IDH1* or *IDH2* mutant chondrosarcoma patients should be included in clinical trials assessing the effect of demethylating compounds specifically in *IDH1* and *IDH2* mutant tumours (NCT03666559).

IDH1 and *IDH2* mutated cells produce very high concentrations of D2HG. For this process high amounts of alpha ketoglutarate are needed, which can be generated either by glycolysis or glutaminolysis. In *IDH1* and *IDH2* mutant cells generation of alpha ketoglutarate by glutaminolysis has been shown as the most important mechanism [48-50], making targeting glutamine metabolism an interesting vulnerability to specifically target *IDH1* or *IDH2* mutated cells. *In vitro* studies in chondrosarcoma cell lines show sensitivity towards glutamine pathway inhibitors, however in contrast to studies in genetically engineered cell lines [49, 51], no correlation to *IDH1* or *IDH2* mutation status was observed [52]. Clinical trials currently conducted in *IDH1* and *IDH2* mutated tumours, including chondrosarcoma, aim at inhibiting glutamine metabolism using either CB-839 (NCT02071862) or metformin or chloroquine (NCT02496741) [53].

The NAD+ synthesis pathway has been identified using metabolic profiling strategies as another *IDH1* and *IDH2* mutation specific vulnerability [54]. Although chondrosarcoma cells were extremely sensitive to inhibition of NAD+ synthesis, no correlation with mutation status could be identified [55]. Unfortunately phase I clinical trials including FK866 and GMX1778 (and prodrug GMX1777) were discontinued due to dose-limiting toxicities [56].

Another proposed vulnerability in *IDH1* and *IDH2* mutant cells is the increase in reactive oxygen species (ROS) levels due to a decrease in NADPH levels. NADPH is important for the reduction of ROS and in addition it is important for DNA damage repair. A decrease in NADPH, and consequently more ROS and less efficient DNA damage repair will increase sensitivity towards chemo and radiotherapy [57]. A better overall survival in *IDH1* and *IDH2* mutated cancers, most likely due to increased therapy sensitivity, has been reported in glioma [58] and glioblastoma [59], but not for acute myeloid leukaemia [60]. For chondrosarcoma the general consensus is that there is no change in outcome although literature studies show conflicting results [47, 61].

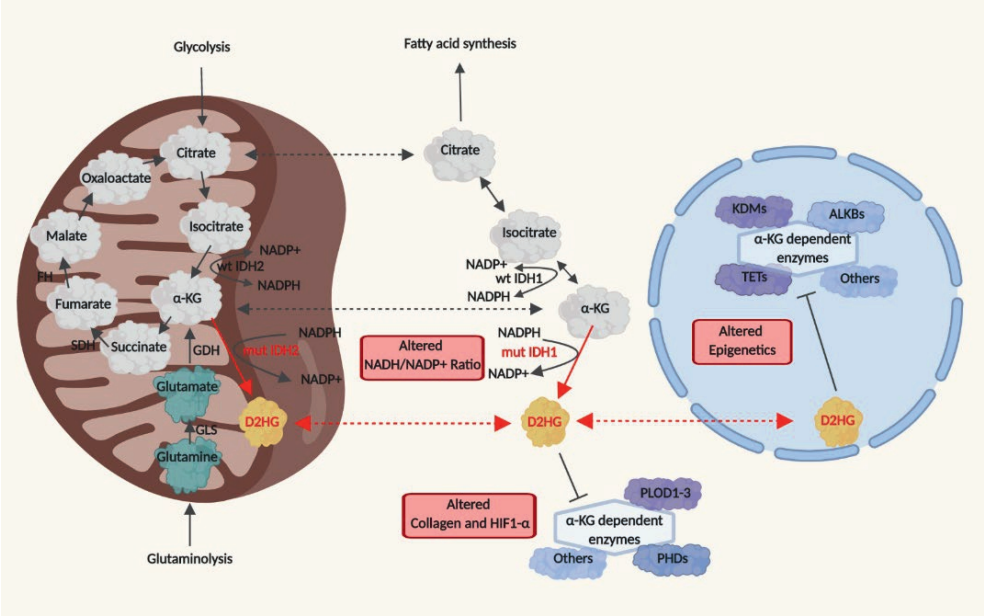


Figure 3. Citric acid cycle and (possible) consequences of *IDH1* or *IDH2* mutations on downstream cellular processes. Wild type IDH1 (cytoplasm) or IDH2 (mitochondria) converts isocitrate to α-ketoglutarate (α-KG), while mutant IDH1 or IDH2 converts α-KG to the oncometabolite D2HG. This process results in an altered NADH/NADP+ ratio. Furthermore the produced oncometabolite D2HG competes with α-KG to bind to α-KG dependent dioxygenases in the nucleus, resulting in altered epigenetics. In the cytoplasm inhibition of α-KG dependent dioxygenases results in alterations in collagen maturation and degradation of HIF1-α. In addition other not yet investigated enzymes are likely to be affected by the production of D2HG. Figure based on [52, 62].

Targeting anti-apoptosis proteins to induce chemosensitization

Apoptosis is a tightly regulated and controlled process of programmed cell death to remove damaged or unnecessary cells from the body. Apoptosis can be activated by either the intrinsic or the extrinsic pathway (figure 4). By upregulating anti-apoptotic proteins, cells can increase the threshold of DNA damage that can accumulate in a cell, and consequently cells can continue to divide in the presence of otherwise too much DNA damage [63]. As briefly mentioned above, chondrosarcoma cell lines express high levels of Bcl-2 and Bcl-xl, and upon inhibition with ABT-737 (inhibiting Bcl-2, Bcl-xl and Bcl-w) conventional and dedifferentiated chondrosarcoma cells could be sensitized towards doxorubicin and cisplatin [41].

Targeting death receptors is another strategy exploited in chondrosarcomas. It was shown that chondrosarcoma cell lines were not sensitive towards monotherapy with a death receptor inhibitor (TRAIL inhibitor), however they could be sensitized towards doxorubicin [64, 65]. Furthermore, clinical trials have been conducted investigating the use of death receptor inhibitors (PRO95780) in treatment of chondrosarcoma (NCT00543712), and although the phase I trial seemed promising, the phase II trial was terminated due to low efficacy. Future studies focussing on combination strategies will determine whether there is a role for TRAIL inhibitors in future chondrosarcoma treatment [66].

TP53 mutations have been observed in 20 percent of chondrosarcomas and are only found in the more malignant high-grade subtypes [67-69]. As can be observed in figure 3 P53 is a main component of the apoptosis pathway and its absence is thought to lead to deregulation of apoptosis activation, although P53 has a broad range of other functions as well [70]. Clinical trials with a mutant P53 inhibitor (APR-246) [71] are ongoing in *TP53* mutated cancers and first results look promising [72, 73] (NCT02098343, NCT03072043, NCT02999893).

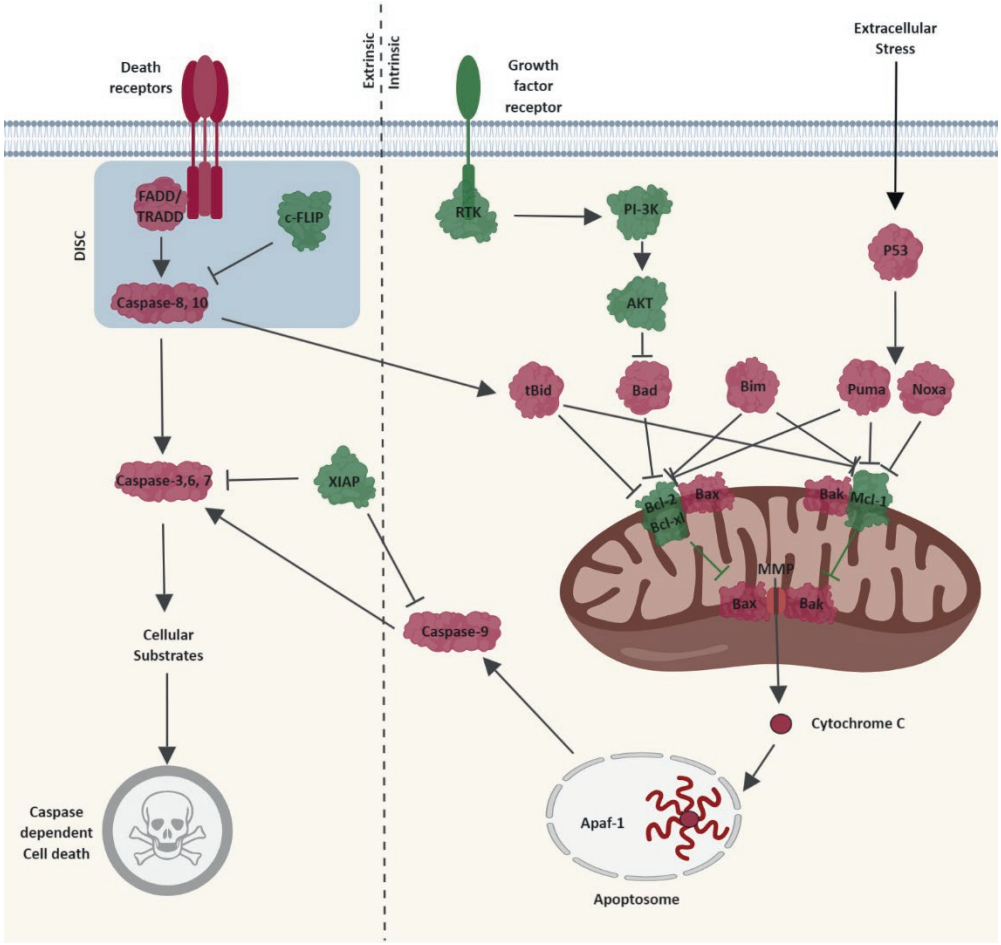


Figure 4. Apoptosis pathway. Schematic overview of the intrinsic and extrinsic apoptosis pathways. The intrinsic apoptosis pathway is activated upon extracellular stimuli, resulting in an upregulation of pro-apoptotic family members and a downregulation of anti-apoptotic family members. This eventually lead to mitochondrial membrane permeabilization (MMP) and the release of cytochrome C. This will bind to apaf-1 and the apoptosome is formed, this is able to cleave caspase-9 which in turn activates caspase 3, 6 and 7. The extrinsic apoptosis pathway is activated by death receptors and the formation of a death-inducing signalling complex (DISC) will result in activation of caspase 3, 6 and 7 either directly or through the activation of Bid, leading to MMP. Figure based on [74].

Upregulated pro-survival mechanisms as possible targets

Survival pathways in cancer cells are usually up or de-regulated and small molecules specifically inhibiting these pathways can serve as possible treatment options. In chondrosarcoma several pro-survival pathways have been shown to be upregulated (see figure 5). Kinome profiling of chondrosarcoma cell lines and primary cultures revealed that the SRC pathway was one of the most active pathways in chondrosarcoma [75]. Treatment of cell lines with dasatinib resulted in a dose dependent decrease in viability and sensitization towards doxorubicin especially in *TP53* mutant cells. In addition, SRC inhibition slowed down migration of chondrosarcoma cell lines.[76]. Treatment of low to intermediate grade chondrosarcoma with dasatinib resulted in stable disease for a small subset of patients indicating that treatment might be beneficial in some specific patient groups [77].

One growth factor receptor found to be active in chondrosarcoma is the platelet-derived growth factor receptor (PDGFR) and expression of PDGFR-alpha was correlated with adverse outcome [78, 79]. Although there was activation of the pathway, no increase in survival was observed in a phase 2 clinical study in which chondrosarcoma patients were treated with imatinib [80]. Regorafenib, a multi-kinase inhibitor, targeting VEGFR-1, 2, 3, TIE2, KIT, RET, RAF, PDGFR and FGFR is tested in a phase 2 study (NCT02389244) in metastatic bone sarcomas and first results in osteosarcoma look promising [81]. Expression of VEGF and presence of microvasculature has been shown in high grade chondrosarcoma, indicating that angiogenesis inhibition could be a potential therapeutic option for these patients [82-84]. A small case series including 10 patients treated with either pazopanib or ramucirumab suggests a potential benefit for patients with metastatic chondrosarcoma [85]. The current trial testing pazopanib in unresectable chondrosarcoma should prove whether inhibition of angiogenesis is a viable therapeutic strategy to treat high grade chondrosarcoma (NCT01330966).

Activating mutations in NRAS have been identified in 12% of chondrosarcoma samples indicating a role for MAP kinase inhibitors in the treatment of chondrosarcoma [86]. No clinical studies have been described so far including chondrosarcoma patients.

Another important pro-survival pathway upregulated in chondrosarcoma is the mTOR pathway. This pathway is active in 69% of conventional and 44% of dedifferentiated chondrosarcoma as shown by S6 phosphorylation [86]. Treatment of chondrosarcoma cell lines with a dual PI3K/mTORC1/2

inhibitor (dactolisib) resulted in a dose dependent decrease in viability. In addition a reduction in tumour growth was observed in an orthotopic mouse model after treatment [86]. Everolimus treatment in a rat chondrosarcoma model showed a block in cell proliferation and a decrease in Glut1 and HIF1a expression, two genes regulated by mTOR [87]. Combination treatment of rapamycin and cyclophosphamide showed a partial response in 1 and stable disease in 6 out of 10 patients. Confirmation of these results is currently ongoing in a phase II clinical trial (NCT02821507).

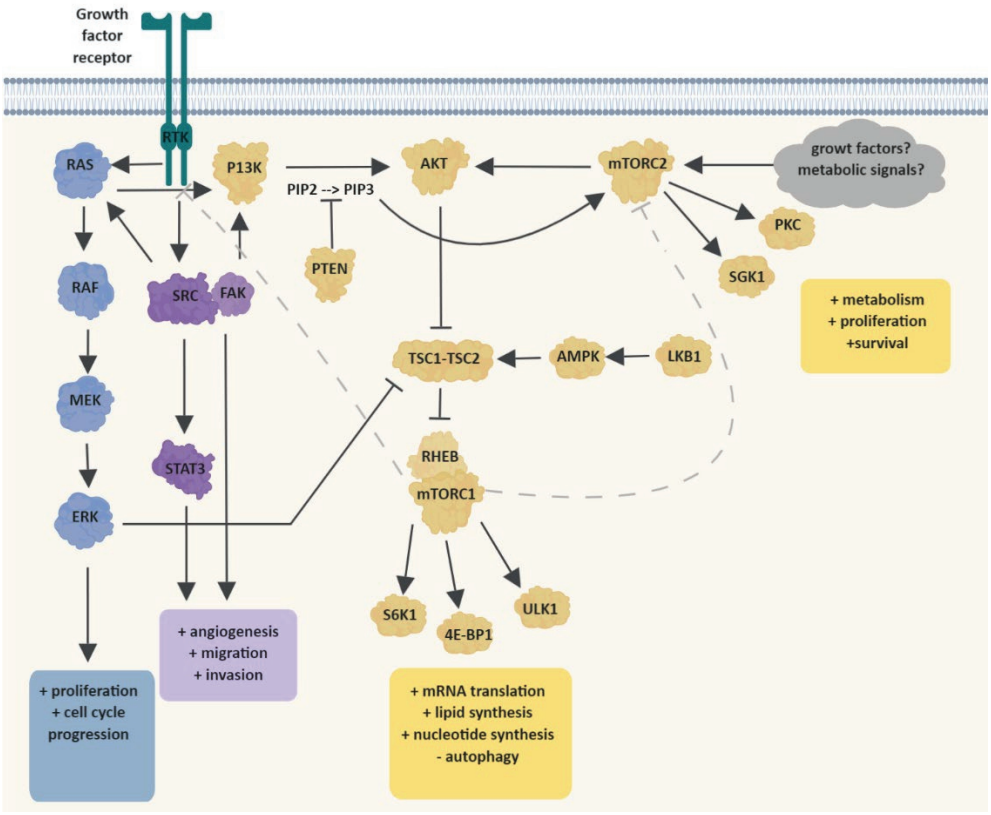


Figure 5. Survival pathways shown to be deregulated in chondrosarcoma. Simple representation of the mTOR pathway (yellow), RAS pathway (blue) and SRC pathway (purple) and their crosstalk. The mTOR pathway is activated by several growth factor receptor tyrosine kinases that can activate Pi3K. Phosphorylation of Phosphatidylinositol 4,5-biphosphate (PIP2) to generate Phosphatidylinositol 3,4,5-triphosphate (PIP3) activates AKT kinases, which in turn phosphorylate tuberous sclerosis protein 1 and 2 (TSC1-TSC2) leading to dissociation of the complex. This complex negatively regulates mTORC1, and therefore its dissociation leads to activation of mTORC1, which leads to an increase in mRNA translation, lipid and

nucleotide synthesis and a decrease in autophagy through the activation of amongst others; S6K1, 4E-BP1 and ULK1. The grey lines represent a negative feedback loop in which mTORC1 prevents the overactivation of the pathway. mTORC2 is less studied, but its activation is caused by PIP3 and growth factors as well as metabolic stimuli. mTORC2 activation leads to activation of amongst others AKT, PKC and SGK1 and will lead to activation of mTORC1, and an increase in metabolism and proliferation and survival. Activation of the Ras-Raf-Mek-Erk pathway is also induced by growth factor receptors and activation leads to an increase in proliferation and cell cycle progression. Ras can also activate Pi3K, and Erk has also the capacity to dissociate the TSC1-TSC2 complex leading to activation of mTORC1. SRC kinases show a lot of crosstalk and are regulating several important survival pathways. They can activate the RAS as well as mTOR pathways and thereby promoting proliferation and cell survival. Activation of SRC kinases also leads to angiogenesis, migration, invasion and metastasis trough activation of focal adhesion kinase (FAK) and transcriptional factors (for example STAT3). Figure based on [88-90].

Developmental pathways that play a role in chondrosarcoma development

During bone development several pathways regulate the differentiation of chondrocytes in the growth plate. One pathway particularly important in the Indian hedgehog (IHH) pathway. Together with parathyroid hormone-like hormone (PTHLH) a gradient is created in which immature chondrocytes are able to terminally differentiate. When IHH is constitutively active terminal differentiation cannot be achieved and this can result in the development of an enchondroma [91]. Mutations in *IHH* genes are identified in 18% of chondrosarcomas suggesting a role for this pathway in development and progression of chondrosarcoma [69]. A phase II clinical trial studying the effect of IHH inhibition (GDC-0449) showed only modest activity [92]. Although good results were obtained in a tumour xenograft model [93], treatment with another IHH pathway inhibitor IPI-926 did not show an improvement in progression free survival in chondrosarcoma patients [94].

Targeting the immune micro-environment

Targeting the immune micro-environment has become a big focus in cancer research and also in the sarcoma field more research has been investigating the applicability of immune therapy [95, 96]. In conventional chondrosarcoma immune infiltrate was found to correlate with prognosis; a higher amount of immune infiltrate correlated with a better prognosis [97]. Although immune infiltrate was detected in conventional chondrosarcoma, in another study immunohistochemical staining showed that there was no expression of the immune checkpoint ligand Programmed death ligand 1

(PDL-1) in conventional chondrosarcoma, but this was only present in 44% of dedifferentiated chondrosarcoma. In addition a high amount of immune suppressive immune infiltrate was found in the latter chondrosarcoma subtype [98]. In a retrospective case study including 2 patients with chondrosarcoma a promising response was observed for one of the patients with metastatic dedifferentiated chondrosarcoma; a partial response was maintained even after 26 cycles with PDL-1 inhibitor nivolumab [99]. A large phase 2 clinical trial assessing the safety and activity of PDL-1 inhibitor Pembrolizumab included five chondrosarcoma patients of which one showed an objective response [100]. In addition, a case report was published describing a patient with conventional chondrosarcoma showing a near complete response by nivolumab treatment [101]. Clinical trials currently ongoing are investigating the effect of combined mTOR and PD-L1 inhibition (NCT03190174) or combined CTLA-4 and PD-L1 inhibitors (NCT02982486) in sarcoma. Future studies should determine how many and which chondrosarcoma patients will benefit from PDL-1 inhibition.

Other immunogenic cancer specific markers identified in chondrosarcoma include MAGE-A3, NYESO-1/LAGE-1a and PRAME antigens that might be targetable using T-cells specific for these antigens [102-104].

Cell cycle regulators in high grade chondrosarcoma

One of the characteristics of cancer is uncontrolled proliferation, caused by a deregulation in the cell cycle and identifying and targeting these proteins can be a good strategy to treat cancer. The cell cycle is regulated by a large amount of proteins that are active during specific phases in the cell cycle (figure 6). Alterations in the retinoblastoma (RB) pathways have been identified in 96% of high grade chondrosarcomas, as shown by overexpression of CDK4, $p16^{INK4A}$ or cyclin D1 in high grade chondrosarcoma tissue samples [105, 106]. In addition higher expression of CDK4 was correlated with a worse overall survival [107]. *In vivo* studies in an orthotopic chondrosarcoma mouse model showed a good response towards CDK4/6 inhibitor palbociclib, which has been FDA approved for the treatment of breast cancer [107]. A clinical trial assessing the efficacy of palbociclib is conducted in sarcoma, although it is unknown if chondrosarcoma patients will be included in these studies (NCT03242382). As mentioned previously, *TP53* mutations are identified in 20% of chondrosarcomas [67-69]. Cells with a defective P53 protein depend more on the G2 checkpoint to halt cell proliferation in case of DNA damage. This gives opportunities to induce synthetic lethality by inhibiting proteins

regulating the G2 checkpoint and select patients with defective P53 function for treatment with these inhibitors.

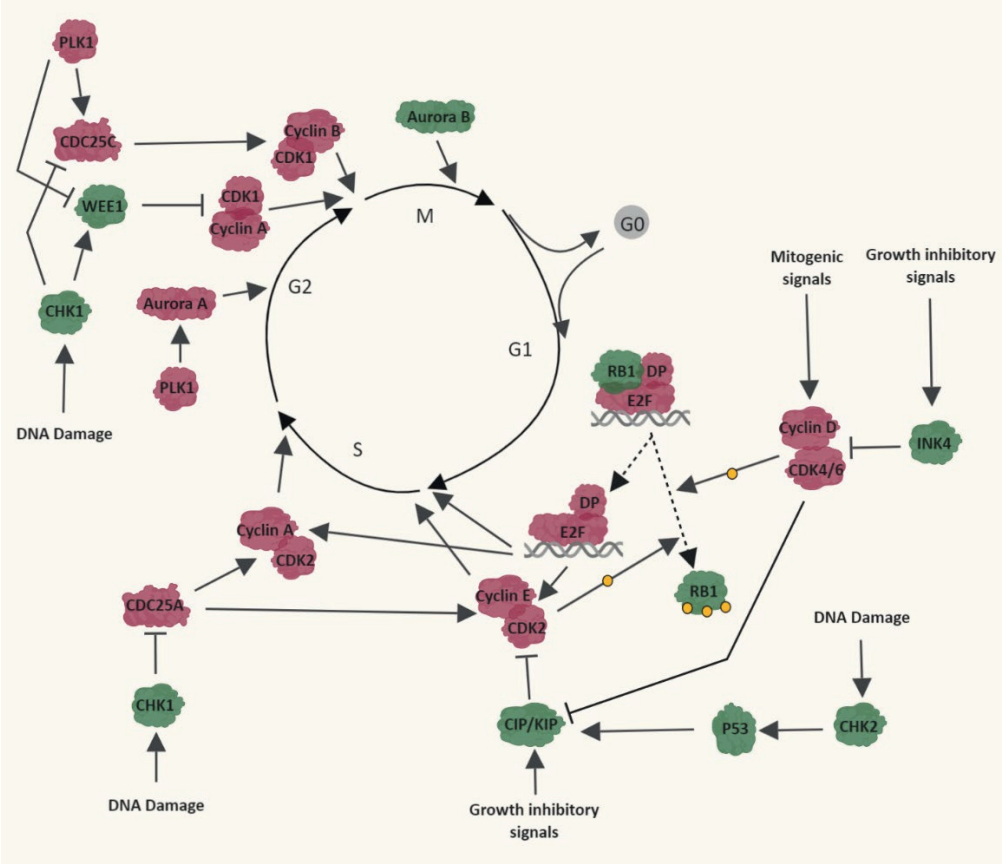


Figure 6. Major proteins involved in cell cycle progression. Activation of cyclin dependent kinases by mitogenic signals results in progression from G1 to S phase. This happens by phosphorylation of retinoblastoma protein 1 (RB1) and subsequent activation of E2F transcription factors. This is negatively regulated by growth inhibitory signals that activate CIP/KIP and INK4, inhibitors of cyclin dependent kinases. Different CDK-cyclin complexes regulate progression through G1-S-G2 and M phase. In addition, several other important proteins such as Polo like kinases (PLK1) and Aurora kinases (Aurora A and B) also regulate cell cycle progression. Cells can exit the cell cycle and go in G0, which can either be reversible of irreversible. DNA damage can trigger cell cycle checkpoints (CHK1 or CHK2), which depends on the type of DNA damage and cell cycle phase. Figure based on [108].

discover new therapeutic targets for chondrosarcoma. For this previously established chondrosarcoma cell lines are used and were chosen based on genetic background and their utility in terms of transfection efficiency. These lines are used to discover new possible targetable vulnerabilities in chondrosarcoma and where possible we tried to validate our findings in more translatable models.

Outline of the thesis

The aim of my thesis was to identify new therapeutic targets for patients with chondrosarcoma. In addition, we tried to explore the biology of chondrosarcoma and tried to find mechanisms that cause chemoresistance in these cells.

Chapter 2 describes the molecular drivers in chondrosarcoma. All known genetic alterations and pathway deregulations are described and discussed in detail for each chondrosarcoma subtype.

In chapter 3 and 4 the role of Bcl-2 family members in chondrosarcoma is studied. **Chapter 3** describes targeting of Bcl-2 family members using ABT-737 as a possible therapeutic strategy in mesenchymal chondrosarcoma, a highly malignant rare chondrosarcoma subtype. In **chapter 4** the role of the different Bcl-2 family members is assessed separately using a selective Bcl-2 and a selective Bcl-xl inhibitor, showing that Bcl-xl is the most promising Bcl-2 family member in targeted treatment of chondrosarcoma.

Screening approaches to identify new therapeutic targets are performed in chapter 5, 6 and 7. **Chapter 5** describes a focussed apoptosis siRNA screen identifying Survivin as a potential therapeutic target for chondrosarcoma patients. The role of kinases is explored in **chapter 6**, where a kinase focussed siRNA screen and compound screen is performed. We show that cell cycle regulators are important in chondrosarcoma cell survival and might be potential targets in a subset of high-grade chondrosarcoma patients. A metabolic compound screen is described in **chapter 7**. Here the role of the mTOR pathway is assessed in chondrosarcoma cell lines and an orthotopic mouse model.

Chapter 8 focusses on radiation resistance in chondrosarcoma. Cell lines and tumour explants are treated with radiotherapy and possible markers predicting radio-sensitivity are assessed using sequencing and immunohistochemistry.

Chapter 9 contains the summary and conclusion and results of different studies will be discussed and put in a broader perspective.

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Chapter 2

Molecular drivers in chondrosarcoma

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Introduction

Chondrosarcoma is a malignant cartilage forming tumour accounting for 20% of all malignant bone tumours [1]. Only a small percentage of chondrosarcomas is found in the skull or spine (2-12%) [2]. Depending on the subtype, different molecular pathways are involved in development and progression of chondrosarcoma. In the skull or spine, chondrosarcoma needs to be distinguished from chordoma, which shows a similar presentation and anatomical location, but a different biological behaviour. The distinction can be made using brachyury immunohistochemistry, as overexpression of brachyury is the hallmark of chordoma [3]. On the other hand, chondrosarcomas represent a heterogeneous group of tumours, in which different cell signalling pathways are involved in tumorigenesis. In this chapter, the most important pathways will be discussed.

Clinicopathological classification of chondrosarcoma

Chondrosarcomas can be divided in conventional chondrosarcoma (85%), and more rare subtypes; dedifferentiated chondrosarcoma (10%), mesenchymal chondrosarcoma (2%), clear cell chondrosarcoma (2%) and periosteal chondrosarcoma (1%) [4-7]. Central conventional chondrosarcoma is the most common in the skull and spine, followed by mesenchymal chondrosarcoma [8].

Conventional chondrosarcoma

Conventional chondrosarcoma can be further subdivided in central (>85%) and peripheral chondrosarcoma (~15%) based on its location in the bone. Central chondrosarcomas develop in the bone either directly or from a previously developed benign enchondroma [4] (see figure 1). Enchondromas can develop as a solitary lesion or as multiple enchondromas in Ollier disease or Maffuci syndrome [9]. Ollier disease and Maffuci syndrome are both rare non-hereditary disorders caused by somatic mosaic mutations in *IDH1* or *IDH2*. In solitary enchondromas ~1% undergoes malignant transformation towards a conventional central chondrosarcoma. Enchondromas from patients with multiple enchondromas have a much higher transformation rate of ~50% [10].

Peripheral chondrosarcomas develop at the surface of the bone from a pre-existing benign osteochondroma. They develop from bones that are formed by endochondral ossification; consequently they are extremely rare findings in the skull. Central and peripheral chondrosarcomas show similar histological features and are graded using a three tiered scale. Atypical cartilaginous tumours (previously chondrosarcoma grade I) show low cellularity, do not metastasize, but behave locally aggressive and local recurrences can develop. Grade II and III chondrosarcomas are high grade tumours with an increased cellularity and mitoses. These tumours show a higher metastatic rate and poor patient survival. Patients with atypical cartilage tumours show a 10 years overall survival rate of 83%, while patients with grade II chondrosarcomas show a 10 years survival rate of 64% and patients with a grade III chondrosarcoma show the poorest 10 years survival rate of 29% [4, 11] So far, histological grade is the most important predictor of prognosis. Upon recurrence of low grade tumours, progression towards a higher histological grade can occur in ~13% of the cases [12, 13], resulting in a poorer prognosis. Chondrosarcomas are resistant to conventional chemo- and radio therapy, leaving surgical removal as the only treatment option [14]. This has major implications for patients with inoperable disease to whom no treatment options can be offered.

Rare chondrosarcoma subtypes

Dedifferentiated chondrosarcoma comprises ~10% of all chondrosarcomas [5]. It is a high grade chondrosarcoma variant that is characterized by a mixed histological appearance of dedifferentiated cells and a cartilaginous component. Patients with dedifferentiated chondrosarcoma show a 5 years overall survival between 7 and 24% [5, 15]. Like conventional chondrosarcoma, dedifferentiated chondrosarcoma is also resistant to conventional chemo- and radiotherapy. Mesenchymal chondrosarcoma is a rare high grade chondrosarcoma consisting of differentiated cartilage mixed with undifferentiated small round cells [7]. Ten years survival rates are reported between 27 and 67% [16, 17]. The small round cell component of mesenchymal chondrosarcoma may respond better to chemotherapy compared to other chondrosarcoma subtypes [17, 18]. Clear cell chondrosarcoma is a rare low grade chondrosarcoma mainly found in the epiphysis of the humoral or femoral head [11]. Histologically, clear cell chondrosarcoma shows malignant cells with clear empty cytoplasm surrounded by hyaline cartilaginous matrix [6]. Surgery is the only treatment option for patients with clear cell chondrosarcoma. The prognosis

is relatively good with a mortality rate of 15% [6]. Periosteal chondrosarcoma is the rarest chondrosarcoma subtype accounting for less than 1% of the cases and originates from the periosteum [19, 20].

Molecular drivers in chondrosarcoma

In this chapter the molecular pathways and mutations in chondrosarcoma are discussed for the different chondrosarcoma subtypes, however most research has been focussing on central conventional chondrosarcoma. Mutations in *IDH1* and *IDH2* have been found in central as well as dedifferentiated and periosteal chondrosarcoma. *COL2A1* mutations and alterations in Indian hedgehog genes are seen in a small percentage of central chondrosarcoma. Deregulation of the hedgehog pathway has been found in a large part of chondrosarcomas indicating that it is important for the development of these tumours. Peripheral chondrosarcomas are characterized by mutations in *EXT1* or *EXT2*. Both central and peripheral chondrosarcomas show alterations in p53 and pRb pathways when progressing from low to high grade.

Central, dedifferentiated and periosteal chondrosarcoma

IDH1 and *IDH2* mutations

Mutations in *isocitrate dehydrogenase 1* and *2* (*IDH1* and *IDH2*) have been first identified in glioblastoma multiforme [21] and later also in 87% of enchondromas, 38-70% of primary central chondrosarcomas, 54% of dedifferentiated chondrosarcomas and 15% of periosteal chondrosarcomas [9, 19, 22, 23]. The occurrence of *IDH1* and *IDH2* mutations in enchondroma indicates that this is an early genetic event in the development of central chondrosarcoma (see figure 1). Also in intracranial chondrosarcomas, but not chordomas, *IDH1* and *IDH2* mutations have been reported, making it a good diagnostic marker in the differential diagnosis between the two different entities [24, 25].

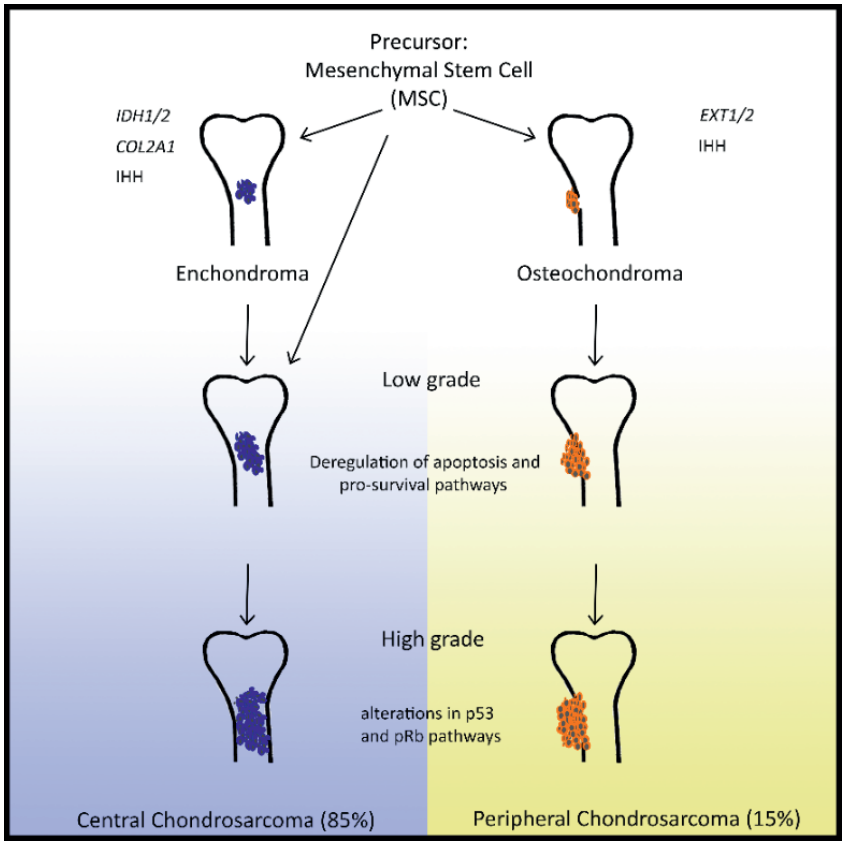


Figure 1. Progression model of conventional chondrosarcoma
Enchondromas, located in the medulla of the bone, can develop into central chondrosarcoma, while osteochondromas, located at the surface of the bone, can progress into peripheral chondrosarcoma. Early driver gene alterations include mutations in *IDH1* or *-2* in enchondroma and *EXT1* or *-2* in osteochondromas. Upon progression towards chondrosarcoma, similar molecular defects in apoptosis and survival pathways are observed in central and peripheral subtypes. Deregulation of p53 and pRb pathways is observed in the majority of high grade chondrosarcomas.

Cellular consequences of an *IDH1* or *IDH2* mutation

IDH1 and IDH2 are enzymes that convert isocitrate to alpha ketoglutarate (αKG) in the Kreb's cycle. IDH1 is located in the cytoplasm and IDH2 in the mitochondria and they both convert isocitrate to αKG while meanwhile NADPH is produced from NADH+ [26]. Mutations in *IDH1* and *IDH2* are heterozygous and affect only a single amino acid. In chondrosarcoma, the *IDH1* mutation is always located on the R132 position in exon 4, and the *IDH2* mutation on R172, which are the active sites of the enzymes. *IDH1* and

IDH2 mutations are mutually exclusive. When IDH1 or IDH2 are mutated, isocitrate cannot be converted to αKG anymore, because affinity for isocitrate is reduced. However, affinity for αKG and NADPH is increased, which leads to the conversion of αKG to the oncometabolite D2-hydroxyglutarate (D2HG) which consumes instead of produces NADPH. Also the enzyme activity of the wild type IDH is inhibited by the mutant [27, 28].

D2HG production has major consequences for enzymes that depend on αKG as a co-substrate (2-OG-dependent dioxygenases). D2HG and αKG are structurally the same, except that the C2 carbonyl group of αKG is replaced by a hydroxyl group in D2HG. Because of the similarity of D2HG to αKG, D2HG acts as a competitive inhibitor by binding to the αKG binding pocket in the active site of the enzymes that depend on αKG [27, 29].

One group of enzymes affected by increased D2HG levels are the ten-eleven translocation (TET) enzymes (Tet 1/2/3). Tet enzymes are demethylating enzymes that convert 5-methylcytosine (5-mC) to 5-hydroxymethylcytosine (5-hmC), which is the first step in demethylation of cytosines [30-32]. Several studies have been conducted investigating the role of Tet enzymes and 5-hmC, showing that they play an important role in stem cell development and differentiation. Simultaneous knock down of all three Tet proteins in mouse ES cells resulted in low levels of 5-hmC, impaired differentiation, promoter hypermethylation and deregulated expression of genes involved in embryonic development [33].

JMJC domain containing histone lysine demethylases (JHKDMs) are also inhibited by D2HG. These enzymes remove methyl groups from the lysine residues of histone proteins, which will affect the histone code leading to alterations in chromatin structure and transcription of genes [29, 34, 35].

Another group of αKG dependent enzymes influenced by D2HG are collagen and HIF prolyl-4hydroxylases (PHDs) [29, 36]. Collagen PHDs are important for collagen folding and maturation. When these enzymes are inhibited collagen cannot be properly folded and accumulates in the endoplasmic reticulum (ER). Hydroxylated collagen is part of the extracellular matrix (ECM), which can influence proliferation, differentiation and invasion [37].

HIF PHDs are oxygen sensing enzymes that hydroxylate HIF1α and HIF2α under normoxic conditions, marking them for recognition by the Von Hippel Lindau (VHL) protein, which targets them for proteosomal degradation. Under hypoxic conditions there is no hydroxylation and HIF1α and HIF2α can dimerize with HIF1β to form complete transcription factors targeting

genes that function to increase oxygen delivery or enhance angiogenesis [38]. Since HIF PHDs need αKG to hydroxylate HIF1α and HIF2α, the hypothesis is that *IDH1* or *IDH2* mutant tumours show a higher frequency of HIF1α and HIF2α activation, because they will not be recognized by VHL and targeted for degradation. However conflicting results have been shown about the role of HIF PHDs in IDH mutant tumours, especially in glioblastomas, reporting both up and down regulation of HIF1α and HIF2α in *IDH1* or *IDH2* mutant tumour cells compared to wild type tumour cells [29, 36, 38]. This indicates that a more complex mechanism is involved in the role of HIF PHDs in *IDH1* or *IDH2* mutant tumours [39].

Mutations in *IDH1* or *IDH2* are expected to alter the metabolism of cells, because IDH1 or IDH2 are involved in the Krebs cycle converting isocitrate to αKG or the other way around. Mutations in IDH1 or IDH2 will inhibit this process and thereby cause differences in metabolic processes that depend on the Krebs cycle, such as glucose and glutamine metabolism as well as fatty acid production [40].

Mutant IDH1 or IDH2 expressing cells may also have an impaired ability to neutralize reactive oxygen species (ROS). This is because the conversion of alpha-ketoglutarate to D-2HG consumes one molecule of NADPH, which will lead to depletion of NADPH and less formation of GSH (glutathione) which is an important antioxidant. Consequently IDH mutant cells remove free radicals less effectively, which can lead to DNA mutations [26, 41]. The increased ROS in *IDH1* or *IDH2* mutant tumours can also influence the sensitivity to chemo- and radiotherapy. This has already been shown in gliomas and can also be of importance in other tumour types with *IDH1* or *IDH2* mutations [42-45].

IDH1 and *IDH2* mutations in enchondroma and chondrosarcoma

In enchondromas with an *IDH1* or *IDH2* mutation DNA hyper methylation is observed [9] and elevated levels of 2HG were shown in mutated cartilaginous tumours compared to wild type tumours [22].

To assess the influence of the *IDH1* or *IDH2* mutation on the development of enchondroma, mesenchymal stem cells (MSCs) have been used as a model system, because the hypothesis is that enchondromas and chondrosarcomas result from altered differentiation of MSCs during bone development. MSCs are the precursors of bone, cartilage and fat tissue and can be forced to differentiate into these specific lineages in vitro, by adding

stimulating growth factors. Both D2HG addition, as well as the introduction of an *IDH1 R132C* mutation have been found to cause a block in osteogenic differentiation and increased differentiation towards the chondrogenic lineage in human MSCs [46, 47]. Also in zebra fish D2HG impairs the development of vertebrate rings, indicating a block in osteogenic differentiation during skeletogenesis [47]. The introduction of *IDH1 R132C* did not only cause an increase in cartilage differentiation, but also resulted in abnormal cartilage formation [46]. This may have something to do with the PHD enzymes involved in collagen maturation, which are affected by the increased D2HG levels [37].

Recently Hirata et al described a conditionally inducible Col2a1 specific *IDH1 132* knock-in mouse model, which showed multiple enchondroma-like cartilage lesions adjacent to the growth plates of the knee after three months. Also after six months the same lesions were still present, without significant differences in size or amount. Cartilage lesions showed patchy expression of Col10a1 indicating deregulated chondrocyte differentiation. Chondrocytes cultured from the lesions of those mice showed increased expression of hedgehog target genes and genes expressed by hypertrophic chondrocytes [48]. A defect in differentiation as well as a hyper methylation phenotype was also observed in *IDH2 R172* knock in murine 10T1/2 mesenchymal progenitor cells. Moreover, subcutaneous injection of these cells in mice led to the formation of undifferentiated sarcomas in vivo indicating that *IDH2 R172* also has tumour promoting potential [49].

In chondrosarcoma cell lines, and in primary tumours of patients with enchondroma and chondrosarcoma with an *IDH1* or *IDH2* mutation, increased levels of DNA methylation are observed compared to wild type cell lines. This hypermethylation was only found at CpG islands, but not at other regions [49, 50].

Increased levels of D2HG were found in chondrosarcoma cell lines with *IDH1* or *IDH2* mutations compared to wild type cell lines and could be diminished by treatment with a mutant specific IDH1 inhibitor (AGI-5198), however no effect was observed on cell viability, proliferation and migration [50, 51]. Likewise, cells cultured for longer periods in the presence of AGI-5198 did not show any reduction in cell viability [50]. Only after treatment with extremely high concentrations, an effect was observed on colony formation, migration and apoptosis [51].

The minor effects observed after inhibition of IDH1 mutant function and consequently D2HG levels, suggests that mutations in *IDH1* and *IDH2* are

not essential for the proliferation and metastasis capacity of malignant chondrosarcoma cells and that *IDH1* or *IDH2* mutations are no driver mutations anymore once progression towards malignant chondrosarcoma is completed.

COL2A1 mutations

Collagen type II alpha 1 (*COL2A1*) mutations have been identified in 37% [52] and 19.3% of central chondrosarcomas and 31.7% of enchondromas [53]. Type II Collagen is the major extracellular component of cartilage and is important for the strength and flexibility of the tissue. Mutations in the *COL2A1* gene can lead to abnormalities in the formation of collagen. Immunohistochemical analysis revealed that only a small part of chondrosarcomas showed complete loss of the Col2a1 protein, while most others still showed focal staining. Mutations in *COL2A1* are associated with several developmental disorders called type II collagenopathies; however no increased incidence of enchondromas or chondrosarcomas is seen in these disorders. Also in mice expressing a mutant *COL2A1* no increased chondrocyte proliferation was observed. However, a disorganized growth plate and an aberrant structure of the collagen network was present [54]. Down regulation of Col2A1 in cultured mouse chondrocytes resulted in an up regulation of Collagen I. Also Hedgehog signalling was affected by knock down of Col2A1, which resulted in a defect in chondrocyte differentiation [55]. Whether mutations in *COL2A1* are drivers of chondrosarcoma formation is still to be determined, since proof of principle studies have not yet been performed.

Dysregulation of the hedgehog pathway

The Indian hedgehog (IHH) pathway is important for bone development. Chondrocytes located in the growth plate undergo differentiation, resulting in longitudinal growth of the bone. This process is regulated by Indian hedgehog and parathyroid hormone-like hormone (PTHLH) in a negative feedback loop. Chondrocytes are differentiating towards hypertrophic chondrocytes after which they will be replaced by osteoblasts. Pre-hypertrophic chondrocytes produce IHH, stimulating proliferation of chondrocytes and stimulating the cells of the perichondrium to produce PTHLH. PTHLH in its turn inhibits IHH and thereby terminal differentiation of chondrocytes. Constitutive activation of the hedgehog pathway will result

in a failure to induce terminal differentiation of growth plate chondrocytes, which can result in the development of enchondromas [56].

Expression studies showed that the hedgehog pathway members were higher expressed in enchondromas as well as in central chondrosarcomas maintaining tumour cells in a less differentiated, but proliferative state. Levels of expression were variable, but there was no correlation with histological grade [57]. Blocking of the hedgehog pathway with triparanol or IPI-926 resulted in reduced tumour volume in chondrosarcoma xenografts [57, 58], while cyclopamine did not decrease proliferation of chondrosarcoma cell lines [59] suggesting that the tumour microenvironment may play a role in this pathway. Also the potency of the different compounds used in these studies can be different.

Patients with enchondromatosis show mutations in one of the genes involved in the IHH pathway in 8% of the cases [60, 61]. Also mice expressing a mutant parathyroid hormone-like hormone receptor specifically in chondrocytes show a deregulated growth plate and formation of cartilage lesions [61].

Hedgehog signalling is activated by binding of hedgehog ligand to the Patched receptor, releasing the Smoothened receptor and thereby Gli transcription factors are no longer inhibited and can travel to the nucleus to activate transcription. Similar to the mutant PTHLH receptor expressing mice, mice constitutively expressing Gli2 in their chondrocytes develop cartilage lesions [61].

Primary cilia have been shown to be involved in IHH signalling and to regulate its activity [61-63]. Primary cilia are also important for the columnar organization of the growth plate [64]. It has been shown that chondrosarcoma cells express less cilia, compared to normal chondrocytes, which correlated with deregulated hedgehog signalling [65]. Inhibition of the hedgehog pathway in enchondromas and central chondrosarcomas can be a possible therapeutic strategy to treat patients with inoperable or metastatic disease. A phase II clinical trial was conducted investigating the efficacy of GDC-0449 Smoothened inhibitor in 45 patients with progressive advanced chondrosarcoma, however only modest activity was observed, with stable disease in only ten patients with grade I tumours after six months [66].

Recently mutations in IHH genes have been identified in 18% of chondrosarcomas using whole exome sequencing [52]. It would be

interesting to see whether these patients will respond better to treatment with hedgehog pathway inhibitors, making it possible to preselect patients for treatment.

P53 and pRb pathway alterations in the progression from low to high grade chondrosarcoma

Mutations in the *TP53* gene are the most frequently observed mutations in cancer, often associated with a more aggressive phenotype and worse prognosis [67, 68]. Mutations in *TP53* can either lead to loss of function or gain of function [69]. Wild type p53 has important functions in controlling cell proliferation and apoptosis, and loss of function mutations lead to cancer as was proven by a *TP53* knock out mouse model [70]. However, point mutations in *TP53* will lead to pathogenic accumulation of p53 in the nucleus, and mutant *TP53* knock-in mice present with highly invasive tumours [71, 72].

In chondrosarcoma *TP53* mutations are observed in 20% of the cases and associated with more malignant tumours and a higher histological grade. Also increased nuclear p53 expression is correlated with a higher histological grade [52, 73, 74] as well as with a worse survival [75]. Mice constitutively expressing Gli, crossed with p53 knock out mice, developed low grade chondrosarcomas, indicating that deregulation of p53 is important in the progression of enchondromas to chondrosarcoma [76].

The retinoblastoma (pRb) pathway is important in the control of the cell cycle. It is the governor of the restriction point transition (R-Point), and decides whether to progress to S-phase or retreat to G0. Mutations are found in several cancer types; however in chondrosarcoma no mutations in the RB gene have been identified [74]. Also pRb expression was normal in chondrosarcomas [77].

A consistent genomic alteration identified in central chondrosarcomas is the amplification of 12q13 [74, 78]. In this region both *cyclin dependent kinase 4 (CDK4)* and *mouse double minute 2 homolog (MDM2)* are located, which play important roles in the pRb and p53 pathways. CDK4 forms a complex with cyclin D1 and together they control the transition through the G1 restriction point by inactivating pRb. Consistent with the 12q13 amplification observed in a subset of chondrosarcomas, CDK4 and Cyclin D1 expression has been shown to be upregulated in 62-73% of the high grade chondrosarcomas [78, 79]. Furthermore inhibition of CDK4 resulted

in a decrease in cell proliferation in chondrosarcoma cell lines [79]. CDKN2A/p16 is a negative regulator of the CDK4-cyclin D1 complex and is down regulated in 20% of chondrosarcomas, which also correlated with histological grade [80]. Moreover, in 28% of chondrosarcomas mutations in *CDKN2A* are observed [52]. However no loss of *CDKN2A*/p16 was found in enchondromas [80-82]. Decreased cell growth, but not apoptosis was observed in cells were CDKN2A/p16 was re-expressed [79]. MDM2 is a negative regulator of p53, targeting it for proteosomal degradation. MDM2 has been shown to be overexpressed in 34% of high grade chondrosarcomas indicating that p53 is degraded with a higher rate in these tumours [79].

The combined incidence of mutations in either the p53 or pRb pathways in high grade chondrosarcomas is 96% [79]. This suggests that these pathways are important in the progression of chondrosarcoma towards a more malignant histological phenotype.

Apoptosis and pro-survival pathways implicated in chondrosarcoma

A downstream target in the hedgehog pathway is Bcl-2, which is upregulated in the growth plate and inhibits differentiation of proliferating chondrocytes after binding of PTHrP to its receptor. In conventional chondrosarcoma Bcl-2 is highly expressed [83, 84] as well as in rare chondrosarcoma subtypes [85].

By up regulating anti-apoptotic proteins of the Bcl-2 family, chondrosarcoma cells can cause resistance to chemotherapy, which is a major problem in the treatment of these tumours. This has been shown in a cell model where chondrosarcoma cells could be sensitized to chemotherapy when treated in combination with a Bcl-2 family inhibitor (ABT-737) [85, 86].

Another protein highly expressed in chondrosarcoma is Survivin [87-89]. Survivin plays an important role in the cell cycle as well as in the apoptotic pathway. Furthermore it is expressed at very low levels in normal tissue, making it an ideal therapeutic target [90]. In preclinical studies a small molecule Survivin inhibitor (YM155) was shown to decrease cell viability in chondrosarcoma cell lines, which was dependent on *TP53* mutation status [91]. Also down regulation of Survivin using siRNAs showed decreased viability and increased sensitivity towards chemotherapeutics [87].

Several studies have shown the importance of the Akt-Pi3K-mTOR pathway in chondrosarcoma. In 69% of conventional chondrosarcoma and 44% of dedifferentiated chondrosarcoma S6 was phosphorylated indicating an active PI3K/mTOR activity. Also treatment with a dual PI3K/mTOR inhibitor (Bez235) resulted in reduced cell viability in chondrosarcoma cell lines and inhibition of tumour growth in a mouse xenografts model [92]. In a rat chondrosarcoma model treatment with everolimus resulted in inhibition of tumour growth [93].

Peripheral chondrosarcoma

EXT1 and *EXT2* mutations in osteochondroma and peripheral chondrosarcoma

Multiple osteochondromas (MO) is an autosomal dominant condition in which mutations in tumour suppressor genes *Exostosin glycosyltransferase 1* or *Exostosin glycosyltransferase 2* (*EXT1* and *EXT2*) have been identified [94-96]. Patients with this disease develop osteochondromas at the surface of their bones and show heterozygous germline *EXT1* or *EXT2* mutations, with loss of the wildtype allele in the osteochondroma cartilaginous cap at least in a subset of the cases [97]. Also homozygous deletions in *EXT1* have been identified in a subset of sporadic osteochondromas [98]. Both hereditary and sporadic osteochondromas can progress towards peripheral chondrosarcoma (see figure 1). This occurs in ~1% of sporadic and up to 5% of hereditary osteochondromas [99].

Exostosin-1 and exostosin-2 are both type II glycosyltransferases which function in the biosynthesis of heparan sulfate chains of proteoglycans [100]. Proteoglycans are molecules that can regulate the signalling of several growth factors in many different ways. They can act as receptors or coreceptors in signalling pathways, regulate receptor trafficking by endo- and exocytosis, facilitate ligand secretion, function as a signal to other cells, or act by presenting a ligand to a receptor. In addition, they can regulate the structure of the extracellular matrix by providing a signalling gradient to ligands [101]. In the growth plate, heparan sulfate is important for the establishment of a gradient of IHH (as discussed above). When the biosynthesis of heparan sulfate is deregulated, the diffusion of IHH signalling is disturbed. This will have consequences for the polar organization of growth plate chondrocytes and the formation of the bony collar, leading to the formation of an osteochondroma [64].

In a conditional inducible mouse model, where both *Ext1* alleles were inactivated specifically in chondrocytes, the development of osteochondromas was observed, mimicking the human situation. The osteochondromas consisted of a mixture of wild type and mutated cells indicating that cells need normal heparan sulfate synthesis to survive [102]. In another mouse model with a heterozygous loss of both *Ext1* and *Ext2* a decrease in heparan sulfate was observed as well as more exostoses compared to mice with heterozygous loss of *Ext1* or *Ext2* [103]. Zebrafish with homozygous loss of *Ext2* in their chondrocytes show osteochondroma-like lesions [104]. In addition, they show altered mesenchymal stem cell differentiation [105].

Osteochondromas can progress into peripheral chondrosarcomas in a small percentage of cases. It has been shown that the majority of peripheral chondrosarcomas has at least one wild type allele for *EXT1* and *EXT2*, indicating that functional EXT is required for cells to survive and to undergo malignant progression [106]. Most likely additional genetic alterations are needed in order to progress to peripheral chondrosarcoma and they probably take place in the *EXT1* and *EXT2* wild type cells.

p53 and pRb pathway alterations

In peripheral chondrosarcoma pRb and p53 pathway alterations are also found in the majority of the cases, especially in tumours with higher histological grade. A mouse model with conditional inducible *Ext1* inactivation specifically in chondrocytes combined with conditional loss of *Trp53* or Ink4a/Arf showed peripheral chondrosarcoma development from Ext-null and Ext mutated cells, mimicking the human situation. Also loss of primary cilia and polar organization was observed in these lesions. This indicates that an alteration in one of the pathways regulating the cell cycle in addition to the *EXT* mutation is needed for the development of peripheral chondrosarcoma from an osteochondroma [107].

Mesenchymal chondrosarcoma: HEY1-NCOA2 fusion

Mesenchymal chondrosarcoma is characterized by a specific gene fusion between *HEY1* (*Hes-Related Family BHLH Transcription Factor with YRPW Motif 1*) and *NCOA2* (*Nuclear Receptor Coactivator 2*) [108]. *HEY1* is a basic helix-loop-helix transcription factor and downstream of the Notch signalling pathway [109, 110]. In rhabdomyosarcoma inhibition of Hey1 resulted in

downregulation of Notch1 and myogenic differentiation [111]. *NCOA2*, also known as *SRC2* (steroid receptor co-activator 2), is a nuclear hormone receptor co-activator, important for chromatin remodelling [112]. Overexpression of NCOA2 was found in prostate cancer and was correlated with metastatic disease [113]. Rearrangements involving *NCOA2* have been identified in other sarcomas as well [114, 115]. The exact mechanism by which the *HEY1-NCOA2* fusion leads to the formation of mesenchymal chondrosarcoma is currently unknown.

Clear cell chondrosarcoma

Clear cell chondrosarcomas are rare chondrosarcomas subtypes, and limited genetic defects have been identified in these tumours. Array-CGH analysis was performed, identifying hemizygous loss of the *CDKN2A*/p16 locus in four out of twelve clear cell chondrosarcomas. However loss of p16 protein expression has been identified in 95% of cases, indicating that aberrations in the pRb pathway are important for the development and/or progression of this tumour type [116].

Summary/conclusion

Treatment of chondrosarcoma patients is mainly by surgery, since these tumours are resistant to conventional chemo and radiotherapy. Therefore it is important to understand the molecular pathways involved in the development and progression of chondrosarcoma. Chondrosarcomas in the skull or spine present a challenge to remove surgically due to their location. Especially for these patients new therapeutic options are needed. In table 1 the most important genetic alterations and pathways that are deregulated in chondrosarcoma are listed. The most frequently observed subtype is central chondrosarcoma, which is characterized by mutations in *IDH1* or *IDH2* in 38-70 % of the cases (see figure 1). Preclinical research exploring the possibility of specifically inhibiting the mutant IDH1 did not lead to a decrease in cell proliferation, indicating that this will not be a therapeutic target for chondrosarcoma patients. However future studies investigating synthetic lethal approaches might provide alternative options exploiting IDH mutation induced vulnerabilities. Promising targets for therapy include the Bcl-2 family members and mTOR, which are highly active in chondrosarcoma. Currently a phase II clinical trial investigating the effect of

mTOR inhibition in combination with cyclophosphamide has started, of which results will be awaited with great interest.

Targeting the IHH pathway may offer another strategy to treat chondrosarcoma patients, since this pathway is deregulated in chondrosarcoma, although a phase II clinical trial did not show promising results. However, recently mutations in IHH genes have been identified in 18% of chondrosarcomas using whole exome sequencing, and perhaps patients with these mutations will benefit from treatment. In peripheral chondrosarcoma *EXT1* and *EXT2* mutations have been found in peripheral chondrosarcoma, influencing the IHH pathway as well, however in contrast to conventional chondrosarcoma this pathway is not activated in peripheral chondrosarcoma making it not suitable for treatment.

Future research will focus on identification of additional molecular pathways that are involved in the development and progression of chondrosarcoma to find therapeutic targets to treat patients with unresectable chondrosarcoma.

Table 1. Genetic alterations and deregulated pathways found in different subtypes of chondrosarcoma. CS= Chondrosarcoma

	Gene/Pathway	***Subtype***	***Reference***
Genetic alterations			
	IDH1 or *IDH2*	Enchondroma Central CS Dedifferentiated CS Periosteal CS	9,19,22,23
	EXT1 or *EXT2*	Osteochondroma Peripheral CS	98 106
	COL2A1	Enchondroma Central CS	52,53
	IHH pathway members	Central CS Peripheral CS	52
	HEY1-NCOA2 fusion	Mesenchymal CS	108
	TP53	Central CS Peripheral CS	52,73,74
	CDKN2A	Central CS Peripheral CS	52
Pathway deregulation			
	p53 and pRB pathway	Central CS Peripheral CS Clear cell CS	79 107 116

	Bcl-2 family members	Central CS Dedifferentiated CS Mesenchymal CS Clear cell CS	83,84,85
	Survivin	Central CS Dedifferentiated CS Mesenchymal CS Clear cell CS	87,88,89,91
	Pi3K/mTOR	Central CS Peripheral CS Dedifferentiated CS	92

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Chapter 3

Inhibition of Bcl-2 family members sensitizes mesenchymal chondrosarcoma to conventional chemotherapy; report on a novel mesenchymal chondrosarcoma cell line

This chapter is based on the publication:
de Jong Y, van Maldegem AM, Marino-Enriquez A, de Jong D, Suijker J, Briaire-de Bruijn IH, Kruisselbrink AB, Cleton-Jansen AM, Szuhai K, Gelderblom H, Fletcher JA, Bovée JVMG, Inhibition of Bcl-2 family members sensitizes mesenchymal chondrosarcoma to conventional chemotherapy: report on a novel mesenchymal chondrosarcoma cell line. Lab Invest 2016 Oct;96(10):1128-37

Abstract

Mesenchymal chondrosarcomas are rare and highly aggressive sarcomas occurring in bone and soft tissue, with poor overall survival. Bcl-2 expression was previously shown to be up regulated in mesenchymal chondrosarcomas. We here report on a newly derived mesenchymal chondrosarcoma cell line, MCS170, in which we investigated treatment with the BH3 mimetic ABT-737 alone or in combination with conventional chemotherapy as a possible new therapeutic strategy.

The presence of the characteristic *HEY1-NCOA2* fusion was confirmed in the MCS170 cell line using FISH, RT-PCR and sequencing. The MCS170 cell line was treated with ABT-737 alone or in combination with doxorubicin or cisplatin. Cell viability and proliferation was determined using WST-1 viability assays and the xCELLigence system. Expression of Bcl-2 family members was studied using immunohistochemistry. Apoptosis was determined using the caspase-glo 3/7 assay and Western blot for PARP cleavage.

The MCS170 cell line was sensitive to doxorubicin treatment with an IC_{50} of 0.09 μM after 72 hours, but more resistant to cisplatin treatment with an IC_{50} of 4.5 μM after 72 hours. Cells showed little sensitivity towards ABT-737 with an IC_{50} of 1.8 μM after 72 hours. Combination treatments demonstrated ABT-737 synergism with cisplatin as well as doxorubicin as shown by induction of apoptosis and reduction in cell proliferation.

Restoration of the apoptotic machinery by inhibition of Bcl-2 family members sensitizes MCS170 mesenchymal chondrosarcoma cells to conventional chemotherapy. This indicates that combining the inhibition of Bcl-2 family members with conventional chemotherapy can be a possible therapeutic strategy for patients with mesenchymal chondrosarcoma.

Introduction

Chondrosarcomas are cartilage forming tumors accounting for 20% of all the primary bone malignancies [1]. Chondrosarcoma consists of different subtypes with conventional chondrosarcoma being the most common (75%). More rare chondrosarcoma subtypes include dedifferentiated (10%) [2], mesenchymal (3%) [3] and clear cell chondrosarcoma (2%) [4]. Patients with chondrosarcoma are mainly treated by surgical removal of the tumor, because chemo- and radiotherapy are generally ineffective [5].

Mesenchymal chondrosarcoma is an aggressive, high grade chondrosarcoma subtype with a reported 10 year survival rate between 27 and 67% [6-8]. Recently the survival of mesenchymal chondrosarcoma was determined using the Surveillance, Epidemiology, and End Results (SEER) database, showing that in a group of 205 patients the 10 years survival was 43% [9]. Genetically, it is characterized by a specific characteristic gene fusion between the bHLH domain of *HEY1* (Hes-Related Family BHLH Transcription Factor with YRPW Motif 1) and the C-terminal transcriptional activation domains of *NCOA2* (Nuclear Receptor Coactivator 2) [10]. Fusions involving NCOA2 have also been identified in other soft tissue tumor subtypes including spindle cell rhabdomyosarcoma (*SRF-NCOA2* and *TEAD1-NCOA2*) [11] and soft tissue angiofibroma (*AHRR-NCOA2*) [12] as well as in acute myeloid leukemia (*ETV6-NCOA2 [13]* and *MYST3-NCOA2* [14])

Histologically mesenchymal chondrosarcoma is characterized by variable amounts of differentiated cartilage admixed with small round undifferentiated cells [3]. In 1983 Huvos et al. described that patients with mesenchymal chondrosarcoma in which the small cell component predominated responded better to combination chemo- and radiotherapy [8]. In a more recent study, investigating the effect of chemotherapy in 113 mesenchymal chondrosarcoma patients, it was shown that treatment with chemotherapy reduces the risk of recurrence and improves overall survival in patients with localized disease [7]. Also in previous smaller case reports, patients with mesenchymal chondrosarcoma treated with chemotherapy showed a better survival compared to untreated patients [6, 15-18]. However, in a recent systematic review of 18 studies including 107 patients no correlation between anthracycline based chemotherapy treatment and survival was found [19].

Conventional chondrosarcomas are resistant to chemotherapy. Low grade chondrosarcomas consist of a large amount of cartilage matrix and slowly dividing cells, which was for long thought to be the cause of their

insensitivity to chemotherapy. However, high grade chondrosarcomas have less matrix and more rapidly dividing cells, and are also resistant to chemotherapy. Expression of multidrug resistance pumps has been shown on chondrosarcoma cell lines and patient tissues [20-22]; however this does not prevent doxorubicin from accumulating in the cell nuclei, indicating other mechanisms are involved in chondrosarcoma chemoresistance [21].

Our group has previously shown that Bcl-2 family members play a key role in chemoresistance in conventional and dedifferentiated chondrosarcoma [21, 23]. Pro- and anti-apoptotic proteins of the Bcl-2 family play important roles in initiation of the intrinsic routes of apoptosis, changing the balance from anti- to pro-apoptotic, and activating Bak and Bax to facilitate mitochondrial outer membrane permeabilization. Anti-apoptotic proteins, such as Bcl-2, Bcl-xL and Bcl-w, prevent apoptosis by binding to pro-apoptotic proteins, thereby impeding Bak and Bax activation [24]. Treatment of different chondrosarcoma cell lines with an inhibitor of the Bcl-2 family, including Bcl-2, Bcl-xL and Bcl-w (ABT-737) in combination with doxorubicin or cisplatin resulted in a synergistic effect, as was determined by viability and apoptosis assays [21].

Since we previously demonstrated high expression of Bcl-2 and Bcl-xL in a panel of 23 mesenchymal chondrosarcomas [23], we hypothesized that Bcl-2 family members are also important in mesenchymal chondrosarcoma. So far, however, no mesenchymal chondrosarcoma cell lines were available for functional studies. We here report a novel mesenchymal chondrosarcoma cell line, MCS170, carrying the characteristic HEY1-NCOA2 fusion product, in which we functionally evaluated the role of Bcl-2 family members in chemosensitivity.

Material and methods

Compounds

The BH3-mimetic ABT-737 (S1002, Selleckchem, Munich, Germany) is an inhibitor of Bcl-2, Bcl-xL and Bcl-w and was dissolved in DMSO to a stock concentration of 20 mM. Doxorubicin (DXR) and cisplatin (CDDP) were obtained from the in-house hospital pharmacy in a 0.9% NaCl solution in a 1mg/ml concentration. Z-vad-FMK (550379, BD biosciences, San Jose, CA, USA) was used as a general caspase inhibitor and dissolved in DMSO to a stock concentration of 20 mM.

Cell culture

The MCS170 cell line was generated from a recurrent mesenchymal chondrosarcoma of the spine resected from a 33 year-old man at the department of Pathology, Brigham and Women's Hospital and Harvard Medical School located in Boston, USA and was cultured in IMDM medium (Gibco, Invitrogen Life Technologies, Scotland, UK) supplemented with 15% heat inactivated fetal calf serum (Gibco, Invitrogen Life Technologies, Scotland, UK) and 1% Penicillin-streptomycin (100 U/ml) (Gibco, Invitrogen Life Technologies, Scotland, UK). Cells were grown at 37 °C in a humidified incubator with 95% air and 5% CO_2. Cells were confirmed bi-monthly for absence of mycoplasm infection and cell line identity was confirmed before and after experiments by STR profiling using the GenePrint10™ System from Promega (Promega Benelux BV, Leiden, The Netherlands).

Molecular analysis

The presence of the characteristic *HEY1-NCOA2* fusion between exon 4 of *HEY1* and exon 13 of *NCOA2* was confirmed on RNA isolated using TRIzol (Invitrogen, Carlsbad CA, USA) from the MCS170 cell line and a previously published mesenchymal chondrosarcoma patient sample as a positive control according to methods described previously [10]. Products were purified using MinElute 96 UF PCR purification kit (Qiagen, Venlo, the Netherlands) according to the manufacturer's instructions and Sanger sequenced by Macrogen (macrogen.com).

Fluorescent in situ hybridization was performed as described previously [25] using labeled BAC clones RP11-152C15 and RP11-888F10 [10]. MCS170 cells were screened for mutations in 50 frequently mutated cancer related genes using the Ion AmpliSeq™Cancer Hotspot Panel v2 (Life Technologies, Thermo Fisher Scientific, USA, catalog number 4475346) according to the manufacturer's instructions.

Cell viability assay

When cells reached 75-80% confluence they were washed with PBS, trypsinized, and counted using a Burker Turk counting chamber. MCS170 cells were plated in 96 well plates (10.000 cells/well) and allowed to adhere overnight. Cells were incubated with concentrations of ABT-737, doxorubicin or cisplatin ranging between 0.1 μM to 100 μM for 24, 48 and 72h after which a WST-1 assay was performed according to the

manufacturer's instructions (Roche diagnostics GmbH, Penzberg, Germany). Combination assays were performed 72h after simultaneous addition of ABT-737 (0, 125, 500 nM or 1 μM) and doxorubicin (0, 50, 125 nM or 1 μM) or cisplatin (0, 500 nM, 1 or 5 μM). Results of the WST-1 assay were measured at 450 nm using a spectrophotometer (Victor3V, 1420 multilabel counter, Perkin Elmer, Netherlands). All viability assays were performed in triplicate and at least three times.

Cell proliferation measurement

The RTCA xCELLigence system (Roche Applied Sciences, Almere, the Netherlands) was used to measure cell proliferation in real time. MCS170 cells were plated at a density of 15.000 cells/well, 20 hours before addition of either ABT-737 (1 μM), doxorubicin (125 nM or 1 μM) or cisplatin (5 μM) or the combination between ABT-737 with doxorubicin or cisplatin. After 96 hours the measurement was stopped and the results were analyzed using RTCA software. Experiments were performed in duplicate and repeated at least three times.

Immunohistochemistry

Protein expression of Bcl-2 (Dako, 124), Bcl-xL (Cell signaling clone 54H6) and Bcl-w (Abcam 6C1) was determined using immunohistochemistry on paraffin embedded cell pellets of MCS170 cells. Tonsil was used as a positive control for Bcl-2, kidney for Bcl-xl and cerebellum for Bcl-w. Immunohistochemistry was performed according to standard laboratory methods, using citrate (pH 6) as antigen retrieval method, as previously described [26].

Western blot

PARP (clone 46D11, Cell Signaling Technology, Leiden, the Netherlands) cleavage was determined after 24 hours of compound treatment. Cells were treated with 1 μM ABT-737, 125 nM doxorubicin, 5 μM cisplatin or a combination of ABT-737 and doxorubicin or cisplatin. As a loading control α-tubulin (clone DM1A, Cell Signaling Technology, Leiden, the Netherlands) expression was determined. Cells were harvested using hot SDS buffer (1% SDS, 10 mM Tris/EDTA with complete inhibitor and phosSTOP). Of each sample, 20 μg protein was separated by using SDS-PAGE and transferred to PVDF membranes. Blocking was performed using 5% BSA in PBS/ 0.1%

Tween solution. Membranes were incubated with primary antibody overnight at 4 degrees Celsius, followed by HRP labelled secondary antibody for 30 minutes at room temperature. Blots were developed using ECL solution (Pierce™ ECL Western Blotting Substrate, Fisher Scientific, Landsmeer, the Netherlands).

Apoptosis assay

Apoptosis was detected using the caspase-glo 3/7 assay (Promega, Madison, USA) according to the manufacturer's instructions. In brief, 10.000 MCS170 cells were plated into white walled 96 well plates (Corning B.V. Life Sciences, Amsterdam, the Netherlands) and the following day cells were treated with either 1 μM ABT-737, 125 nM or 1 μM doxorubicin, 5 μM cisplatin or a combination of ABT-737 and doxorubicin or cisplatin for 24 hours. Z-vad treatment was used as a control. Thereafter the substrate provided with the kit was added in a 1:1 dilution and incubated for 30 minutes at room temperature. Luminescence was measured using a luminometer (Victor3V, 1420 multilabel counter, Perkin Elmer, Netherlands). In parallel a viability assay was performed by using the method described in the viability assay section.

Statistical analysis

To evaluate synergy between treatment combinations the Bliss independence model was used. In this model the assumption is made that each drug acts independently of the other on the cells [27]. This is used to predict the combined response (C) for the two single compounds (A and B) by the formula: $C = A + B – A * B$. [28].

Results

MCS170 as a model to study mesenchymal chondrosarcoma behavior in vitro

MCS170 cells morphologically show an elongated shape, and they predominantly grow in groups of cells (Figure 1A). Using FISH we confirmed the presence of the *HEY1-NCOA2* intra-chromosomal inversion in all cells similar to the positive control (Figure 1B), colocalization of one set of red and green probes was observed while the other set of red and green probes were at limited distance. Expression of the HEY1-NCOA2 fusion product was confirmed using RT-PCR and Sanger sequencing revealed that exon 4 of

HEY1 was fused to exon 13 of *NCOA2* (Figure 1C). The expression of the HEY1-NCOA2 fusion product was variable between different passage numbers of the cell line. No mutations were detected in any of the 50 oncogenes and tumor suppressor genes, including *TP53*, *IDH1* and *IDH2,* in the Ion AmpliSeq™Cancer Hotspot Panel v2. Cell identity as determined by the Cell ID GenePrint 10 system (Promega) is available on request.

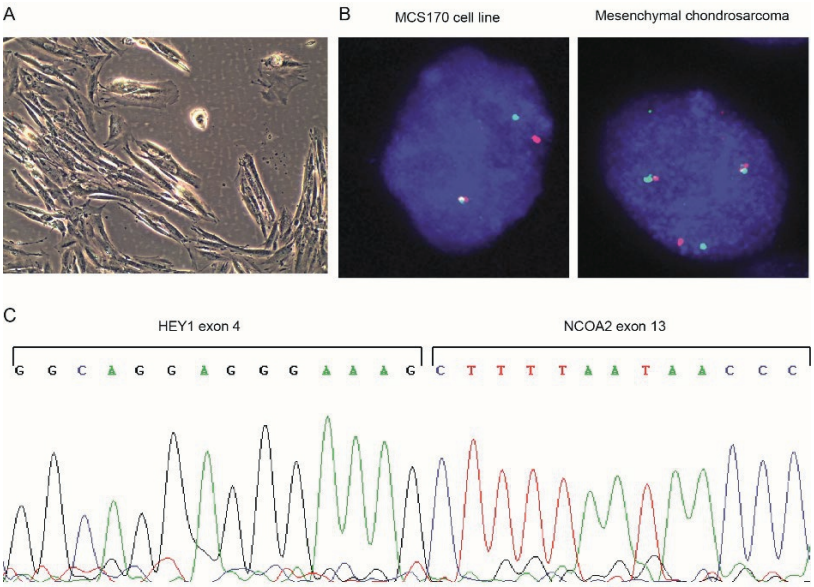


Figure 1: Characteristics of the MCS170 cell line.
A) Morphology of MCS170 cells in culture demonstrated elongated cytoplasm. Cells are growing predominantly in groups. Picture was taken at passage 31 with a 10 times objective. B) Interphase FISH using BAC clones RP11-152C15 and RP11-888F10 on the MCS170 cells and a Mesenchymal chondrosarcoma positive control sample C) Sequence showing the fusion of exon 4 of *HEY1* to exon 13 of *NCOA2* in MCS170 cells.

MCS170 cells are sensitive for doxorubicin, while resistant for cisplatin

To assess chemosensitivity, we treated MCS170 cells with the conventional chemotherapeutics doxorubicin or cisplatin. Cells were sensitive to doxorubicin treatment, especially after longer incubation periods, with an IC_{50} of 4.5 µM after 24h of treatment which decreased to an IC_{50} of 0.08 µM after 72h of exposure (Figure 2A and Table 1). Cells were resistant to cisplatin with an IC_{50} of 60 µM after 24 hours and 4.5 µM after 72 hours (Figure 2B).

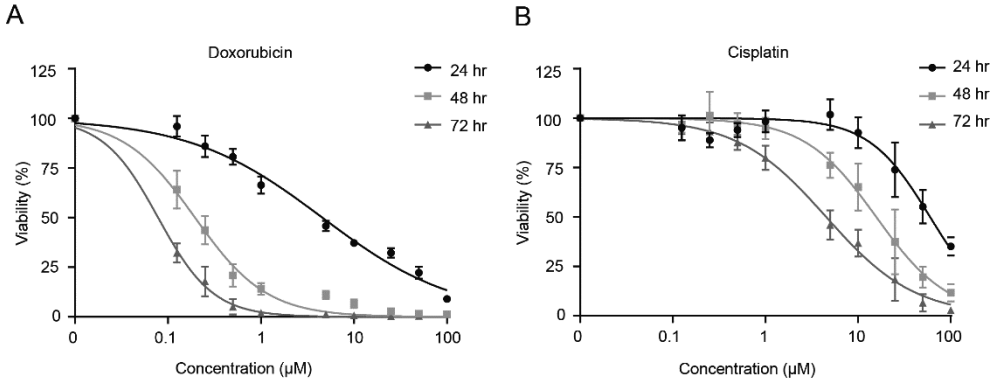


Figure 2: Sensitivity of MCS170 cells to doxorubicin and cisplatin
A, B) Dose response curves of MCS170 cells exposed to doxorubicin, cisplatin and ABT-737 at 24, 48 or 72 hours. Cells are sensitive for doxorubicin, especially when incubated for 72 hours (A), while more resistant for cisplatin (B)

Table 1. IC_{50} values of MCS170 cells treated with doxorubicin, cisplatin and ABT-737 for 24, 48 and 72 hours of exposure.

	IC_{50} 24 h	**IC_{50} 48 h**	**IC_{50} 72 h**
Doxorubicin	4.5 μM	0.2 μM	0.08 μM
Cisplatin	60 μM	15.8 μM	4.5 μM
ABT-737	5.5 μM	2.3 μM	1.8 μM

Bcl-2 family members are not essential for survival of MCS170 cells

Since previous studies indicated that high expression of Bcl-2 and Bcl-xL is a hallmark of mesenchymal chondrosarcoma [23], we determined the protein expression of Bcl-2 family members in the MCS170 cell line. Bcl-2 was expressed in MCS170 (Figure 3A) cells although variable staining intensities were observed between different cells. Bcl-xl was highly expressed in MCS170 cells (Figure 3C), however only very weak Bcl-w expression was observed (Figure 3E). To assess whether these Bcl-2 family members are essential for mesenchymal chondrosarcoma cells, these were inhibited in MCS170 cells using the BH3 mimetic ABT-737. After 72 hours of treatment an IC_{50} of 1.8 μM was observed (Figure 3G), showing that cells are not sensitive for Bcl-2 family member inhibition.

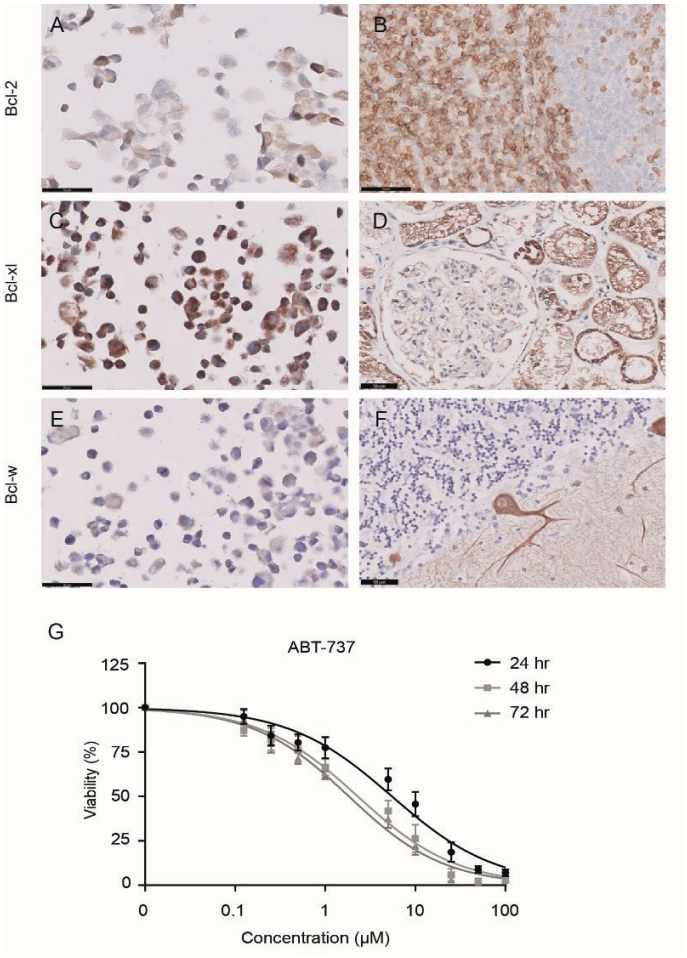


Figure 3: Bcl-2 and Bcl-xl are expressed in mesenchymal chondrosarcoma
A-F) Expression of Bcl-2(A, B) and Bcl-xl (C,D), but only low expression of Bcl-w (E,F) was found in paraffin embedded MCS170 cells (A, C, E), compared to expression in control tissues tonsil (B), kidney (D) and cerebellum (F) G) MCS170 cells show little sensitivity towards ABT-737 treatment after 24, 48 or 72 hours of exposure.

Inhibition of Bcl-2 family members sensitizes towards conventional chemotherapy

To evaluate a functional role for Bcl-2 family members in the response to chemotherapy MCS170 cells were incubated for 72 hours with ABT-737 and either doxorubicin or cisplatin. Compared to single treatment, a reduction in viability was observed when ABT-737 was combined with doxorubicin or cisplatin for 72 hours. (Figure 4A, B). To assess synergy the excess over Bliss

score was calculated. An excess over Bliss score of more than 12% (indicating synergy) was observed when 1 µM ABT-737 was used in combination with 125 nM doxorubicin or when 0.5 or 1 µM ABT-737 was combined with 5 µM cisplatin pointing towards a small synergistic effect (Table 2). Real time measurement of proliferation using the xCELLigence real time cell proliferation assay demonstrated an early significant reduction in cell index when 1 µM ABT-737 was combined with 1 µM doxorubicin, as compared to single treatment. Consistent with the viability assay, combining 1 µM ABT-737 with 125 nM doxorubicin or 5 µM cisplatin resulted in a reduction in cell viability at 72 hours of incubation and persisted until 96 hours (Figure 4C, D).

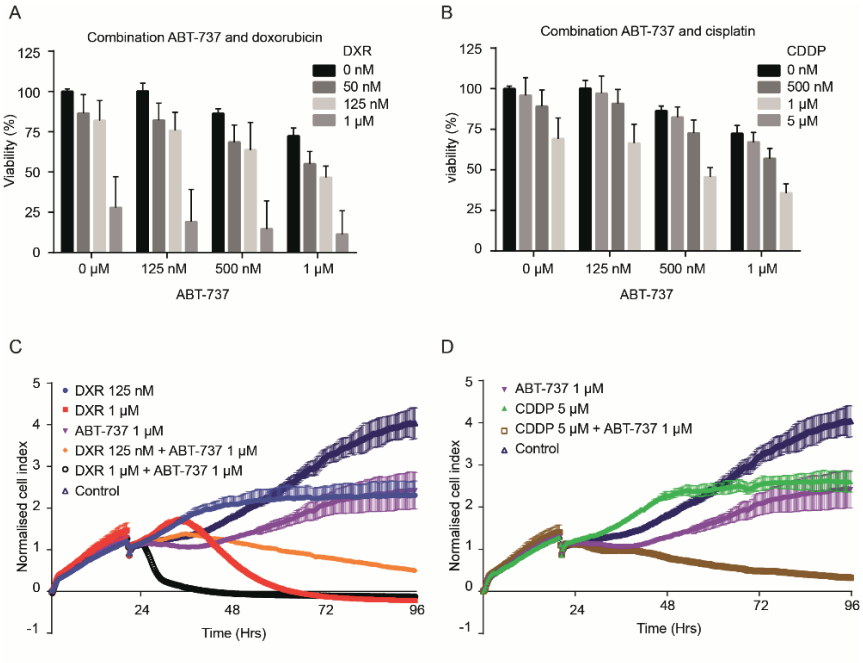


Figure 4: Combination treatment of ABT-737 and doxorubicin or cisplatin in MCS170 cells.
A,B) Viability after treatment of MCS170 cells for 72h with ABT-737 and doxorubicin (A) or ABT-737 and cisplatin (B). Combination treatment shows a larger reduction in viability compared to single treatment after 72 hours. C, D) Real time proliferation assay with the xCELLigence system of MCS170 cells treated with ABT-737 and doxorubicin (C) or ABT-737 and cisplatin (D). The combination of 1 µM ABT-737 and 1 µM doxorubicin is most effective and shows a faster reduction in cell index, compared to single treatment. The combinations of 1 µM ABT-737 and 125 nM or 5 µM cisplatin show a similar reduction in cell index and are more effective compared to single treatments.

Table 2. Excess over Bliss calculations of MCS170 cells treated with combinations of ABT-737, doxorubicin or cisplatin showing highest percentages when 1 μM ABT-737 was used in combination with 125 nM doxorubicin or when 1 or 0.5 μM ABT-737 is combined with 5 μM cisplatin.

Excess over Bliss (%)		**ABT-737**		
		1 μM	0.5 μM	0.125 μM
Doxorubicin	1 μM	8.9	9.4	8.8
	0.125 μM	12.6	7.0	6.3
	0.05 μM	7.5	6.1	4.4
Cisplatin	5 μM	14.4	14.1	2.8
	1 μM	7.6	4.4	-1.5
	0.5 μM	-5.5	-8.9	-12

Combination of ABT-737 with doxorubicin or cisplatin increases apoptosis

To assess if treatment with ABT-737 and combination with chemotherapy resulted in apoptosis, caspase 3/7 activity was measured after 24h of treatment (Figure 5A). Combination treatment of ABT-737 with doxorubicin or cisplatin for 24 hours substantially increased caspase 3/7 dependent apoptosis. This effect was inhibited by the pan caspase inhibitor Z-vad-FMK, confirming that the effect was caspase dependent (Figure 5A). Consistent with results obtained using xCELLigence, combining 1 μM ABT-737 with 1 μM doxorubicin resulted in a large reduction of cell viability, after 24 hours (Figure 5B). However this effect could not completely be restored by adding z-vad, indicating that additional mechanisms that reduce viability, besides caspase dependent apoptosis, may be operable. PARP cleavage was also measured using western blot (Figure 5C), confirming that combination treatment results in activation of apoptosis.

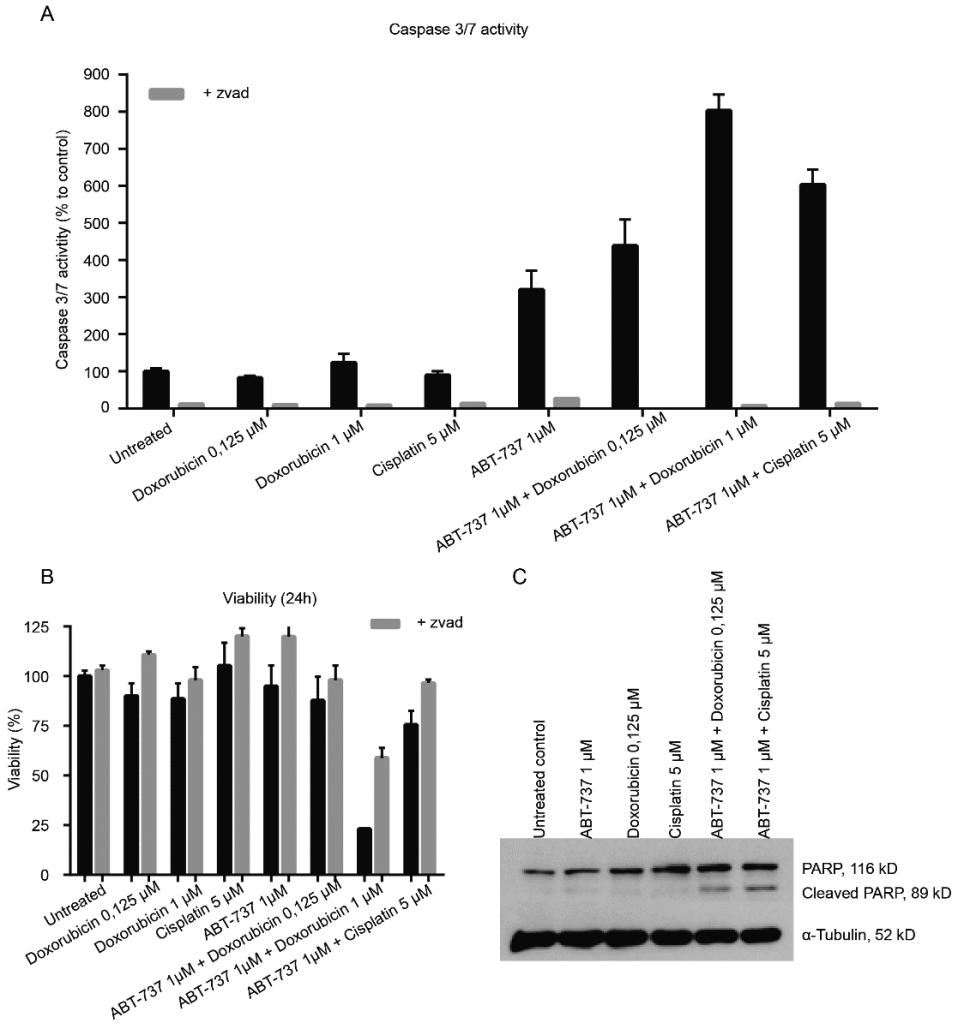


Figure 5: Apoptosis activation after combining ABT-737 and doxorubicin or cisplatin in MCS170 cells.
A, B) Caspase 3/7 activity (A) and viability (B) measured after 24 hours of treatment with doxorubicin, cisplatin or ABT-737 and the combination showing highest caspase 3/7 activity and lowest viability in MCS170 cells treated with 1 µM ABT-737 combined with 1 µM doxorubicin. Pan-caspase inhibitor Z-vad-FMK inhibits all the caspase 3/7 activity and restores part of the viability reduction observed after treatment with 1 µM ABT-737 and 1 µM doxorubicin. C) Western blot image showing PARP cleavage in cells treated with 1 µM ABT-737 and 125 nM doxorubicin or 5 µM cisplatin.

Discussion

We here present the first mesenchymal chondrosarcoma cell line; MCS170, in which we could demonstrate that inhibition of Bcl-2 family members, can restore chemosensitivity. Mesenchymal chondrosarcoma is an aggressive chondrosarcoma subtype, with a poor prognosis. There is an urgent need for alternative treatment strategies, as the current prognosis is very poor with reported 10 years survival rates between 27% and 67% [6-9].

The generation of the MCS170 cell line as a model for mesenchymal chondrosarcoma will give the opportunity to perform more preclinical research to identify possible therapeutic candidates to treat patients with mesenchymal chondrosarcoma, as well as to study the functional role of the HEY1-NCOA2 fusion. The presence of the HEY1-NCOA2 fusion gene was confirmed at the DNA level and at the RNA level. As expected in translocation driven sarcomas, a low mutational burden was found, as no additional mutations were found in 50 oncogenes or tumour suppressor genes frequently mutated in cancer.

Preclinical studies on mesenchymal chondrosarcoma are rare, and only few possible therapeutic options have been described based on expression analysis in small sample groups. A small study investigating a panel of different proteins in three mesenchymal chondrosarcoma cases identified PKC-α, PDGFR, Bcl-2 [29] and mTOR [30] as potential therapeutic targets. In a larger study including 23 cases of mesenchymal chondrosarcoma TGF-β, Bcl-2 and Bcl-xL were identified as possible therapeutic targets in mesenchymal chondrosarcoma [23]. No mutations in hot spot regions of *TP53* were identified in a panel of 33 mesenchymal chondrosarcomas; however 61.3% showed nuclear overexpression of the p53 protein [31].

In this study we focused on evaluating the role of Bcl-2 family members and conventional chemotherapy in mesenchymal chondrosarcoma. The anti-apoptotic proteins Bcl-2 and Bcl-xL were found to be highly expressed in chondrosarcoma, especially in the mesenchymal subtype, suggesting an important role for these proteins in mesenchymal chondrosarcoma chemoresistance and thereby a possible novel treatment strategy [23].

The MCS170 cell line was sensitive for doxorubicin, especially when cells were treated for 72 hours, but resistant for cisplatin. This is in line with a recently published retrospective study including 119 patients, showing that mesenchymal chondrosarcoma patients with localized disease have reduced risk of recurrence when treated with anthracycline based chemotherapy

suggesting that it is beneficial to add adjuvant chemotherapy to the standard treatment regimen [7]. However, a recent analysis of the available literature could not confirm these data [19]. Also the study of Xu et al. did not detect a difference in overall survival when patients with localised disease were compared with patients that showed metastasis.

Bcl-2 and especially Bcl-xL were found to be highly expressed in the MCS170 cell line, which is consistent with the known high expression of these Bcl-2 family members in mesenchymal chondrosarcoma primary tumours [23]. To investigate the role of Bcl-2 family members in mesenchymal chondrosarcoma, the BH3 mimetic ABT-737 was used to inhibit the anti-apoptotic proteins Bcl-2, Bcl-xL and Bcl-w in MCS170. While ABT-737 monotherapy was ineffective, combination of ABT-737 and doxorubicin resulted in a synergistic induction of apoptosis, and reduction in viability, suggesting a role for Bcl-2 family members in resistance to chemotherapy in mesenchymal chondrosarcoma.

Induction of apoptosis is important for the effectiveness of many therapies, which can be prevented by high levels of anti-apoptotic proteins or down regulation of pro-apoptotic proteins. By directly targeting the intrinsic apoptotic pathway using BH3 mimetic ABT-737, more pro-apoptotic proteins will be available to activate Bak and Bax, resulting in higher efficacy of other therapies that induce apoptosis [24].

The combination of Bcl-2 family protein inhibitors and chemotherapeutics has activity in vitro in a wide variety of tumours [32], including conventional as well as dedifferentiated chondrosarcoma [21, 23]. Now that the MCS170 cell line is available, we had the opportunity to show this experimentally in a mesenchymal chondrosarcoma model as well, indicating that patients with mesenchymal chondrosarcoma might also benefit from this treatment combination.

ABT-263 (Navitoclax) is an orally available derivative of ABT-737 with the same working mechanism, which makes it a more attractive compound compared to ABT-737. ABT-263 is currently under evaluation in clinical trials, however due to side effects caused by the high Bcl-xL expression on platelets, only low doses could be administered [33]. Here we demonstrate that treatment with BH3 mimetics can sensitize mesenchymal chondrosarcoma for conventional chemotherapy, while single agent administration is not effective. This raises the possibility to administer these agents in combination, increasing the activity, while lowering toxicity in mesenchymal chondrosarcoma.

Mesenchymal chondrosarcoma is a rare aggressive chondrosarcoma subtype with a poor prognosis. In this study we describe the first mesenchymal chondrosarcoma cell line; MCS170, containing the characteristic *HEY1-NCOA2* fusion, that can be used as a model to identify new therapeutic targets. As mesenchymal chondrosarcoma is known to have high Bcl-2 and Bcl-xl expression [23], we used this cell line to functionally investigate the role of Bcl-2 family members in chemosensitivity. We demonstrate that inhibition of Bcl-2 family members restores the apoptotic machinery in mesenchymal chondrosarcoma, rendering the cells sensitive to conventional chemotherapy, especially doxorubicin.

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Chapter 4

Bcl-xl as the most promising Bcl-2 family member in targeted treatment of chondrosarcoma

This chapter is based on the publication:
de Jong Y, Monderer D, Brandinelli E, Monchanin M, van den Akker BE, van Oosterwijk JG, Blay JY, Dutour A , Bovée JVMG, Bcl-xl as the most promising Bcl-2 family member in targeted treatment of chondrosarcoma. Oncogenesis. 2018 Sep 21;7(9):74

Abstract

Chondrosarcomas are malignant cartilage tumors showing relative resistance to conventional chemo- and radiotherapy. Previous studies showed that chondrosarcoma cells could be sensitized to chemotherapy by inhibiting the Bcl-2 family members Bcl-2, Bcl-xl and Bcl-w using ABT-737. In this study we explored the specific role of Bcl-2 family members to identify the most important player in chondrosarcoma cell survival and chemo resistance. Immunohistochemistry was performed on tissue microarrays containing 137 conventional chondrosarcomas of different grades. Selective inhibition of Bcl-2 (S55746) or Bcl-xl (WEHI-539 or A-1155463) and the combination with doxorubicin or cisplatin was investigated in a panel of 8 chondrosarcoma cell lines using presto blue viability assays and caspase 3/7 glo apoptosis assays. In addition Bcl-2 and Bcl-xl inhibition was investigated in an orthotopic Swarm Rat Chondrosarcoma (SRC) model. Bcl-2 and Bcl-xl were most abundantly expressed in the primary tumors, and expression increased with increasing histological grade. A subset of chondrosarcoma cell lines was sensitive to selective inhibition of Bcl-xl, and synergy was observed with doxorubicin or cisplatin in 3 out of 8 chondrosarcoma cell lines resulting in apoptosis. Conversely, selective inhibition of Bcl-2 was not effective in chondrosarcoma cell lines and could not sensitize to chemotherapy. In vivo, selective inhibition of Bcl-xl, but not Bcl-2 resulted in a decrease in tumor growth rate, even though no sensitization to doxorubicin was observed. These results suggest that among the Bcl-2 family members, Bcl-xl is most important for chondrosarcoma survival. Further research is needed to validate whether single or combination treatment with chemotherapy will be beneficial for chondrosarcoma patients.

Introduction

Chondrosarcomas are malignant primary bone tumors characterized by the production of a hyaline cartilage matrix, with poor vascularization [1]. Histologically chondrosarcomas can be subdivided into conventional, dedifferentiated, mesenchymal, clear cell and periosteal chondrosarcoma. Conventional chondrosarcoma is the most frequent subtype found in 85% of the cases and can either be found in the medulla of the bone (central subtype) or at the surface of the bone (peripheral subtype). The 10-year survival rate for patients with conventional chondrosarcoma depends on the histological grade. Atypical cartilage tumors (previously grade I) show a 10-year survival of 83%, patients with grade II chondrosarcomas show 64% survival and patients with grade III tumors show a 10-year survival rate of 29% [1]. Chondrosarcomas show relative resistance to conventional chemo- and radiotherapy leaving surgery as the only treatment option. As such the 10-year survival rate of chondrosarcoma patients has remained unchanged for the last four decades [2]. Therefore, new treatment options are urgently needed, especially for patients with inoperable or metastatic disease. Apoptosis is a form of programmed cell death, eliminating damaged or unnecessary cells from the body. The intrinsic apoptosis pathway is regulated by B-cell lymphoma-2 (Bcl-2) family of proteins that comprises of anti- and pro-apoptotic and BH3-only proteins. Under stress conditions the BH3-only proteins inhibit the anti-apoptotic proteins Bcl-2, Bcl-xl, Bcl-w and Mcl1 leading to mitochondrial outer membrane permeabilization (MOMP) through Bax and Bak oligomerization, activating the caspase cascade. Increased expression of anti-apoptotic proteins is a widely used strategy by cancer cells to increase the threshold for caspase activation and thereby prevention of apoptotic cell death [3]. Previously we already showed the importance of Bcl-2 family members in chondrosarcoma. Up regulation of Bcl-2 family members is an important mechanism causing chemo resistance, and combined inhibition of Bcl-2, Bcl-xl and Bcl-w with the BH3-mimetic ABT-737 [4] successfully induced sensitization of chondrosarcoma cell lines of all different subtypes to the chemotherapeutic agents doxorubicin and cisplatin [5-7]. Moreover, the anti-apoptotic protein Bcl-2 is up regulated in conventional chondrosarcoma, while expression of Bcl-xl and Bcl-w has not been studied [8]. In dedifferentiated and mesenchymal chondrosarcoma, Bcl-2 and Bcl-xl are highly expressed [7]. Unfortunately inhibition of Bcl-2 family members with the orally available derivative of ABT-737, ABT-263 resulted in a high toxicity rate (most significantly thrombocytopenia) in lymphoid malignancies and solid tumors [3].

Our aim was to further unravel the role of the separate Bcl-2 family members, and to investigate whether selective inhibition of the separate Bcl-2 family members with possible lower toxicity could serve as an alternative to ABT-737. We evaluated the expression of the Bcl-2 family members Bcl-2, Bcl-xl and Bcl-w using immunohistochemistry on tissue microarrays containing 137 conventional chondrosarcomas. Since Bcl-2 and Bcl-xl were most abundantly expressed, we assessed the anti-tumoral effect of selective Bcl-2 inhibition using a novel BH3-mimetic, S55746 [9], which binds to the BH3-binding groove of Bcl-2, and selective Bcl-xl inhibition, using WEHI-539 [10] or its structurally related compound A-1155463 [11], in combination with chemotherapy. Studies were performed in vitro using a panel of chondrosarcoma cell lines of different histological subtypes, and in vivo in the orthotopic Swarm Rat Chondrosarcoma (SRC) model.

Material and methods

Compounds

The selective Bcl-2 inhibitor S55746 was kindly provided and developed by Servier (Suresnes, France). For in vitro experiments S55746 was dissolved in phosphate buffered saline (PBS). ABT-737 (S1002, Selleckchem, Houston, TX, USA), WEHI-539 (ApexBio Technology, Houston, TX, USA) and Z-vad-FMK [22] (550377, BD biosciences, San Jose, CA, USA) were dissolved in DMSO and stored at -20°C. The animal experiments were performed with freshly dissolved S55746 according to the manufacturer's indications at 30 mg/mL in a solution of 40% polyethylene glycol (Sigma-Aldrich, St Louis, MO, USA), 10% ethanol (Sigma-Aldrich) and 50% sterile water for injection (B. Braun, Melsungen, Germany). A-1155463 (S7800, Selleckchem, Houston TX USA) was dissolved in 5% DMSO, 10% ethanol, 20% PEG 35 and 65% sterile water as recommended by the manufacturers. Doxorubicin (2 mg/ml in a 0.9% NaCl solution) and cisplatin (1 mg/ml in a 0.9% NaCl solution) were obtained from the in house hospital pharmacy from the Leiden University Medical Centre for in vitro experiments or Centre Léon Bérard for in vivo experiments.

Cell culture

Chondrosarcoma cell lines JJ012 [23], SW1353 (ATCC), CH2879 [24], L2975 [25] and NDCS1 [26] and control cell lines HeLa (ATCC) and HL-60 (ATCC) were cultured in RPMI-1640 (Gibco, Invitrogen Life-Technologies, Scotland,

UK) containing 10% Fetal Calf Serum (Gibco, Invitrogen Life-Technologies, Scotland, UK). Chondrosarcoma cell lines CH3573 [27], L3252 [25] and L835 [25] were cultured in RPMI-1640 containing 20% Fetal Calf Serum. Mesenchymal chondrosarcoma cell line MCS170 [5] was cultured in IMDM medium (Gibco, Invitrogen Life-Technologies, Scotland, UK) supplemented with 15% Fetal Calf Serum. JJ012 and L835 cell lines are known to contain an *IDH1* mutation, while SW1353 and L2975 show a mutation in *IDH2*. All other cell lines are *IDH1* and *IDH2* wild type [28]. Cell lines were cultured in a humidified incubator (5% CO_2) at 37°C. Before and after completion of the experiments cell identity was confirmed using the Cell ID Gene Print 10 system (Promega Benelux BV, Leiden, The Netherlands).Mycoplasma negativity was confirmed on a regular basis.

Swarm rat chondrosarcoma model

All animal experiments were performed in accordance with European and French regulations and protocols were authorized by the animal ethical evaluation committee C2EA-UCBL55, (protocol number: DR2014-49). All experiments were conducted in the pathogen-free animal facilities SCAR (Faculté de Médecine Rockefeller, Université Claude Bernard Lyon 1, Lyon, France) at the Rockefeller Medicine faculty (Agreement # A 69 388 10 01). The Swarm rat chondrosarcoma model (SRC) is a transplantable in vivo model which has been described previously [29]. It mimics the aggressiveness and chemo-resistance observed in human chondrosarcoma as well as histological features and it is classified as a grade II chondrosarcoma. [30]. Tumor fragments of 10 mm^3 were grafted on the right posterior tibia of one month old Spraque-Dawley rats. Before transplantation rats underwent periosteal abrasion. Palpable tumors were observed after approximately 10 days upon which rats were randomly divided into treatment groups consisting of 5-6 rats in each group. Treatment was given for a period of 10-14 days until tumors reached 2,500 mm^3. Treatment response was evaluated by monitoring tumor growth by regular visual inspection and tumor dimensions were measured every 2–3 days (no blinding was done). Calculation of the tumor volume was performed using the following formula: Volume = (longest tumor diameter × (shortest tumor diameter)^2)/2. Rats were sacrificed when tumors reached 2,500 mm^3 or after completion of the treatment period. Tumors were harvested and fixed in 10% formalin for Immunohistochemical analysis. The following treatments were given twice a week either by IP administration or per oral

gavage (S55746): S55746 (25, 50, 100 or 150 mg/kg), A-1155463 (5 mg/kg;) and / or Doxorubicin (1 mg/kg).

Immunohistochemical analysis

Previously constructed tissue micro arrays (TMAs) containing 137 conventional chondrosarcomas (92 central of which 42 grade I, 36 grade II, 14 grade III and 45 peripheral including 31 grade I, 11 grade II, 3 grade III) [31] were stained for Bcl-2 (Dako, clone 124, Agilent Technologies, Santa Clara, CA, USA), Bcl-xl (Cell signaling, clone 54H6, Danvers MA, USA) and Bcl-w (Abcam, clone 6C1) protein expression. Tonsil was used as a positive control for Bcl-2, prostate for Bcl-xl and cerebellum for Bcl-w. Cytoplasmic staining was scored separately by two observers (JVMGB, YDJ) using a scoring system assessing intensity (0= no, 1=weak, 2=moderate, 3=strong) and percentage of staining (0=no, 1=1-24%, 2=25-49%, 3=50-74%, 4=75-100%) [7] and discrepancies were discussed to reach consensus. *IDH* mutation status was determined for 70 central chondrosarcomas in a previous study [32], and correlation towards Bcl-2, Bcl-xl and Bcl-w immunohistochemistry expression was assessed. The protocol was validated and approved by the medical ethical evaluation committee (protocol number: B17.020). Immunohistochemical analysis of rat chondrosarcomas was performed to determine Bcl-2 and Bcl-xl expression using the following primary antibodies: anti-Bcl-2 (polyclonal rabbit; Bio Vision, Milpitas, USA) and anti-Bcl-xl (clone 54H6, Cell Signaling, Danvers, USA). Goat anti-rabbit antibody (AI-1000; Vector Lab, Burlingame, CA, USA; dilution 1:100) was used as a secondary antibody and detection was performed using avidin-biotin complex and visualization with DAB peroxidase. (VECTASTAIN Elite ABC Reagent, ImmPACT reagent; Vector Lab).

Viability assay

Chondrosarcoma cells were seeded in 96 well plates (3000-20000 cells depending on the cell line) and were treated with increasing concentrations of S55746 (0-1000 nM) or WEHI-547(0-10 uM) for 72 hours. HL-60 cells were used as positive control cells. Combination treatments with doxorubicin or cisplatin where performed with either 0.5 μM of S55746 or 5 or 0.5 μM of WEHI-547. Presto blue reagent (Invitrogen, Life-Technologies, Scotland, UK) was used as described by the manufacturer to measure cell viability. Fluorescence was measured at 590 nm as a read out for cell viability on a victor3V mutilabel reader.(Perkin Elmer, the Netherlands).

Three technical replicates were included in each experiment and assays were repeated 2-3 times.

Caspase assay

The caspase-glo 3/7 assay from promega (Madison, USA) was used to measure apoptosis induction. Chondrosarcoma cell lines CH2879, L835 and L3252 were plated in white walled 96 well plates (Corning B.V. Life Sciences, Amsterdam, the Netherlands). After overnight attachment cells were treated for 24 hours with either WEHI-539, doxorubicin, cisplatin or a combination. To measure caspase 3/7 activity the substrate was added in a 1:1 dilution with medium and incubated for half an hour at room temperature. Cells treated with a combination of ABT-737 and doxorubicin were included as a positive control. As an additional control treatment with pan-caspase inhibitor Z-VAD-FMK was performed. Luminescence was measured with a victor V3 multilabel reader (, Perkin Elmer, Netherlands). Each experiment was performed in triplicate and the assay was repeated three times.

Western blotting

Western blotting was performed for Bcl-xl (Cell signaling, clone 54H6), Bcl-w (Abcam, clone 6C1), Mcl-1, Bak (Cell signaling, clone D4E4) and bax (Cell signaling, clone D2E11). Western blotting for Bcl-2 was performed with two different clones (Cell signaling, clone D55G8 and clone 50E3) of which clone D55G8 was proven to give the most reliable results and was therefore chosen to be used for experiments. Cells were lysed using hot-SDS buffer (1% SDS, 10 mM Tris/EDTA with complete inhibitor and phosSTOP) and 20 µg protein was loaded for each sample. Otherwise the procedure was performed as previously described [33]. α-tubulin (clone DM1A, Sigma-Aldrich Chemie B.V. Zwijndrecht, the Netherlands) expression was determined as a loading control.

Statistical analysis

Results were analyzed using a 2way ANOVA followed by Turkey's multiple comparisons test using GraphPad Prism v6. software (GraphPad Software, Inc., La Jolla, CA, USA). Correlation tests were performed using the Spearman correlation test in graphpad Prism v6. Results are given as mean ± SD and results with $p<0.05$ were considered significant. The Bliss independence model was used to evaluate synergy between treatment

combinations. [34]. Using the formula C = A + B – A * B, in which C represents combined response and A and B the two single compounds, predictions were made to assess synergy [35]. The heatmap figure was generated using the MORPHEUS online tool.

Results

Bcl-2 family members Bcl-2 and Bcl-xl are highly expressed in conventional chondrosarcoma

Protein expression of Bcl-2, Bcl-xl and Bcl-w was determined in a panel of 137 conventional chondrosarcomas. Expression of Bcl-2 was variable and found in 100 out of 110 chondrosarcomas (27 were lost from the TMA) and correlated with histological grade in central as well as peripheral chondrosarcoma (figure 1A). Bcl-2 was significantly higher expressed in grade II (P=0.0022) and grade III (P=0.0002) central chondrosarcoma compared to ACTs. The same correlation with grade was observed in peripheral chondrosarcoma (ACT vs. grade II: P=0.0332, ACT vs. grade III: P=0.0021). Bcl-xl was expressed in 101 out of 102 chondrosarcomas (35 were lost from the TMA) and the extent and intensity significantly correlated with histological grade in central chondrosarcoma (ACT vs. grade II: P=0.0035, ACT vs. grade III: P=0.0034) (figure 1B). Expression of Bcl-w was found in 48 out of 120 chondrosarcomas and was higher in grade III central chondrosarcomas compared to ACTs (P=0.0104) (figure 1C). No statistical differences were observed for Bcl-xl or Bcl-w in the peripheral chondrosarcomas, probably due to the smaller sample size. Examples of different ranges of Bcl-2 family member expression within one tumour are shown in supplementary figure 1A Correlation between Bcl-2 and Bcl-xl was assessed using the Spearman correlation test showing a positive correlation (r=0.5829 P<0.0001) (supplementary figure 1B). The correlation was stronger in high grade chondrosarcomas (grade 2 and grade 3)(r=0.6301 P<0.0001) compared to low grade chondrosarcomas (r=0.3393, P=0.03461). For the central chondrosarcomas, *IDH* mutation status was known for 70 tumors, and there was no correlation between expression of Bcl-2 or Bcl-xl and *IDH1* or *2* mutation status (supplementary figure 1C). Taken together, Bcl-xl shows the highest expression in chondrosarcoma, followed by Bcl-2, suggesting that these two Bcl-2 family members are the most important, and were therefore selected for further functional validation.

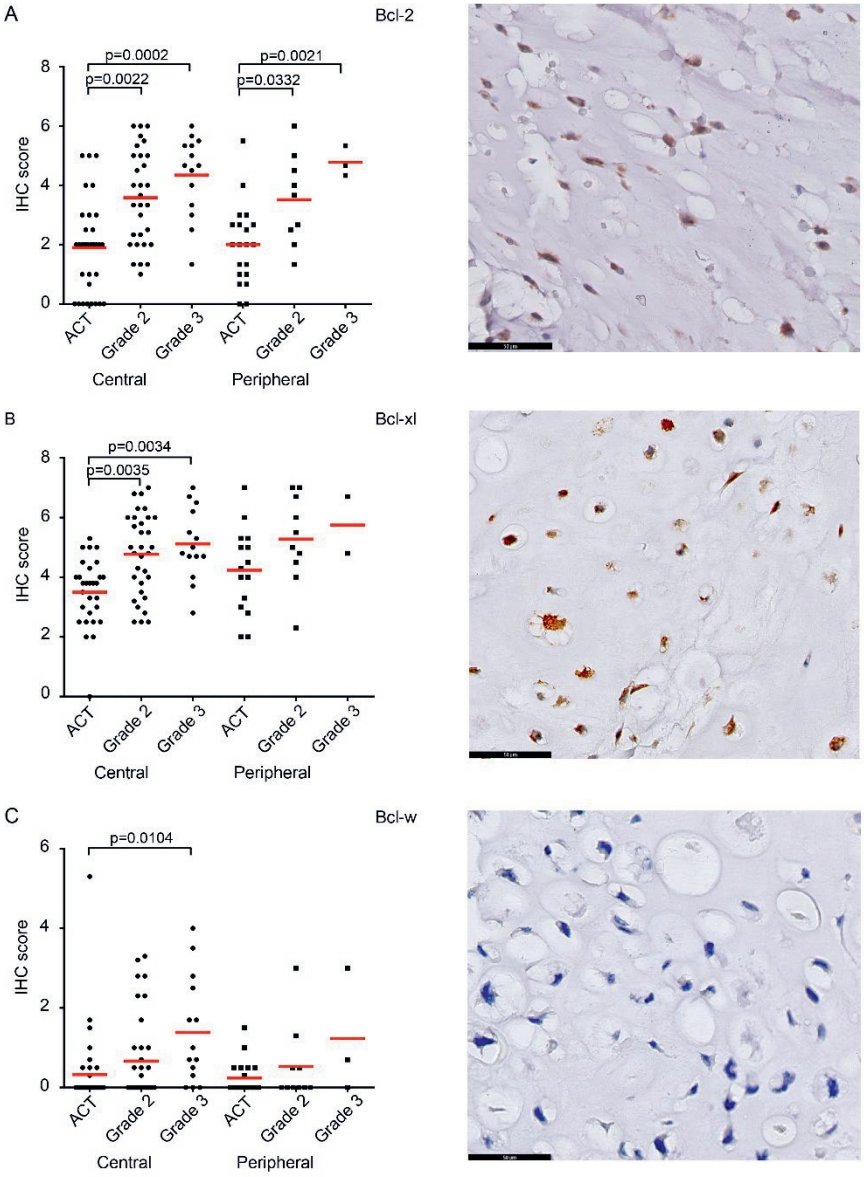


Figure 1. Bcl-2 and Bcl-xl are highly expressed in conventional chondrosarcoma. A, Protein expression of Bcl-2 as detected by immunohistochemistry significantly increased with increasing histological grade, in peripheral as well as in central chondrosarcoma. B, Bcl-xl is highly expressed in conventional chondrosarcoma and is increased in high grade (grade II and III) compared to ACT. C, Expression of Bcl-w is low in conventional chondrosarcoma, but higher expression is observed in grade III chondrosarcoma compared to ACT. Each dot represents one tumor and mean value with standard deviation is shown for each group.

Chondrosarcoma cell lines show minimal sensitivity for selective Bcl-xl inhibition and are not sensitive for selective Bcl-2 inhibition.

Protein expression analysis of apoptotic family members Bcl-2, Bcl-xl, Bcl-w, Mcl-1, Bak and Bax revealed that Bcl-xl was highly expressed in all nine chondrosarcoma cell lines, while Bcl-2 expression was variable (Figure 2A). Bcl-2 was expressed in three conventional and two dedifferentiated chondrosarcoma cell lines and is not correlated to IDH mutation status [12]. Bcl-w expression was variable and was highest in L2975. Bak and Bax are both expressed in all chondrosarcoma cell lines although expression of Bax is variable. Interestingly the same trend is observed as shown with immunohistochemical staining; Bcl-xl is highly expressed in all cell lines. Inhibition of Bcl-xl using WEHI-539 resulted in a reduction in cell viability in a subset of chondrosarcoma cell lines (Figure 2B). The most sensitive chondrosarcoma cell lines were conventional chondrosarcoma cell line CH2879 and dedifferentiated chondrosarcoma cell lines L2975 and NDCS1. In contrast, no sensitivity for Bcl-2 inhibition using S55746 was observed in any of the chondrosarcoma cell lines tested, while the positive control cell line HL-60 showed a dose dependent decrease in viability (figure 2C).

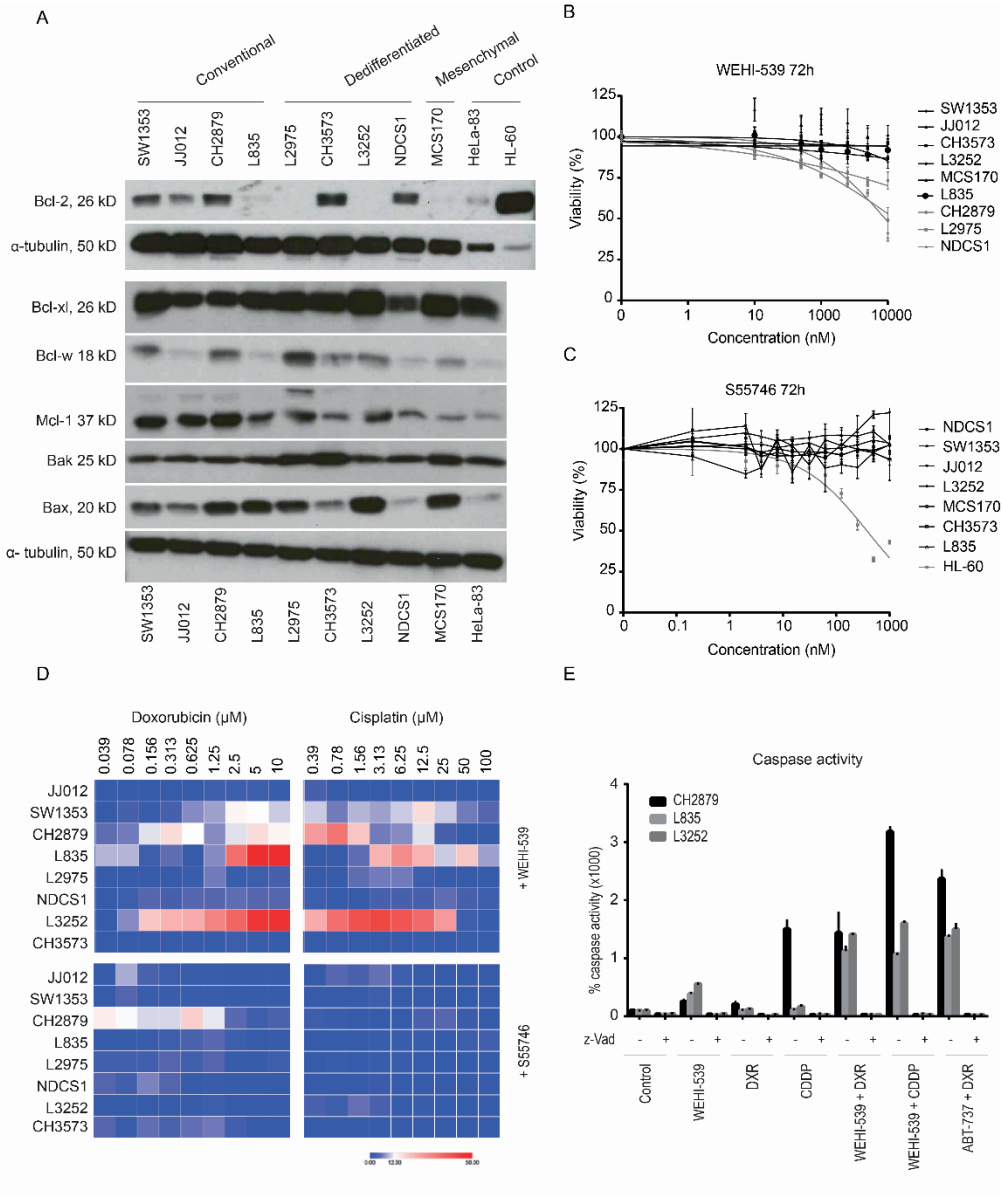


Figure 2. Bcl-xl can sensitize for chemotherapy in a subset of chondrosarcoma cell lines

A, Western blots showing expression of apoptotic regulators Bcl-2, Bcl-xl, Bcl-w, Mcl-1, Bak and Bax in chondrosarcoma cell lines. HeLa-83 and HL-60 cell lines are used as positive controls. Results for Bcl-2 where obtained in a separate experiment compared to the other protein expression analysis. B, Dose response viability curves of chondrosarcoma cell lines after 72h treatment with Bcl-xl inhibitor WEHI-539. Chondrosarcoma cell lines NDCS1, L2975 and CH2879 show minimal sensitivity

towards treatment with Bcl-xl inhibitor WEHI-539. C, Dose response viability curves of chondrosarcoma cell lines after 72h treatment with Bcl-2 inhibitor S55746 show that chondrosarcoma cell lines are not sensitive for S55746, as compared to the positive control HL-60. D, Excess over Bliss percentages of chondrosarcoma cell lines treated with doxorubicin or cisplatin in combination with WEHI-539 or S55746 for 72 hours. Values were obtained by addressing the viability using presto blue assays. Chondrosarcoma cell lines L835 and L3252 show increased sensitivity towards doxorubicin and cisplatin when co-treated with WEHI-539. In addition CH2879 and SW1353 show increased sensitivity towards cisplatin when treated in combination with WEHI-539. A small increase in sensitivity is observed in CH2879 when S55746 is combined with doxorubicin E, Caspase 3/7 activity of CH2879, L835 and L3252 cell lines after treatment with WEHI-539 (0.5 or 5 μM), Doxorubicin (1 μM), Cisplatin (10 μM) or a combination. As a positive control cells have been treated with ABT-737 and doxorubicin. Addition of pan-caspase inhibitor Z-vad showed that measured activity was specific. (DXR=doxorubicin, CDDP=cisplatin).

Selective Bcl-xl but not Bcl-2 inhibition can sensitize for conventional chemotherapy in a subset of chondrosarcoma cell lines.

Inhibition of Bcl-xl with WEHI-539 led to increased sensitivity to doxorubicin or cisplatin treatment in a subset of chondrosarcoma cell lines, while combination treatment with Bcl-2 inhibitor S55746 and chemotherapy did not result in a difference in sensitivity compared to single treatment (Figure 2D., supplementary figure 2 and supplementary figure 3). Combination treatments with chemotherapy were performed with either 1 μM S55746 or 0.5 μM WEHI-539 for the sensitive cell lines (CH2879, L2975 and NDCS1) or 5 μM WEHI-539 for the more resistant cell lines (JJ012, SW1353, L835, L3252 and CH3573). Bliss independence values were calculated and showed that the most chemo resistant cell lines L835 and L3252 could be sensitized for doxorubicin and cisplatin by inhibition of Bcl-xl using WEHI-539. In addition, CH2879 and SW1353 showed an increase in sensitivity towards chemotherapy after Bcl-xl inhibition (Figure 2D and supplementary figure 2). Inhibition of Bcl-2 using S55746 in combination with doxorubicin or cisplatin only showed a small effect in CH2879 cell line, while in all other cell lines no difference was observed (Figure 2D and supplementary figure 3). In addition, an increase in caspase activation was observed when CH2879, L835 or L3252 was treated with WEHI-539 (0.5 μM WEHI-539 CH2879, 5 μM L835 and L3252) and chemotherapy compared to single treatment (Figure 2E). Single treatment with WEHI-539 or doxorubicin or cisplatin resulted in a 200-500% increase in caspase dependent apoptosis compared to the 100% control in all cell lines. The only exception was CH2879 treated with cisplatin which resulted in a 1700% increase. Combination treatment resulted in an increase of 1500-3500% compared to

the control indicating that the sensitizing effect we observe is depending on an increase in caspase dependent apoptosis.

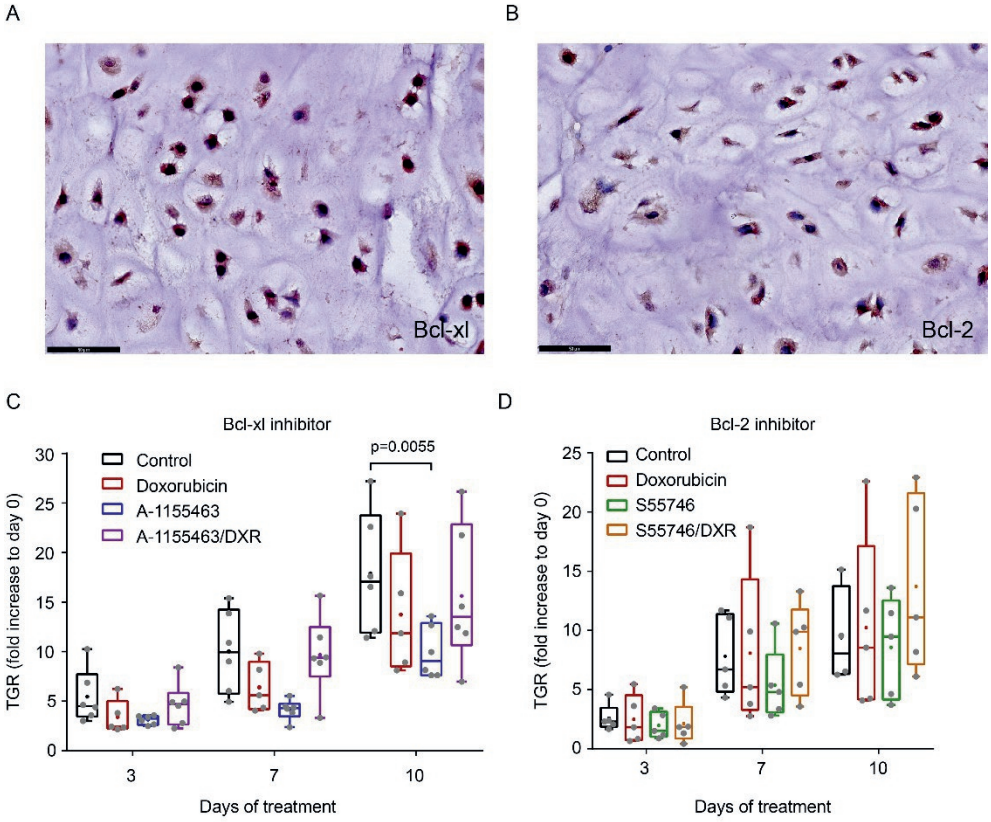


Figure 3. Bcl-xl inhibition is more effective in Swarm rat chondrosarcoma compared to Bcl-2 inhibition.
High protein expression of Bcl-xl (A) and Bcl-2(B) in the SRC tissue determined by immunohistochemistry C, Tumor growth rate (TGR) of tumors orthotopically implanted in rats on day 3, 7 and 10 compared to day 0. Rats were treated with doxorubicin, A1155463 or a combination. Treatment with A1155463 resulted in a significant decrease in TGR compared to control mice D, TGR of tumors orthotopically implanted in rats on day 3, 7 and 10 compared to day 0. Rats were treated with doxorubicin, S55746 or a combination. No statistical difference is observed between the different treatment groups.

Inhibition of Bcl-xl but not Bcl-2 results in a decrease in tumor growth in a Swarm Rat chondrosarcoma model

Expression of Bcl-2 and Bcl-xl was determined in Swarm rat chondrosarcoma to evaluate their suitability as a representative model to study Bcl-2 family member inhibitors. Figure 3A and B show that Bcl-xl as well as Bcl-2 are highly expressed in these tumors as determined by immunohistochemistry, mimicking the human situation. The effect of Bcl-xl inhibition was studied using A-1155463, a structurally related compound to WEHI-539, shown to cause less toxicity in vivo. A concentration of 5 mg/kg was used, since this had shown on target activity before in mice [11]. Inhibition of Bcl-xl showed a significant (P=0.0055) decrease in tumor growth compared to control or doxorubicin treated rats (figure 3C), however no difference was observed when A-115463 was combined with doxorubicin, indicating that single Bcl-xl inhibition is more effective. Since Bcl-2 inhibitor S55746 was never tested in rats before a dose escalation study was performed (25, 50, 100 and 150 mg/kg). A biweekly administration of 50 mg/kg S55746 resulted in the smallest increase in tumor volume (1218 ± 403mm^3 in treated group versus 2033 ± 647 mm^3 in the PBS control group at day 14) and was selected for further experiments (supplementary figure 4). Inhibition of Bcl-2 did not lead to a significant reduction in tumor growth as a single agent nor in combination with doxorubicin. (Figure 3D).

Discussion

Bcl-2 family members have been investigated as therapeutic targets in a large variety of solid tumors as well as in chondrosarcoma. Previously we showed that chondrosarcoma cell lines of all different subtypes can be sensitized to chemotherapy by inhibiting Bcl-2 family members using ABT-737 [5-7]. In this study we investigated whether single Bcl-2 or Bcl-xl inhibition could be an alternative therapeutic strategy for patients with chondrosarcoma, since inhibition of Bcl-2, Bcl-xl and Bcl-w using ABT-263, showed toxicity problems when used in the clinic for other malignancies [3]. Bcl-2 and especially Bcl-xl were highly expressed in chondrosarcoma tissue samples, which correlated with an increased histological grade. Bcl-w was also expressed but not as abundantly, and also correlated with histological grade. In addition a positive correlation between Bcl-2 and Bcl-xl expression was observed, especially in high grade chondrosarcomas. These results are in line with previously published smaller studies, conducted by us as well as others, in which Bcl-2 and Bcl-xl also showed an increased expression with increasing histological grade [8, 13-15]. Selective Bcl-xl inhibition with WEHI-539 was effective at relatively high doses in a subset of chondrosarcoma cell lines, which did not correlate with expression of Bcl-2 or Bcl-xl. These doses were comparable with concentrations obtained in osteosarcoma cell lines [16]. Our in vivo data confirm that tumor growth can be slowed down by selective inhibition of Bcl-xl by A-1155463, a structurally related Bcl-xl inhibitor showing less toxicity *in vivo* [11]. When the combination with chemotherapy was investigated the two cell lines that were most resistant to doxorubicin or cisplatin (L835 and L3252) could be sensitized for these agents when combined with WEHI-539. In addition, we confirmed that the combination treatment induced apoptosis. Interestingly those two cell lines did not show Bcl-2 and low Bcl-w expression. In the SRC model, the combination of selective Bcl-xl inhibition and doxorubicin did not show a sensitizing effect, and thus did not confirm the advantage of combination treatment suggested by the in vitro data. However, while the cell lines show a heterogeneous response with respect to chemo-sensitization, with a preference for chemo-resistant cell lines with high Bcl-xl and low Bcl-2 and Bcl-w expression, the SRC model represents only a subset of the chondrosarcomas.

Although half of the chondrosarcoma cell lines showed Bcl-2 expression, none of the cell lines was sensitive for selective Bcl-2 inhibition with S55746 and only one cell line (CH2879) showed a small increase in sensitivity for doxorubicin when treated in combination with the Bcl-2 inhibitor. In line

with this, in the orthotopic Swarm rat chondrosarcoma model, S55746 also showed no beneficial effect regarding inhibition of tumor growth. These data indicate that Bcl-2 is not essential for the survival of chondrosarcoma cells, and its function might be taken over by other apoptosis proteins. Bcl-xl is essential only in a subset of chondrosarcoma cell lines, and also seems important in the rat chondrosarcoma model. This means that the previously observed effects of combined inhibition of Bcl-2, Bcl-xl and Bcl-w using ABT-737 cannot be substituted by selective inhibition of Bcl-2 alone, not even in the cell lines that highly express Bcl-2. This can be caused by the high Bcl-xl expression observed in all chondrosarcoma cell lines, which has been reported previously as a possible cause of resistance to selective Bcl-2 inhibition [17, 18]. In the majority of the high grade chondrosarcomas high Bcl-2 and Bcl-xl expression is observed, which might suggest that selective Bcl-2 inhibition could be problematic due to high Bcl-xl expression or vice versa. However, high Bcl-2 expression does not confer resistance towards Bcl-xl inhibition as shown in a study by Punnoose et al. in multiple myeloma pointing towards Bcl-xl as a more promising target in tumours expressing both Bcl-2 and Bcl-xl [18]. No difference in sensitivity for Bcl-2 or Bcl-xl inhibition was observed between *IDH1* or *-2* mutant cell lines and wild type cells. This is different from acute myeloid leukaemia in which Bcl-2 inhibition was identified as synthetic lethal to elevated levels of the oncometabolite D-2HG caused by mutations in *IDH1* or *IDH2*. The authors propose a model in which D-2HG inhibits the activity of cytochrome c oxidase (COX) in the mitochondrial electron transport chain, which lowers the threshold to trigger apoptosis after Bcl-2 inhibition [12]. In addition, IDH1 mutant gliomas were more sensitive for Bcl-xl inhibition, which was shown to be dependent on D-2HG, which lowers Mcl-1 expression in mutant gliomas compared to wild type gliomas thereby increasing sensitivity for Bcl-xl inhibition [19]. The different results in these different tumour types sharing a mutation in *IDH* emphasize that these mutations may have a tissue specific effect which hampers the development of a common therapy for *IDH* mutant tumours.

The importance of Bcl-xl over Bcl-2 regarding chemo resistance is in line with results found in several other solid tumours. One study testing ABT-263, Bcl-2 and Bcl-xl selective inhibitors in a panel of breast cancer, non-small lung cancer and ovarian cancer cell lines, showed that only ABT-263 and the Bcl-xl selective inhibitors where able to induce sensitization to docetaxel, indicating that Bcl-xl is the most important protein causing chemo resistance [20]. Also in osteosarcoma cell lines a selective Bcl-2 inhibitor could not sensitize for doxorubicin, whereas WEHI-539 treatment

resulted in a synergistic effect [16]. A correlation between Bcl-xl and chemo resistance has been shown before in a study in which they found a strong correlation between Bcl-xl mRNA expression and the sensitivity of 60 cell lines towards 122 standard chemotherapy agents [21]. No clinical trials have been performed using selective Bcl-xl inhibitors, however the Bcl-xl inhibitor A-1155463 that we tested in vivo and its orally available related compound A-1331852 seem promising in pre-clinical studies in rodents [11, 20]. Furthermore a study by Leverson et al shows that in rodents higher concentrations of Bcl-xl inhibitors in combination with docetaxel can be administered before thrombocytopenia becomes dose limiting, in comparison with combination therapy using ABT-263, in which, because of Bcl-2 inhibition, suppression of granulopoiesis and neutropenia becomes dose limiting at much lower concentrations [20]. Although a clear biomarker to predict sensitivity still needs to be identified, we show that among the Bcl-2 family members, Bcl-xl is most important for chondrosarcoma survival. Further research is needed to validate whether single or combination treatment with chemotherapy will be beneficial for chondrosarcoma patients.

Acknowledgements

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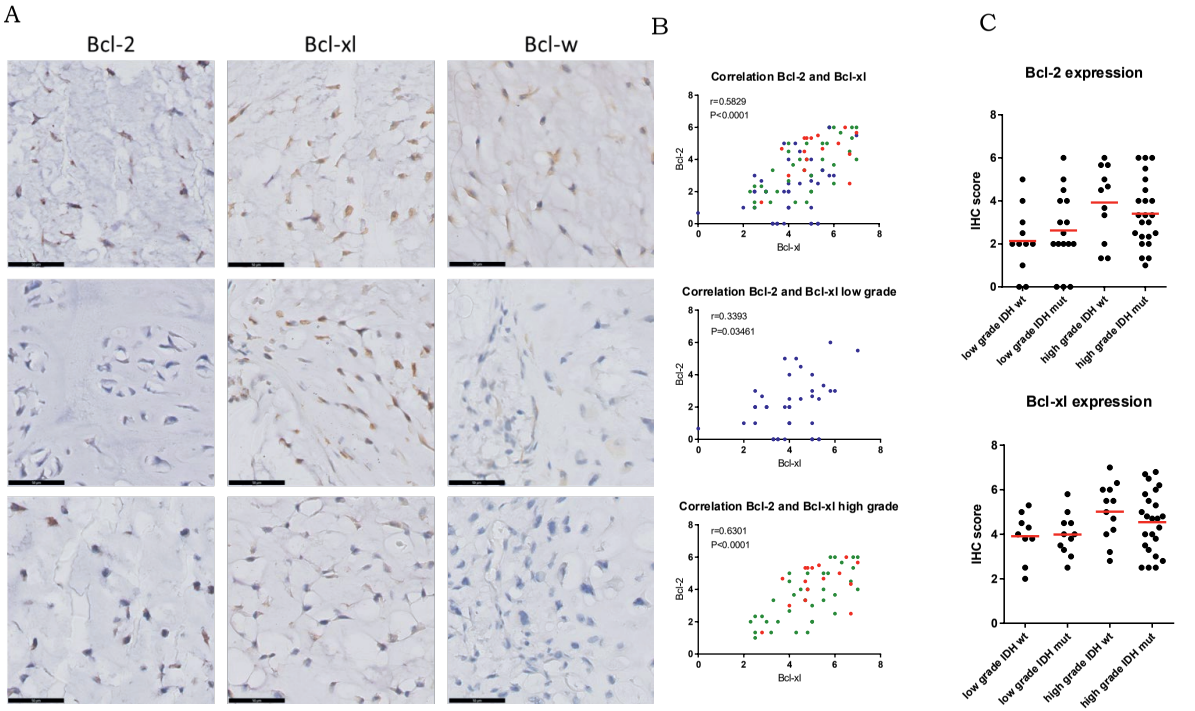
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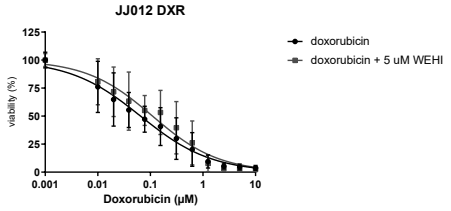
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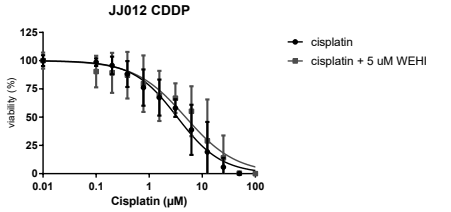
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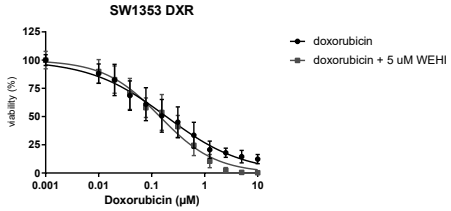
Supplementary materials

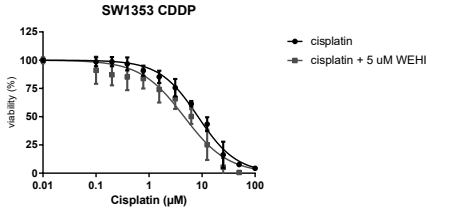


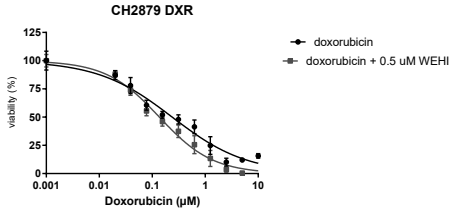
Supplementary figure 1. A. Immunohistochemical staining results of three different cases of high grade chondrosarcoma tumor samples showing different staining patterns for Bcl-2, Bcl-xl and Bcl-w. B. A positive correlation is observed between Bcl-2 and Bcl-xl expression in chondrosarcoma patient tissue samples (r=0.5829, P<0.0001). Blue = Grade I , Green = Grade II, Red = Grade III. Higher grade chondrosarcomas show a stronger correlation (r=0.6301, P<0.0001) compared to low grade chondrosarcomas (r=0.03393, P=0.03461) Correlation is determined using the spearman correlation test. C. Bcl-2 and Bcl-xl expression are not correlated towards *IDH* mutation status

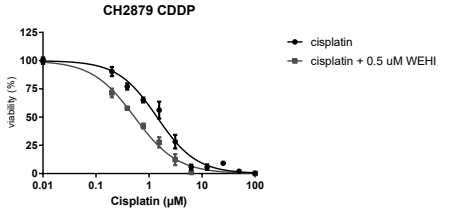

JJ012 DXR
viability (%)
125
100
75
50
25
0
0.001
0.01
0.1
1
10
Doxorubicin (μM)
doxorubicin
doxorubicin + 5 uM WEHI

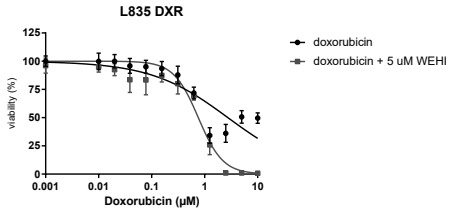

JJ012 CDDP
viability (%)
125
100
75
50
25
0
0.01
0.1
1
10
100
Cisplatin (μM)
cisplatin
cisplatin + 5 uM WEHI

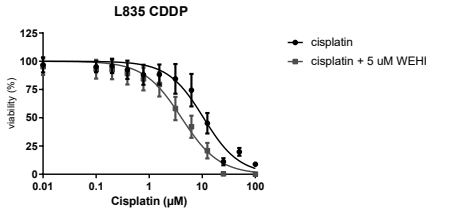

SW1353 DXR
viability (%)
125
100
75
50
25
0
0.001
0.01
0.1
1
10
Doxorubicin (μM)
doxorubicin
doxorubicin + 5 uM WEHI

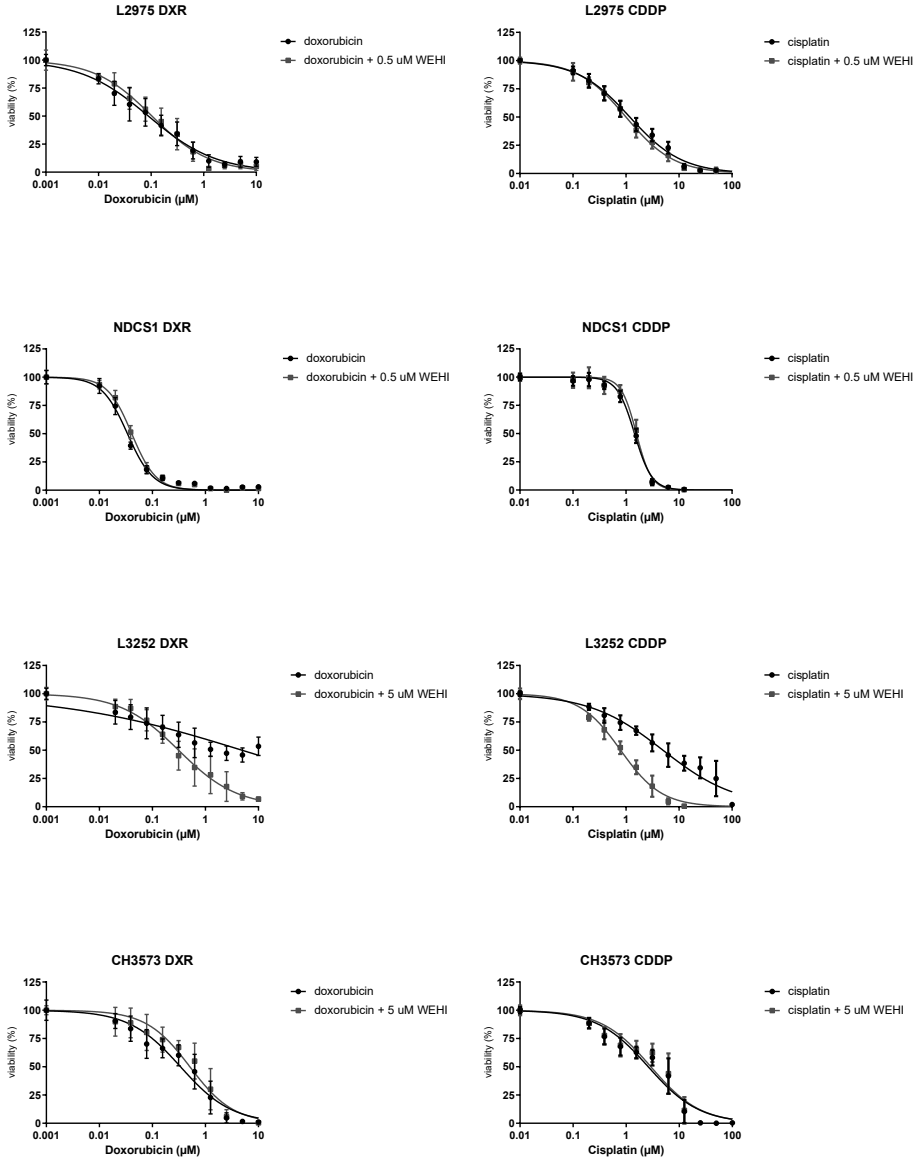

SW1353 CDDP
viability (%)
125
100
75
50
25
0
0.01
0.1
1
10
100
Cisplatin (μM)
cisplatin
cisplatin + 5 uM WEHI


CH2879 DXR
viability (%)
125
100
75
50
25
0
0.001
0.01
0.1
1
10
Doxorubicin (μM)
doxorubicin
doxorubicin + 0.5 uM WEHI

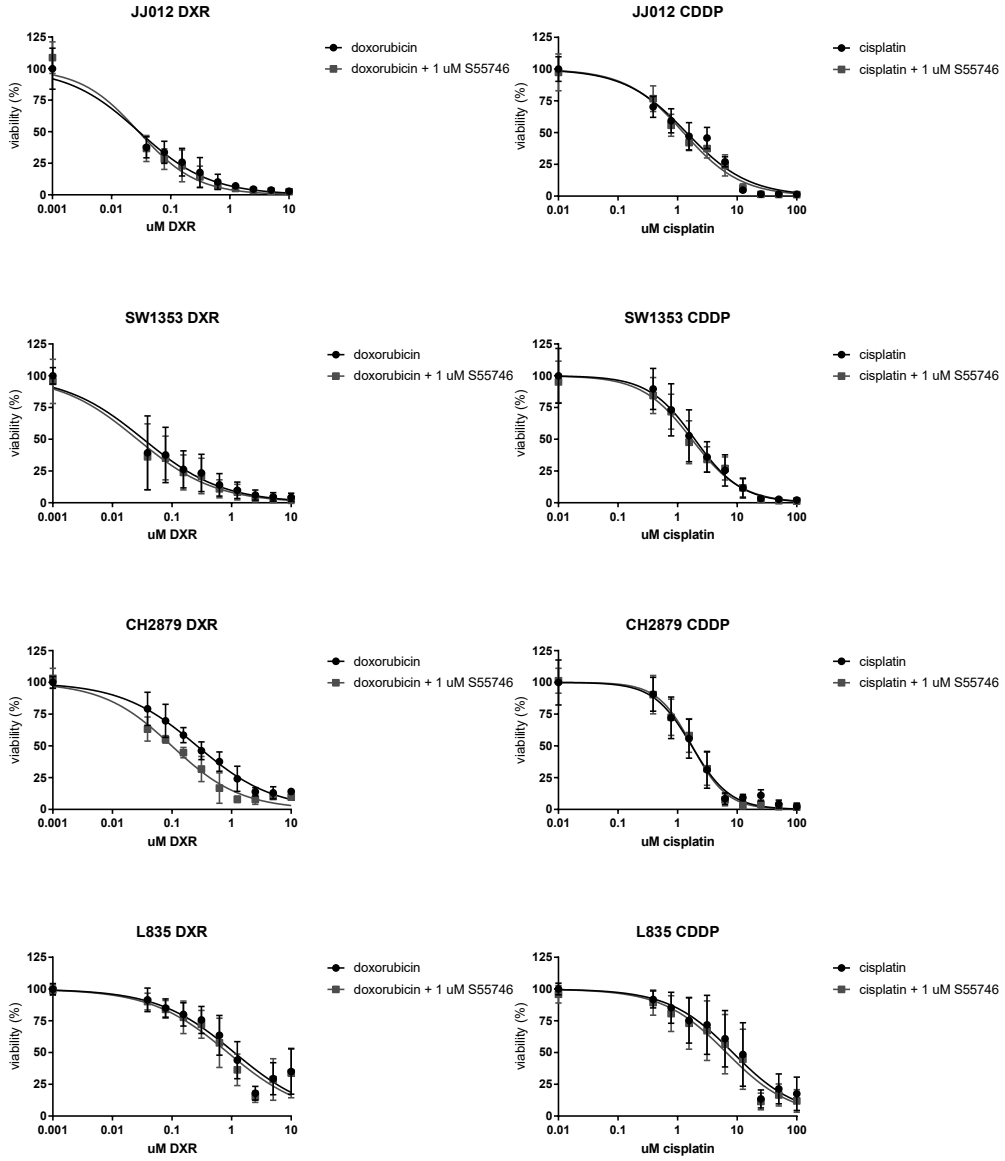

CH2879 CDDP
viability (%)
125
100
75
50
25
0
0.01
0.1
1
10
100
Cisplatin (μM)
cisplatin
cisplatin + 0.5 uM WEHI

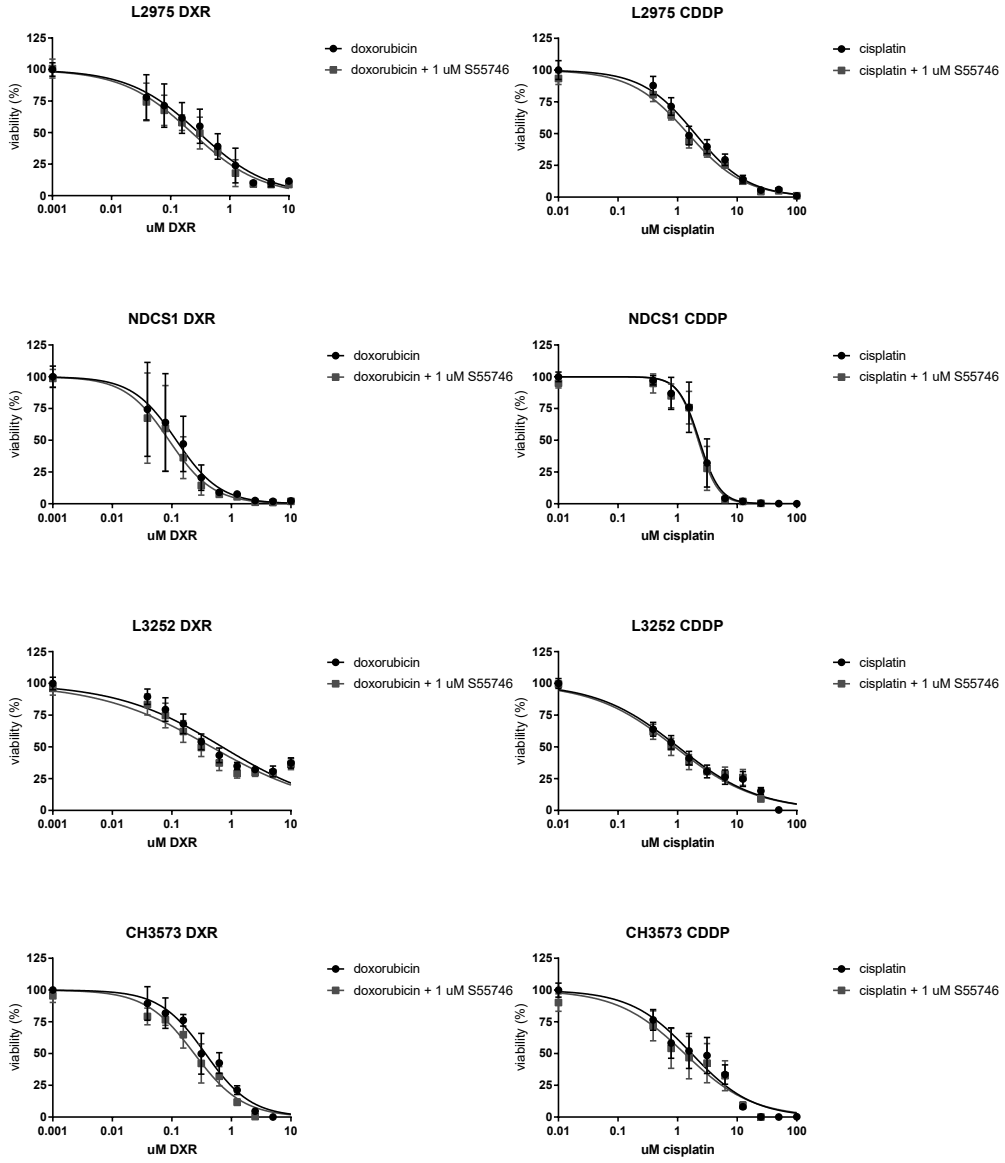

L835 DXR
viability (%)
125
100
75
50
25
0
0.001
0.01
0.1
1
10
Doxorubicin (μM)
doxorubicin
doxorubicin + 5 uM WEHI


L835 CDDP
viability (%)
125
100
75
50
25
0
0.01
0.1
1
10
100
Cisplatin (μM)
cisplatin
cisplatin + 5 uM WEHI

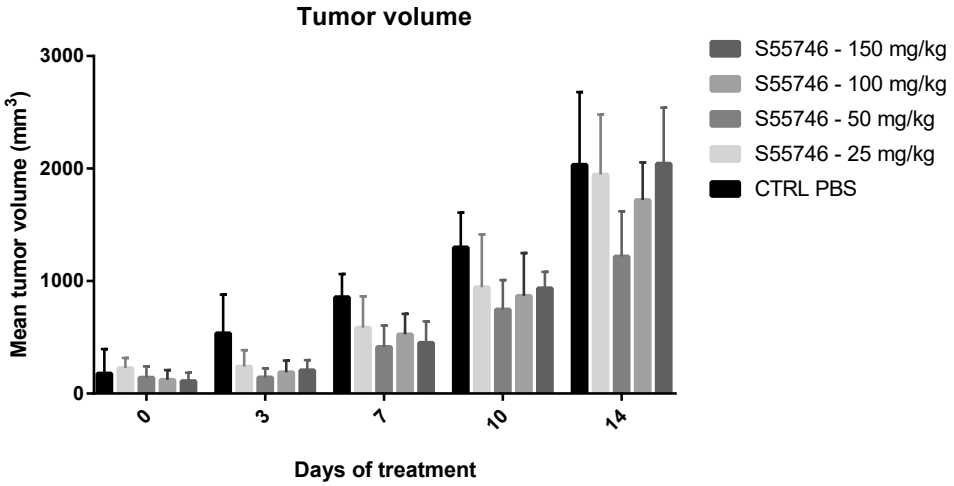


Supplementary figure 2. Inhibition of Bcl-xl with WEHI-539 results in an increased sensitivity for doxorubicin (DXR) and cisplatin (CDDP) in L835 and L3252 cell lines. In addition SW1353 and CH2879 show a small increase in sensitivity for cisplatin.


JJ012 DXR
viability (%)
uM DXR
doxorubicin
doxorubicin + 1 uM S55746
JJ012 CDDP
uM cisplatin
cisplatin
cisplatin + 1 uM S55746
SW1353 DXR
SW1353 CDDP
CH2879 DXR
CH2879 CDDP
L835 DXR
L835 CDDP



Supplementary figure 3. Inhibition of Bcl-2 with S55746 does not result in an increased sensitivity for doxorubicin (DXR) or cisplatin (CDDP) in all chondrosarcoma cell lines. Only CH2879 shows a small increase in sensitivity for doxorubicin.



Supplementary figure 4. Dose escalation study of S55476 in Swarm Rat chondrosarcoma model. (n=3-4 Rats/group).

Chapter 5

Targeting survivin as a potential new treatment for chondrosarcoma of bone

This chapter is based on the publication:
de Jong Y, van Oosterwijk JG, Kruisselbrink AB, Briaire-de Bruijn IH, Agrogiannis G, Baranski Z, Cleven AH, Cleton-Jansen AM, van de Water B, Danen EH, Bovée JVMG, Targeting survivin as a potential new treatment for chondrosarcoma of bone. Oncogenesis 2016 May 9;5:e222

Abstract

Chondrosarcomas are malignant cartilage-forming bone tumours, which are intrinsically resistant to chemo- and radiotherapy, leaving surgical removal as the only curative treatment option. Therefore our aim was to identify genes involved in chondrosarcoma cell survival that could serve as a target for therapy.

siRNA screening for 51 apoptosis related genes in JJ012 chondrosarcoma cells identified *BIRC5*, encoding survivin, as essential for chondrosarcoma survival. Using immunohistochemistry, nuclear as well as cytoplasmic survivin expression was analyzed in 207 chondrosarcomas of different subtypes. Nuclear survivin has been implicated in cell cycle regulation while cytoplasmic localization is important for its anti-apoptotic function. RT-PCR was performed to determine expression of the most common survivin isoforms. Sensitivity to YM155, a survivin inhibitor currently in phase I/II clinical trial for other tumours, was examined in ten chondrosarcoma cell lines using viability assay, apoptosis assay and cell cycle analysis.

Survivin expression was found in all chondrosarcoma patient samples. Higher expression of nuclear and cytoplasmic survivin was observed with increasing histological grade in central chondrosarcomas. Inhibition of survivin using YM155 showed that especially *TP53* mutant cell lines were sensitive, but no caspase 3/7 or PARP cleavage was observed. Rather, YM155 treatment resulted in a block in S phase in two out of three chondrosarcoma cell lines indicating that survivin is more involved in cell cycle regulation than apoptosis.

Thus, survivin is important for chondrosarcoma survival and chondrosarcoma patients might benefit from survivin inhibition using YM155, for which *TP53* mutational status can serve as a predictive biomarker.

Introduction

Chondrosarcoma is a malignant cartilage forming tumour accounting for 20% of all malignant bone tumours [1]. Chondrosarcomas represent a heterogeneous group as different histological subtypes are recognized, including conventional, dedifferentiated, mesenchymal, clear cell and periosteal chondrosarcoma. The conventional subtype is most frequent (85%) [1] and can be further categorized in central chondrosarcoma (>85%)(in medullar cavity) and peripheral chondrosarcoma (at the bone surface) [2]. Histologically, atypical cartilaginous tumours (previously referred to as grade I chondrosarcomas), show low cellularity and are locally aggressive, but do not metastasize. High-grade chondrosarcomas comprise grade II and III chondrosarcomas and show higher cellularity, mitoses and less cartilaginous matrix. Histological grading represents the most important prognostic factor; patients with atypical cartilaginous tumours show a 10 year overall survival rate of 83%, patients with grade II tumours show 64% survival and patients with grade III chondrosarcomas show 29% 10 year survival rate [1, 2]. Dedifferentiated chondrosarcomas comprise 10% of all chondrosarcomas and are characterized by a high grade dedifferentiated component juxtaposed to a low grade cartilaginous component. Patients with dedifferentiated chondrosarcoma show a 5 year overall survival between 7 and 24% [3]. Mesenchymal chondrosarcoma is a rare (<3%) high grade chondrosarcoma subtype with reported 10 year survival rates between 27 and 67% [4, 5]. Histologically it consists of differentiated cartilage mixed with undifferentiated small round cells [6].

Chondrosarcomas are intrinsically resistant to conventional chemo- and radiotherapy, and therefore surgical removal of the tumour is the only curative treatment option. Several studies have been performed investigating possible new therapeutic targets for the treatment of chondrosarcoma. This has led to several discoveries including mTOR [7, 8], Src [9, 10] and Bcl-2 family members [11, 12] as possible targets but still no targeted therapies are available for chondrosarcoma patients. Therefore, there is still an urgent need for novel therapeutic targets that can be easily and rapidly applied in the treatment of patients with high-grade metastatic or inoperable chondrosarcoma.

To identify new cancer drug targets, high throughput RNA interference (RNAi) screens are widely used and have led to several important findings regarding new cancer gene discoveries [13]. Loss of function RNAi screens have led to the identification of important oncogenes in several different

cancer types, such as the identification of Brd4 as a therapeutic target in acute myeloid leukaemia [14] and MED12 as a determinant of drug response to tyrosine kinase inhibitors in non-small cell lung cancer [15]. Since RNAi is a powerful tool to discover survival related genes in a specific manner we performed a siRNA screen targeting 51 genes involved in apoptosis regulation to identify genes involved in survival of chondrosarcoma cells, that could serve as a potential target for therapy for patients with inoperable or metastatic chondrosarcoma. To validate our results we used a unique panel of chondrosarcoma cell lines including conventional, dedifferentiated and mesenchymal subtypes, reflecting the heterogeneity of chondrosarcoma of bone.

Materials and methods

Cell culture

Central conventional chondrosarcoma cell lines SW1353 (ATCC), JJ012 [41], CH2879 [18], OUMS27 [42], CH3573 [43] and L835, and dedifferentiated chondrosarcoma cell lines L3252B, L2975 [20] and NDCS1 [44] were cultured in RPMI-1640 (Gibco, Invitrogen Life-Technologies, Scotland, UK) supplemented with 1% penicillin/streptomycin (100 U/mL) and 10 or 20 % Fetal Calf Serum (Gibco, Invitrogen Life-Technologies, Scotland, UK). Mesenchymal chondrosarcoma cell line MCS-170 was cultured in IMDM medium (Gibco, Invitrogen Life-Technologies, Scotland, UK) supplemented with 1% penicillin/streptomycin (100 U/mL) and 15% Fetal Calf Serum (Supplementary table 1). All cell lines were cultured at a temperature of 37^0C in a humidified incubator (5% CO_2). Identity of cell lines was confirmed using the Cell ID GenePrint 10 system (Promega Benelux BV, Leiden, The Netherlands) before and after completion of the experiments. Mycoplasma tests were performed on a regular basis.

Mutation analysis

Mutations in *TP53*, *IDH1* and *IDH2* were confirmed with Ion AmpliSeq™Cancer Hotspot Panel v2 (Life Technologies, Thermo Fisher Scientific, USA, catalog number 4475346) according to the manufacturer's instructions. Sanger sequencing was performed when validation was necessary (Supplementary table 1). *TP53* mutations identified were analyzed using prediction software Align GVGD and Sorting Intolerant From Tolerant

(SIFT). Also entries in the Catalogue of Somatic Mutations in Cancer (COSMIC) database were analyzed.

Compounds

The survivin inhibitor YM155 (Catalog No.S1130, Selleckchem) and BH3 mimetic ABT-737 (Catalog No.S1002, Selleckchem) were dissolved in DMSO according to the manufacturer's instructions. Doxorubicin and cisplatin were obtained from the in house hospital pharmacy in a 0.9% NaCl solution. Z-vad-FMK (550377, BD biosciences) was used as a general caspase inhibitor and dissolved in DMSO.

siRNA screen

To identify critical genes for chondrosarcoma cell survival, a focused targeted siRNA screen was performed on the JJ012 central chondrosarcoma cell line targeting 51 apoptosis related genes (Dharmacon, GE life sciences, Landsmeer, the Netherlands) (Supplementary table 2). In the primary screen, reverse transfection of SMARTpools of 4 different siRNAs in a final concentration of 50 nM for each gene was performed using DharmaFECT 3 transfection reagent (Thermo Fisher Scientific Inc. Waltham, MA USA,T-2003). Deconvolution confirmation screens were performed where each of four individual siRNAs was transfected separately. A gene was considered as a hit when three out of four individual siRNAs mimicked the SMARTpool. *GFP* or *GAPDH* siRNAs were used as negative and *KIF11* siRNAs were used as positive controls. Mock (no siRNA) transfection served as an additional control. The transfection was performed in duplo in u-clear 96 well black clear bottom plates (Corning B.V. Life Sciences, Amsterdam, the Netherlands) using 7000 cells/well. After one day the medium was replaced. After 5 days cells were fixed with formalin and stained with Hoechst. Nuclei present in each well were imaged using a BD-pathway microscope. Quantification was performed by determining the total Hoechst area using Image Pro analyzer software and normalizing towards negative controls. Z'factor analysis was performed as a quality control using the mean of si*GFP* and si*GAPDH* as a negative and si*KIF11* as a positive control.

Immunohistochemistry

Survivin and p53 expression were evaluated in human primary chondrosarcoma tumour tissue using previously constructed tissue

microarrays containing 137 conventional chondrosarcomas (92 central of which 42 grade I, 36 grade II, 14 grade III and 45 peripheral including 31 grade I, 11 grade II, 3 grade III) [10] and ten chondrosarcoma cell lines. Additionally, survivin expression was evaluated in 20 clear cell, 21 mesenchymal, and 25 dedifferentiated chondrosarcomas [12]. For dedifferentiated chondrosarcoma the well-differentiated and the dedifferentiated component were scored separately. To visualize survivin, the 71G4B7 antibody (Rabbit mAb #2808, Cell Signaling Technology) was diluted 1:100, and placenta was used as a positive control. p53 expression was determined using an antibody from DAKO (M700101) in a 1:800 dilution with tonsil as positive control tissue. Immunohistochemistry was performed according to standard laboratory methods, using citrate (pH 6) as antigen retrieval method, as previously described [45]. Survivin is known to be expressed in the nucleus as well as in cytoplasm displaying different functions and prognostic significance [46]. Therefore, nuclear and cytoplasmic expression was scored separately for each core by two independent observers (JVMGB,GA) using a scoring system assessing staining intensity (0= no, 1=weak, 2=moderate, 3=strong) and percentage of staining (0=no, 1=1-24%, 2=25-49%, 3=50-74%, 4=75-100%) [12]. p53 expression was considered as high when added scores were ≥ 4, as previously described [27]. Cores that were lost from the TMA section were excluded from analysis (n=39 for conventional chondrosarcomas, n=3 for clear cell chondrosarcoma, n= 7 for mesenchymal chondrosarcoma and n=4 for dedifferentiated chondrosarcoma). Slides were scanned using a Philips intellisite pathology scanner and pictures were taken using Philips IMS viewer.

RNA isolation and Quantitative real time PCR

RNA was isolated from fresh frozen tissue of 34 conventional chondrosarcoma primary tumour tissues (supplementary table 3) and six cartilage control tissues, i.e. three growth plate, and three articular cartilage tissues. RNA was also isolated from untreated and YM155 treated cell lines. For isolation TRIzol (Invitrogen, Carlsbad CA) was used followed by RNA clean up using the RNeasy mini kit (Qiagen) according to the manufacturer's instructions. All patient samples were handled according to the ethical guidelines described in 'Code for Proper Secondary Use of Human Tissue in The Netherlands' of the Dutch Federation of Medical Scientific Societies. Q-PCR was performed for; wild type (wt) survivin, survivin 2b and survivin Δex3

with primers described previously [47]. Expression was normalized towards housekeeping genes PPIA, CPSF6 and GPR108, as previously described [48].

Viability assay

Chondrosarcoma cell lines were plated in 96 well plates so as to achieve 50-70% confluency the following day. Dose response curves were performed using concentrations from 0.01 till 1000 nM. Concentrations used for combination studies were 10 and 50 nM doxorubicin, 250 and 1000 nM cisplatin and 1 and 5 nM YM155 and combinations of drugs were added at the same moment. After 72 hours of incubation a presto blue assay (Life-Technologies, Scotland, UK) was performed according to the manufacturer's instructions. Fluorescence was measured reading the plate at 590 nm on a fluorometer (Victor3V, 1420 multilabel counter, Perkin Elmer, Netherlands). Experiments were performed at least three times in triplicate.

Apoptosis

To measure apoptosis the caspase-glo 3/7 assay (Promega, Madison, USA) was used according to the manufacturer's instructions. In brief, cells were plated into white walled 96 well plates (Corning B.V. Life Sciences, Amsterdam, the Netherlands) and incubated with IC_{75} concentrations of YM155 (determined using 72h viability assays). After 24 hours the substrate was added in a 1:1 dilution and incubated for 30 minutes at room temperature. As a positive control MCS-170 cells treated with ABT-737 and doxorubicin were used. Luminescence was measured using a luminometer (Victor3V, 1420 multilabel counter, Perkin Elmer, Netherlands). Experiments were performed at least two times in duplicate.

Western blotting

Western blotting for PARP (Cell Signaling Technology, Leiden, The Netherlands) was performed on lysates obtained using hot-SDS buffer (1% SDS, 10 mM Tris/EDTA with complete inhibitor and phosSTOP) as previously described [9]. For PARP and cleaved PARP detection Jurkat cell lysates treated with 25 μM etoposide obtained from Cell Signaling Technology (#2043) were used as a positive control. Of each sample, 20 μg protein was loaded on the gel. As loading control, α-tubulin (clone DM1A, Sigma-Aldrich Chemie B.V. Zwijndrecht, the Netherlands) expression was determined. Proteins were blotted on a PVDF membrane and detected using

enhanced chemo luminescence (Pierce™ ECL Western Blotting Substrate) followed by exposure of 1 minute and development of the film (ECL hyperfilm, Amersham).

Cell cycle analysis

Cells were plated in T25 flasks in amounts ensuring 70% confluency when harvested. For knock down experiments cells were seeded in 6 well plates. After 48 hours of treatment with YM155 IC_{50} concentrations or BIRC5 or GAPDH siRNA, cells were prepared for cell cycle analysis using methanol and propidium iodide staining as previously described [49]. Cell cycle data analysis was performed using WinList 7.1 (Verity Software House, Topsham, ME). Experiments were performed at least two times in duplicate.

Statistical analysis

Mann-Whitney testing was applied using Prism Graph path software to assess significant differences between survivin expression levels. Bonferroni correction was used to correct for multiple testing. Dose response curves and IC_{50} values were determined using Prism Graph path software.

Results

BIRC5 is an essential survival gene in chondrosarcoma cells

A siRNA screen targeting 51 apoptosis related genes revealed that *HRK* (Harakiri, BCL2 Interacting Protein), *BIRC5* (Baculoviral IAP Repeat Containing 5), *BCL2L1* (B-cell/Lymphoma 2 like 1), *BCL-10* (B-cell/Lymphoma 10) and *CRADD* (CASP2 And RIPK1 Domain Containing Adaptor With Death Domain) were essential genes in the JJ012 chondrosarcoma cell line (Figure 1A). Across the primary screen, transfection with control siRNAs led to a slight (~13%) reduction in viability compared to mock whereas positive control si*KIF11*, caused a very strong (>90%) loss of viability indicating successful gene silencing and a good experimental window (Figure 1B). The average Z'factor was 0.61, which indicates a qualitatively good screen. Deconvolution was performed for selected hits and *BCL2L1*, *BIRC5*, *CRADD* and *HRK* were confirmed (Supplementary figure 1). Identification of *HRK* and *CRADD* is surprising since these encode pro-apoptotic proteins. A role for *BCL2L1* in chondrosarcoma has been previously described by us [11]. Here, we focused

on *BIRC5* (Figure 1C), which encodes the survivin protein, an anti-apoptotic protein overexpressed in human cancers [16].

Survivin is highly expressed in chondrosarcoma tumour tissue and cell lines.

Survivin protein expression and its subcellular localization was determined in 207 chondrosarcomas of different subtypes. Nuclear expression, suggesting a role for survivin in cell cycle progression, was found in 90/98 conventional chondrosarcomas. Cytoplasmic expression, reflecting its function in apoptosis regulation, was found in 97/98 conventional chondrosarcomas. Expression levels were variable. Both nuclear and cytoplasmic survivin expression increased with increasing histological grade in central chondrosarcoma (Figure 2A, B). Cytoplasmic survivin expression was significantly higher in grade II ($P \leq 0.001$) and III ($P \leq 0.01$) central chondrosarcomas compared to ACTs. Also, higher expression of nuclear survivin was observed in grade III chondrosarcomas compared to ACTs ($P \leq 0.05$). No correlation was found with *IDH1* or *-2* mutation status (not shown). Moreover, survivin was also highly expressed in dedifferentiated, clear cell and mesenchymal chondrosarcoma. Survivin expression was significantly higher in nuclei compared to the cytoplasm in the well differentiated part of dedifferentiated chondrosarcoma ($P \leq 0.001$) (Figure 2C, D). Also in clear cell chondrosarcoma nuclear expression was significantly higher ($P \leq 0.001$) compared to cytoplasmic expression (Figure 2C).

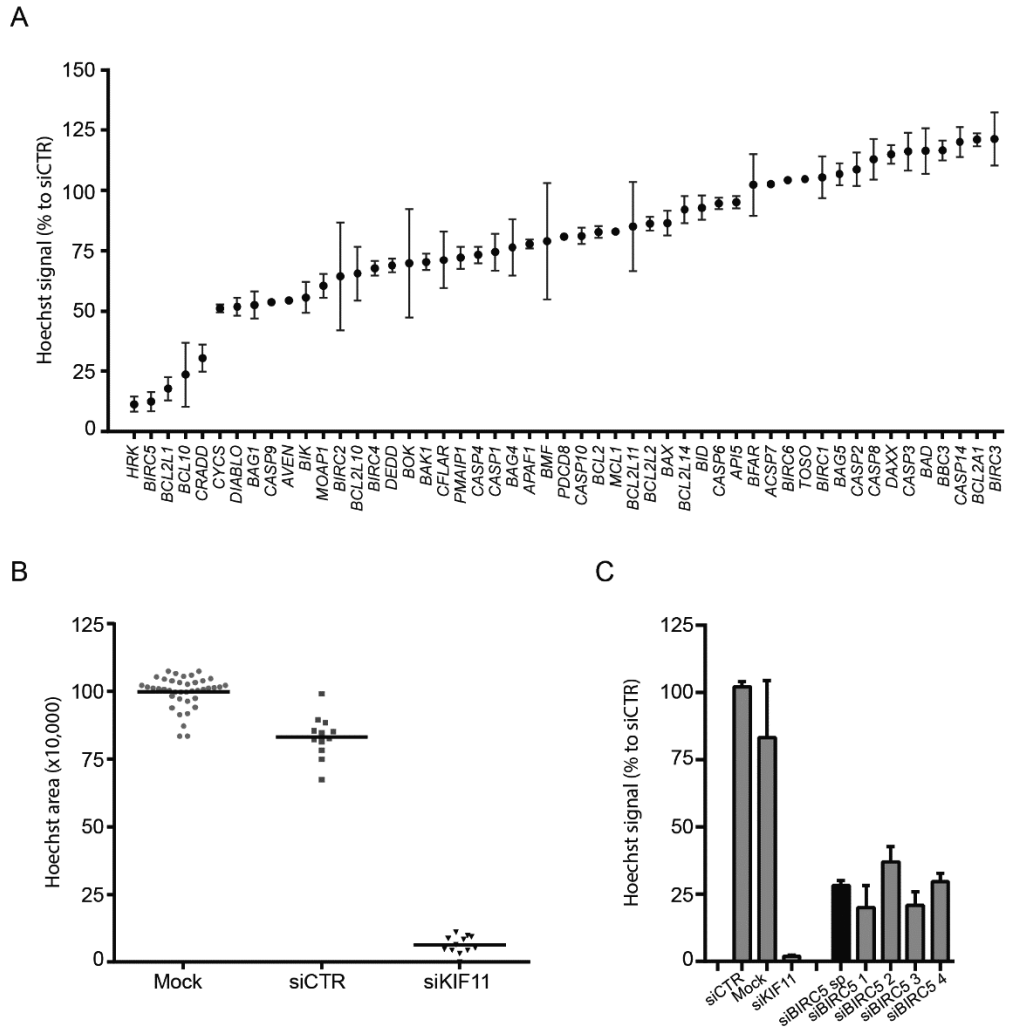


Figure 1. Screening for apoptotic regulators identifies *BIRC5* as an important survival gene in chondrosarcoma cell line JJ012.
A) Occupied area of cells present in the well as a percentage to control siRNA of 51 apoptosis related genes. Mean values and range of duplicates are shown. B) Raw data measured as Hoechst area of controls used in the siRNA screen. Dots indicate individual measurements and mean values are shown for each control. siCTR indicates control siRNA of either GFP or GAPDH. C) Area of cells as a percentage to control siRNA of selected hits. *BIRC5* shows 4/4 siRNAs that mimic the smart pool. Data represent means of duplicate values with range. Black bars represent the smart pool and grey bars represent individual siRNAs.

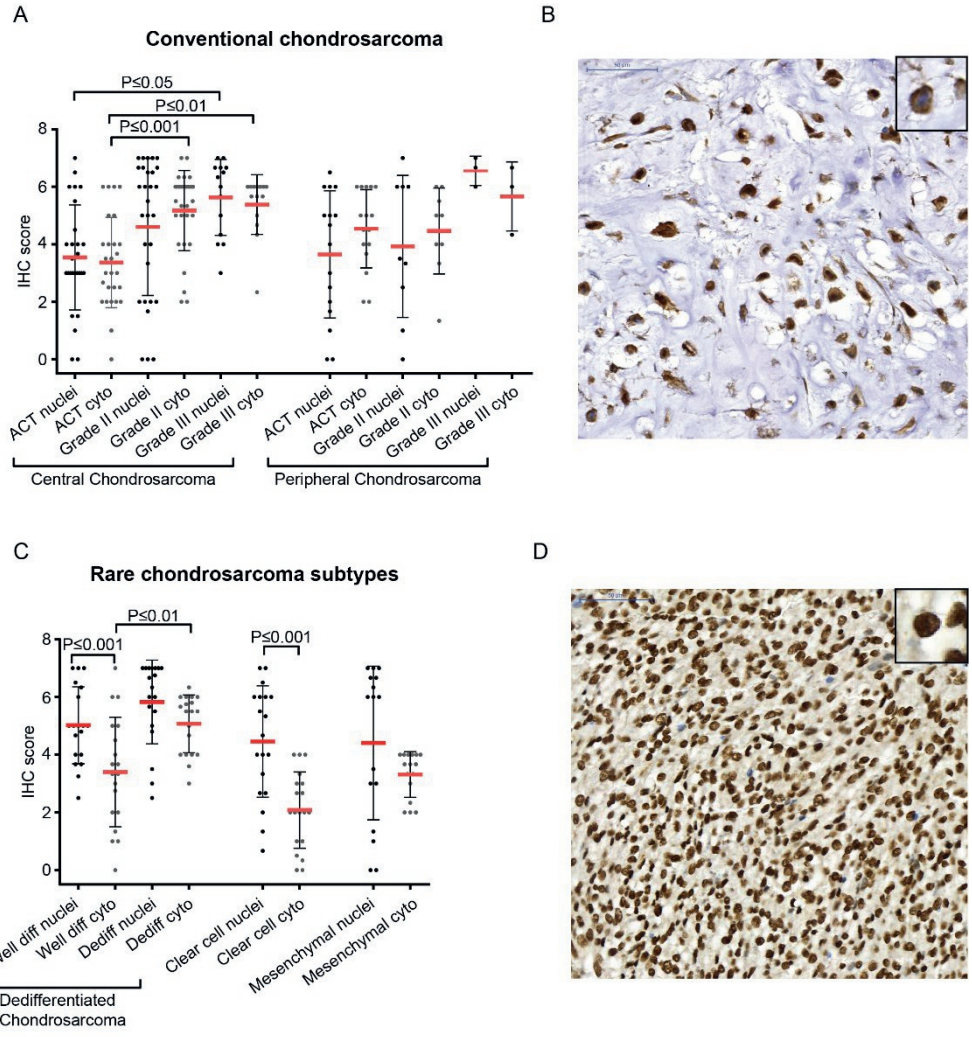


Figure 2. Survivin is highly expressed in high grade chondrosarcoma.
A) Immunohistochemical analysis of Survivin in conventional chondrosarcoma showing higher cytoplasmic expression in grade II and III central chondrosarcoma compared to ACTs. Also higher expression of nuclear survivin is observed in grade III chondrosarcomas compared to ACTs. Each dot represents one patient and mean value with standard deviation is shown for each group. B) High cytoplasmic Survivin expression in a grade III chondrosarcoma. C) Immunohistochemical analysis of nuclear and cytoplasmic survivin in dedifferentiated, clear cell and mesenchymal chondrosarcoma. Nuclear survivin is higher expressed in nuclei of the well differentiated part of dedifferentiated chondrosarcoma and clear cell chondrosarcoma compared to the cytoplasmic part. Each dot represents one patient and mean value with standard deviation is shown for each group. D) High nuclear Survivin expression in dedifferentiated chondrosarcoma (dedifferentiated part).

Nuclear survivin expression is associated with p53 overexpression in high grade conventional chondrosarcoma

Since p53 is a known effector of survivin expression [17], we investigated a possible correlation between p53 overexpression (indicative of a mutated *TP53* gene) and survivin expression using the conventional chondrosarcoma TMA. p53 overexpression was observed in 0/50 grade I, 20/41 grade II and 9/17 grade III conventional chondrosarcomas (Figure 3A, B). In high grade chondrosarcomas (grade II and III) with overexpression of p53, the expression of nuclear survivin was higher as compared to high grade chondrosarcomas without overexpression of p53 (P≤0.01 for grade 3) (Figure 3C). No difference between peripheral and central conventional chondrosarcoma was observed (data not shown).

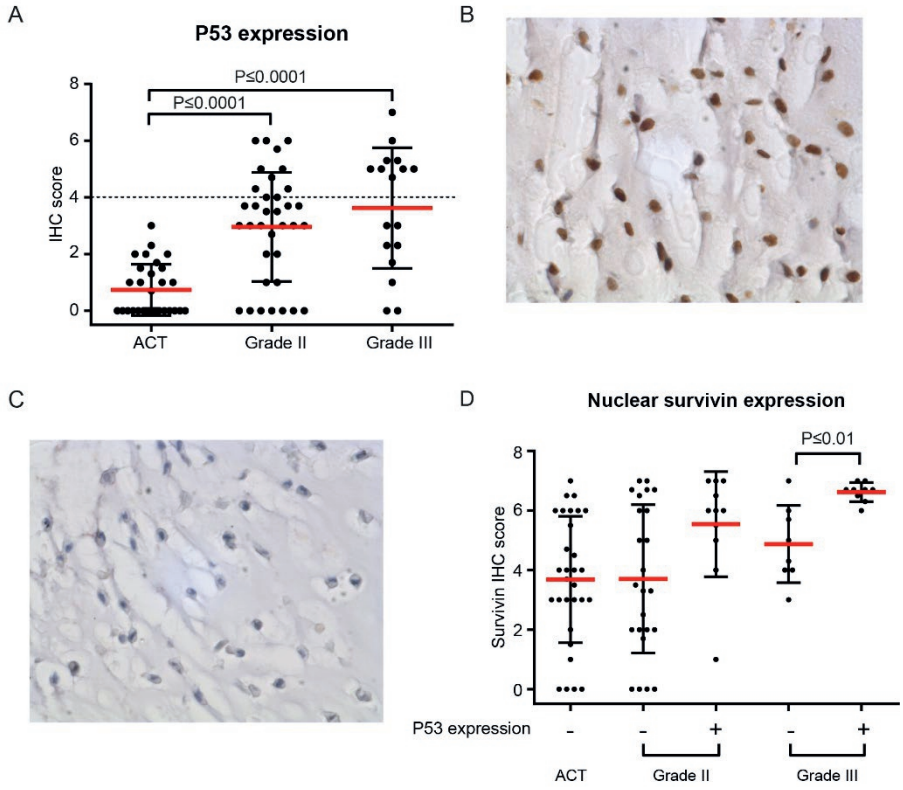


Figure 3. P53 is higher expressed in high grade conventional chondrosarcoma and correlated with nuclear survivin in grade III chondrosarcoma.
A) Immunohistochemical analysis of p53 expression in conventional chondrosarcoma. Grade II and III chondrosarcomas show significantly higher expression compared to grade I chondrosarcoma (P<0.0001). Each dot represents one patient and mean value with standard deviation is shown for each group. B)

High nuclear p53 expression in a high grade chondrosarcoma. C) Low nuclear p53 expression in a high grade chondrosarcoma. D) Correlation between nuclear survivin and p53 overexpression in conventional chondrosarcoma. P53 is considered as overexpressed when it reached a sum score of 4 (indicated by +). Grade I chondrosarcomas did not show p53 overexpression. In grade II chondrosarcoma higher nuclear survivin was seen in p53 overexpressing chondrosarcomas, as well as in grade III chondrosarcoma (P<0.01) compared to low p53 expressing tumours. Each dot represents one patient and mean value with standard deviation is shown for each group.

Survivin isoforms are higher expressed in high grade chondrosarcoma

The three most common survivin isoforms; wt survivin, survivin 2b and survivin Δex3 were all expressed significantly higher in high grade chondrosarcomas (grade II and III) compared to low grade chondrosarcomas (P≤0.001 for wt survivin, P≤0.01 for survivin 2b and P≤0.05 for survivin Δex3) (Figure 4A, B, C). No significant difference in survivin expression was observed between *IDH* wild type and *IDH1* or *-2* mutant chondrosarcomas (not shown). Furthermore, a correlation was found between all different survivin isoforms (Supplementary figure 2).

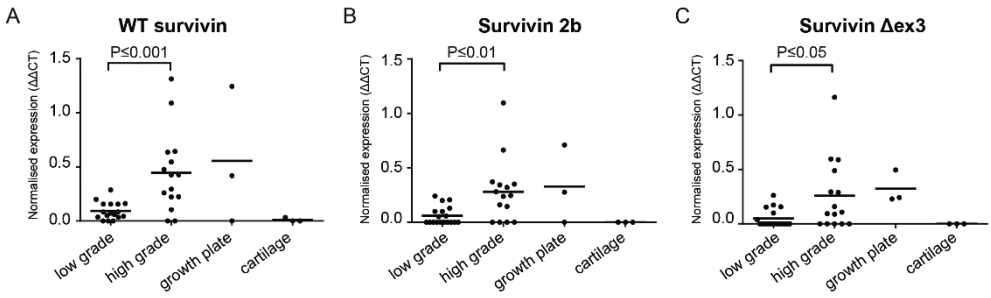


Figure 4. Survivin wt, 2b and Δex3 isoforms are highly correlated and show higher expression in high grade chondrosarcoma as determined by Q-PCR analysis.
A, B, C) Survivin isoforms wt (A), 2b (B) and Δex3 (C) are higher expressed in high grade (grade II and III) chondrosarcoma compared to low grade (ACT) chondrosarcoma. No expression is found in cartilage while variable expression is seen in growth plate tissue. Each dot represents one measurement and mean values are shown for each group.

Chondrosarcoma cell lines highly express survivin, predominantly in the nucleus

We confirmed that all cell lines showed high expression of survivin protein, predominantly in the nucleus (Figure 5A and supplementary figure 3). All

survivin isoforms where highly expressed compared to normal articular cartilage (Figure 5B). Using the Ion AmpliSeq™Cancer Hotspot Panel v2 the *IDH1* or -2 mutation status was confirmed (Supplementary table 1). For *TP53* the use of this highly sensitive targeted next generation sequencing technique provided novel insights for CH2879 and L2975, which were previously reported to be wild type [18-20] (Supplementary table 1). In CH2879 a sub clonal pathogenic mutation was found with a frequency of 17%, which corresponded to the mosaic staining pattern of nuclear p53 observed in the cell pellet and primary tumour (Supplementary figure 4). The mRNA expression levels of survivin were not correlated with *TP53* or *IDH1* or -*2* mutational status (not shown).

Chondrosarcoma cell lines are sensitive to survivin inhibition using YM155

YM155 is a compound currently in phase I/II clinical trial, that represses survivin promoter activity [21]. We first assessed the effect of YM155 on survivin RNA down regulation (Figure 5B). JJ012, SW1353 and L835 cell lines showed clear down regulation of all three survivin isoforms after YM155 treatment. NDCS1 only showed down regulation of survivin Δex3 after YM155 treatment and L2975 only showed down regulation of survivin 2b. Chondrosarcoma cell lines were highly sensitive to YM155 showing IC_{50} values below 5 nM in four out of ten chondrosarcoma cell lines (SW1353, OUMS27, NDCS1, L2975) (Figure 5C,D). JJ012 showed an IC_{50} of 8.2 nM and CH3573 showed an IC_{50} of 12.32 nM. The least responsive cell lines were L835, CH2879, L3252B and MCS-170. Interestingly, three of these are *TP53* wild type (P = 0.033) (Figure 5E and supplementary table 1). There was no relation between YM155 sensitivity and histological subtype or *IDH1* or -*2* mutation status. Combination treatment of YM155 with doxorubicin and cisplatin did not show a synergistic effect in JJ012 and SW1353 cell lines (Supplementary figure 5).

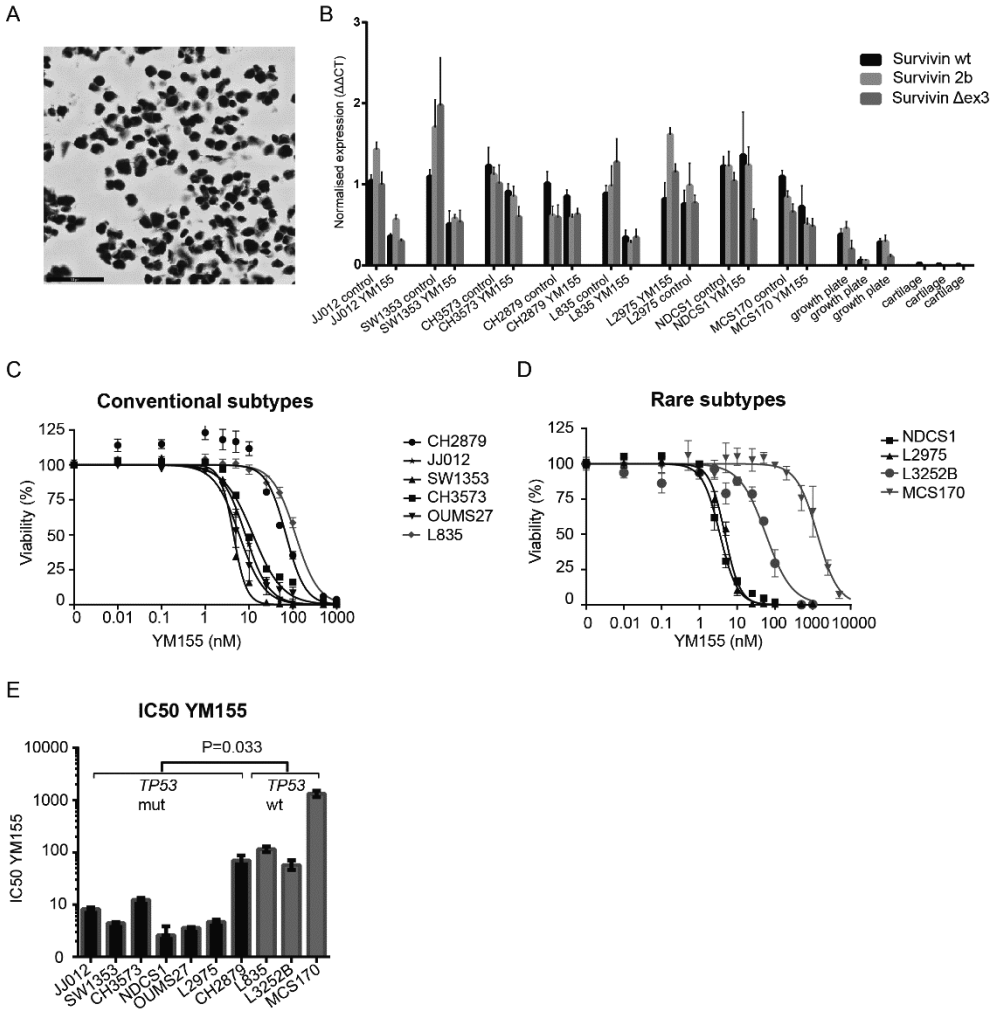


Figure 5. Chondrosarcoma cell lines are sensitive to YM155, which is p53 dependent.
A) JJ012 cell line showing strong survivin expression B) Normalized RNA expression of three survivin isoforms in 8 chondrosarcoma cell lines. JJ012, SW1353 and L835 show a clear reduction in survivin expression after YM155 treatment. Growth plate and cartilage are taken as a control. Bars represent mean with standard deviation. C, D) Dose response curves for YM155 (72h) in conventional subtypes (C) and rare chondrosarcoma subtypes (D). Error bars are shown for three experiments performed in triplicate. E) IC50s determined for *TP53* wild type and mutant cell lines showing a significantly increased sensitivity of *TP53* mutant cell lines towards YM155. Bars represent mean values of three experiments performed in triplicate.

The survivin inhibitor YM155 does not induce caspase 3/7 or PARP dependent apoptosis in chondrosarcoma cells.

No activation of apoptosis was found in chondrosarcoma cell lines after treatment with IC_{75} concentrations of YM155. Caspase 3/7 activation was not observed after 24 or 48 hours of treatment (Figure 6A and data not shown). Furthermore, treatment with pan-caspase inhibitor z-VAD could not restore the reduction in viability observed after treatment with YM155 (Figure 6B). PARP expression, but not cleaved PARP was observed in CH2879 and NDCS1 cell lines (Figure 6C), however not in SW1353 and only low expression was observed in JJ012.

YM155 deregulates the cell cycle in a subset of chondrosarcoma cell lines

Because no activation of apoptosis was found in chondrosarcoma cell lines treated with YM155, we evaluated its effect on the cell cycle (Figure 6D). A large increase in S phase and a reduction in G1 was observed in JJ012 cells after 48 hours of treatment. CH2879 showed only minor effects in cell cycle distribution after YM155 treatment and NDCS1 cells did not show any change in cell cycle distribution after treatment with YM155. Knock down of BIRC5 in JJ012 cells also resulted in a deregulation of the cell cycle (Supplementary figure 6).

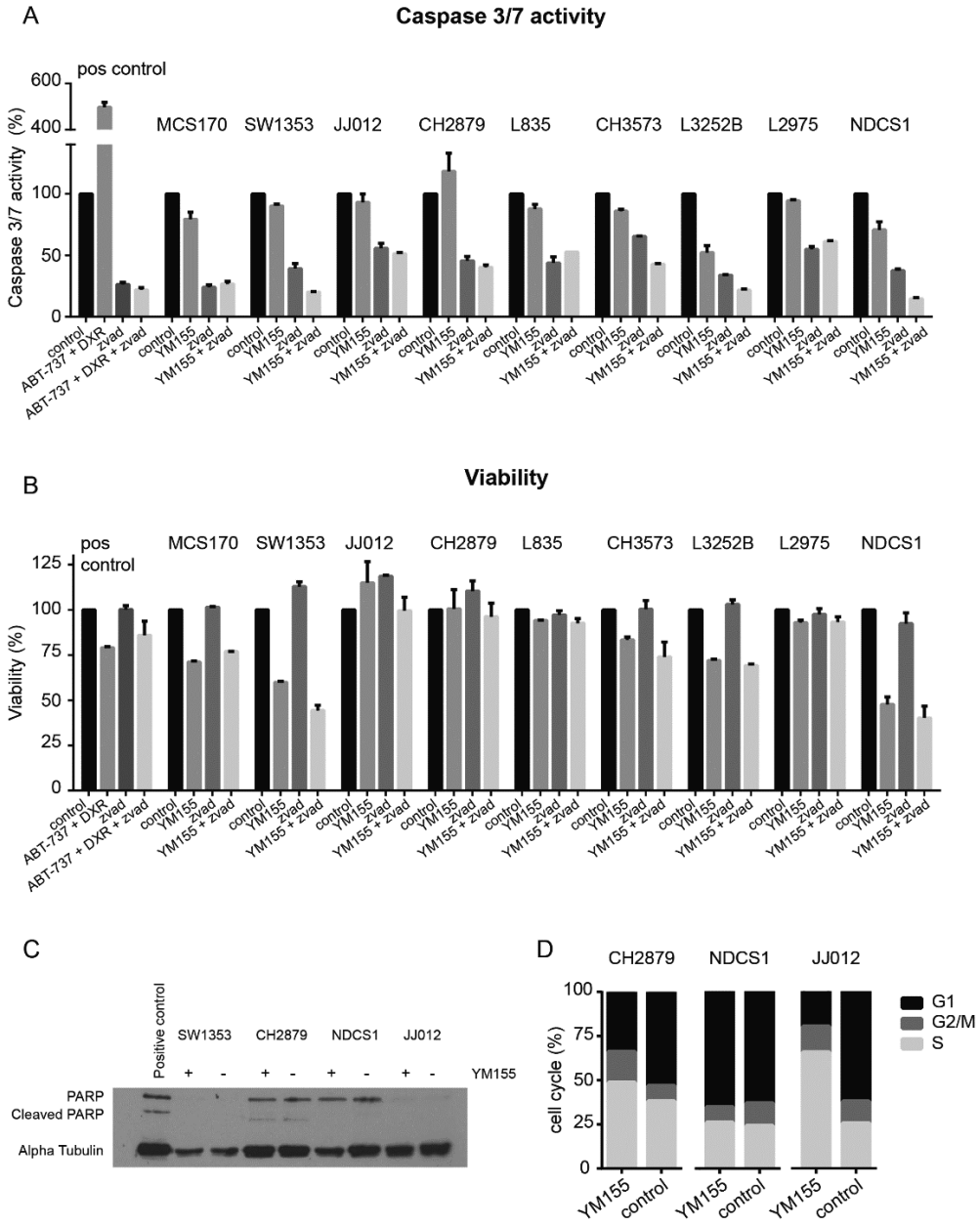


Figure 6. YM155 does not cause caspase 3/7 and PARP dependent apoptosis, but cell cycle deregulation in chondrosarcoma cell lines.
A,B) Caspase 3/7 activity (A) and viability (B) after 24h as percentage to untreated control measured in nine chondrosarcoma cell lines by caspase glo assay and presto blue viability assay. No caspase 3/7 activity is seen after YM155 treatment in all cell lines. Pan Caspase inhibitor z-vad was not able to rescue the YM155 dependent

reduction in viability. MCS-170 cells treated with doxorubicin and ABT-737 were used as a positive control. Error bars are shown for two independent experiments performed in duplicate. C) Western blot analysis for PARP and cleaved PARP expression in four chondrosarcoma cell lines. No differences are seen between treated (+) and untreated (-) samples. Alpha tubulin was used as a loading control. Jurkat cell lysates treated with 25 μM etoposide obtained from cell signaling were used as a positive control. D) FACS analysis of three chondrosarcoma cell lines treated with YM155 for 48 hours. JJ012 and CH2879 show a reduction in G1 and increase in S-phase after treatment with YM155. NDCS1 is not showing a difference in cell cycle distribution.

Discussion

For patients with inoperable or metastatic chondrosarcoma no treatment options are available resulting in a 10 years survival rate below 30% [1]. By using a focused RNAi screen targeting core apoptosis machinery components we identified *BIRC5* as an important player in chondrosarcoma survival and we here show that survivin inhibition using YM155 could be a promising novel treatment strategy for this malignant tumor.

BIRC5 encodes the survivin protein which is part of the inhibitor of apoptosis family, and is involved in a large variety of cellular processes in the nucleus as well as in the cytoplasm. Nuclear survivin functions in the cell cycle as an essential mitotic regulator being a member of the chromosomal passenger complex. On the other hand, cytoplasmic survivin is predominantly involved in preventing apoptosis [16]. Overexpression is found in many tumor types including osteosarcomas [22] and soft tissue sarcomas [23]. Moreover, survivin up regulation was previously shown in small series of conventional chondrosarcomas [24-26]. We here demonstrate high survivin expression in conventional as well as rare chondrosarcoma subtypes in a large panel of >200 chondrosarcomas. Expression was found both in the nucleus as well as in the cytoplasm in high grade conventional chondrosarcomas suggesting a function both in the cell cycle and as an anti-apoptotic protein. In contrast, in dedifferentiated and clear cell chondrosarcomas, survivin was predominantly expressed in the nucleus, suggesting a more prominent role for survivin in cell cycle regulation.

We also show a positive correlation between nuclear survivin and p53 overexpression in conventional chondrosarcoma. High p53 expression is known to correlate with increasing histological grade in chondrosarcoma [27] and is suggestive for mutated *TP53* [28]. Wild type p53 was shown to be able to repress survivin expression [17], which may explain the higher survivin expression in chondrosarcomas with high, probably mutated, p53 expression.

Survivin isoforms are differentially expressed in different types of cancer correlating with survival, depending on the isoform. In chondrosarcoma we found increased expression of all three isoforms with increasing histological grade. SurvivinΔEx3 has been most extensively studied and has been associated with a worse prognosis in breast, colon and cervical cancer [29]. In soft tissue sarcomas, all survivin transcripts were strongly overexpressed compared to non-malignant control tissue and elevated expression of survivinΔEx3 was correlated with a worse survival [30]. Survivin was

expressed in normal growth plate tissue, suggesting a role for survivin in endochondral ossification. This is in concordance with the role for survivin described in tissue development and its down regulation with differentiation [31]. No survivin expression was detected in normal articular cartilage, which is in concordance with the low expression levels observed in other normal adult tissues [31], rendering survivin a very attractive therapeutic target [32].

We here show for the first time that survivin inhibition using YM155 could be a promising novel treatment strategy for chondrosarcoma as YM155 was highly potent in reducing cell viability. YM155 is already in phase I and II clinical trials, so could be readily applicable in clinical trials for chondrosarcoma patients. It is an indirect inhibitor of survivin, that blocks the survivin promoter region by binding and disrupting the RNA binding protein ILF3/NF110, thereby preventing survivin transcription [33]. Another proposed mechanism is disruption of binding of zinc transcription factor SP1 to the survivin promoter by YM155 [34]. Interestingly, *TP53* mutant cell lines were more sensitive to YM155 as compared to *TP53* wt cell lines, suggesting that the *TP53* mutation status could be a predictive biomarker. This was also found in other cancer cell lines [35]. In *TP53* wt cell lines, mechanisms other than survivin may dominate tumor cell proliferation and survival.

YM155 does not induce apoptosis in the chondrosarcoma cell lines, as shown by the absence of caspase 3 and 7 activity and of PARP cleavage. In previous studies YM155 was shown to induce apoptosis in leukaemia, prostate cancer and breast cancer [36]. However, in merkel cell carcinoma, YM155 did not induce apoptotis but inhibited DNA synthesis [37]. Survivin is predominantly expressed during G2/M phase of the cell cycle [38], and its inhibition is expected to cause a G2/M arrest. G2/M phase cell cycle arrest has been described by Lechler et al. in two chondrosarcoma cell lines after siRNA knock down of survivin [26], however Yang et al showed a decrease in S-phase after knock down of survivin in the same cells [24]. We found an increase in S phase after treatment with YM155 in two of our chondrosarcoma cell lines and an increase in G2/M phase in JJ012 cells after BIRC5 knock down. This inconsistency can be caused by several issues including different confluency of the cells, different methods to inhibit survivin and different incubation times before the measurement.

Consistent with our findings, Brun et al. also observed a block in S phase after treatment with YM155 in sonic hedgehog driven medulloblastoma [39].

We did not observe a difference in cell cycle distribution in all cell lines, which shows that chondrosarcoma cell lines show a heterogeneous response towards YM155 treatment.

In our hands, combination treatment of YM155 with doxorubicin or cisplatin did not result in synergistic inhibition of viability in two chondrosarcoma cell lines, suggesting that survivin is not involved in chondrosarcoma chemo resistance. However, Lechler et al. previously reported on a synergistic induction of apoptosis after combining survivin siRNA with doxorubicin treatment in two chondrosarcoma cell lines [26]. This can possibly be attributed to the different cell lines and the different mechanism of inhibition used to target survivin.

Phase I and II clinical trials have already been conducted for YM155 as a single agent as well as in combination with chemotherapy for a few tumour types [36]. Phase I studies show that YM155 is well tolerated, however larger phase II studies in diffuse large B-cell lymphoma, non-small cell lung cancer, melanoma and prostate cancer do not show very promising results regarding anti-tumour activity [36]. This does not necessarily mean that this will also be the case for chondrosarcoma, especially since we show here that in chondrosarcoma instead of the induction of apoptosis, YM155 causes a block in the cell cycle, and that patients can be preselected based on *TP53* mutation status. New survivin inhibitors, for example antisense oligonucleotides and survivin based vaccines, are in development and some already reached clinical trials [40].

In conclusion, we show that survivin is essential for chondrosarcoma cell survival and is highly expressed in high grade chondrosarcomas and absent in normal cartilage. Chondrosarcoma cell lines are highly sensitive for treatment with YM155, especially if *TP53* is mutant, indicating that survivin could be a possible new therapeutic target for patients with chondrosarcoma.

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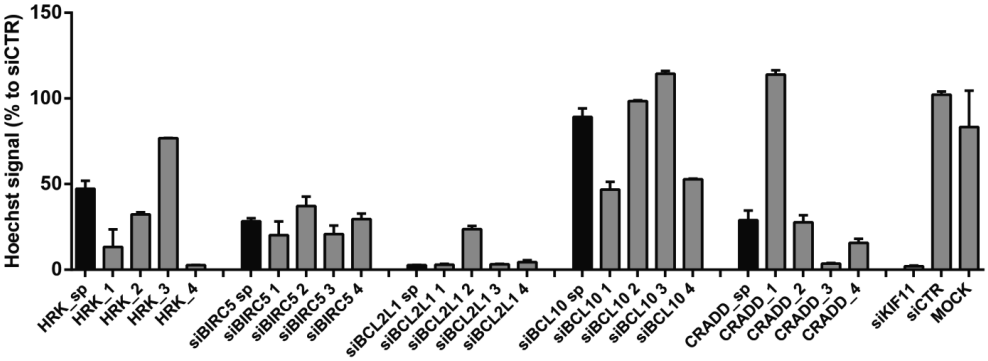
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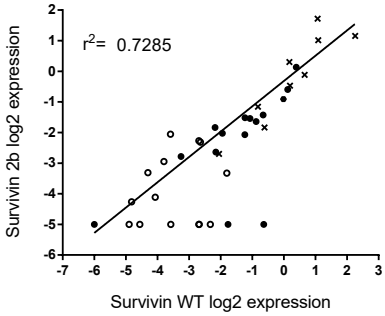
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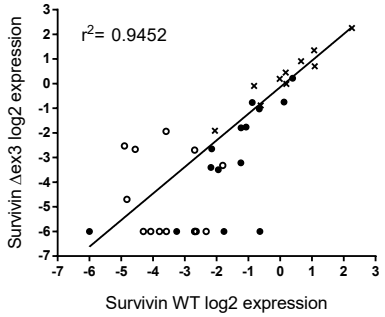
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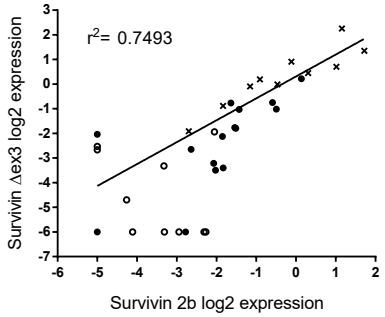
Supplementary materials



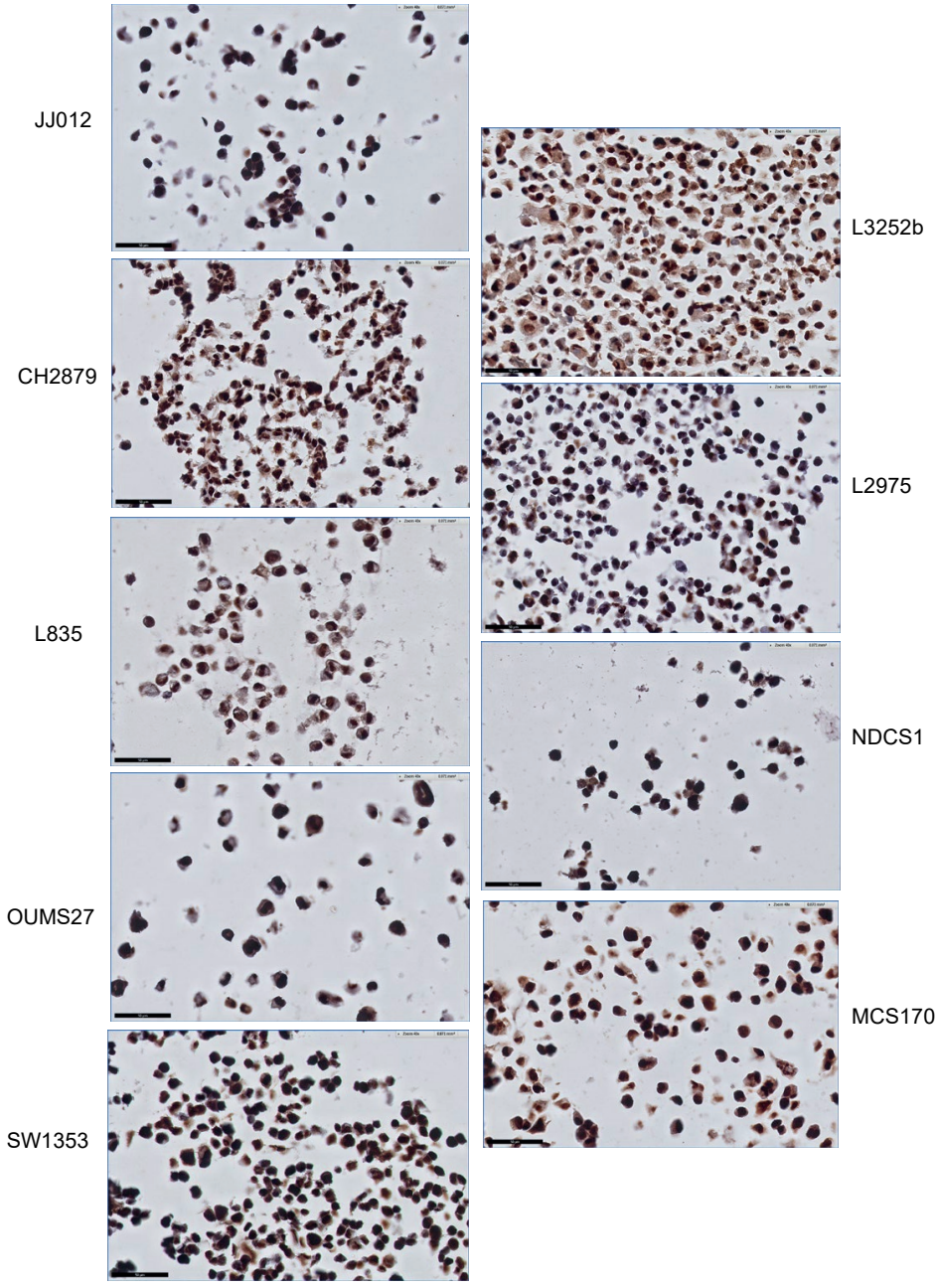
Supplementary figure 1: Results of deconvolution.
Area of cells as a percentage to control siRNA. *BCL2L1*, *BIRC5*, *CRADD* and *HRK* all show 3/4 or 4/4 siRNAs that mimic the smart pool. Data represent means of duplicate values with range. Black bars represent the smart pool and grey bars represent individual siRNAs.



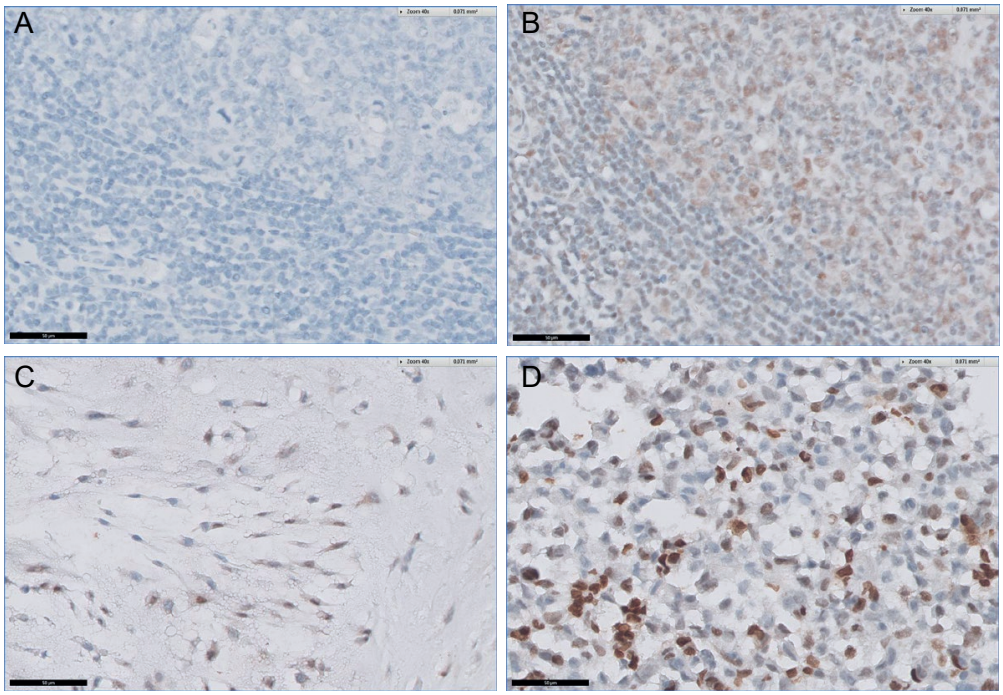




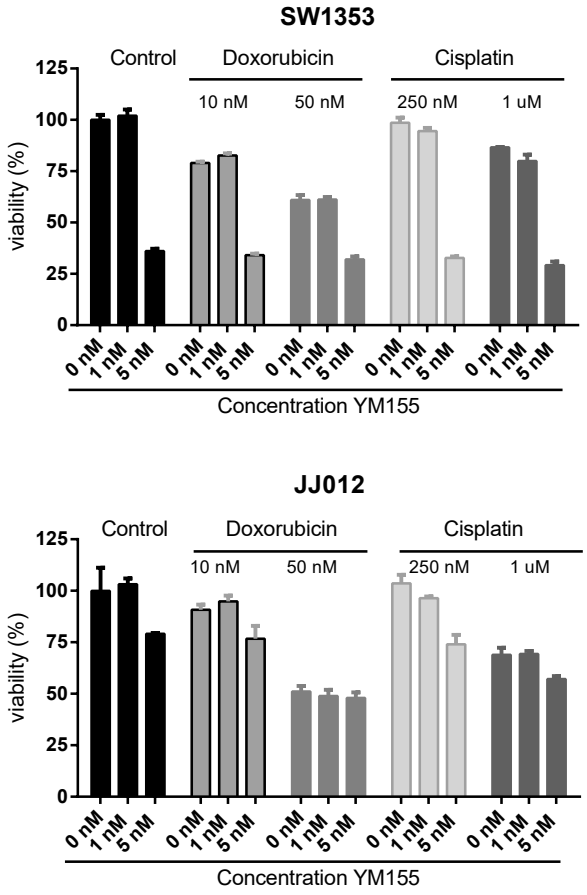
Supplementary figure 2: Correlation survivin expression
A, B, C) Survivin expression of all three isoforms was correlated in chondrosarcoma tissues and cell lines. Expression is plotted as log2 and r^2 was determined to assess correlation. Black dots indicate high grade chondrosarcoma, white dots indicate low grade chondrosarcoma and crosses indicate chondrosarcoma cell lines.



Supplementary figure 3. Survivin staining in chondrosarcoma cell lines. Survivin is highly expressed in the nucleus of all chondrosarcoma cell lines.



Supplementary figure 4. Mosaic staining pattern of P53 protein in CH2879.
A, B) Negative (A) and positive control (B) tonsil tissue. C, D) Mosaic P53 staining in primary tumor tissue (C) and cell pellet (D) of CH2879 cell line.



Supplementary figure 5. No synergistic effects of YM155 combined with doxorubicin or cisplatin are observed in JJ012 or SW1353 cell lines as determined by presto blue assay. Values represent percentages to non-treated control cells.

Supplementary Table 1 Chondrosarcoma cell line panel

Cell line	**Subtype**	***TP53* status**	**Prediction** *Align GVGD*	*SIFT*	**Hits in Cosmic**	**P53 staining** *Intensity*	*percentage*	***IDH1* or *-2* status**	**Ref**
JJ012	CCS	p.Gly199Val	Pathogenic	Deleterious	10	Strong	100%	*IDH1* p.Arg132Gly	[41]
SW1353	CCS	p.Val203Leu	Tolerated	Tolerated	5	Strong	100%	*IDH2* p.Arg172Ser	ATCC
CH3573	CCS	p.Leu201Cysfs*46	Pathogenic	Deleterious	3	No	0%	WT	[43]
OUMS27	CCS	p.Ser149Tyrfs*31	Pathogenic	deleterious	0	No	0%	WT	[42]
L835	CCS	WT				moderate	70%	*IDH1* p.Arg132Cys	[20]
CH2879	CCS	p.Ser366Ala p.Arg273Cys#	Uncertain Pathogenic	Tolerated Deleterious	2 628	strong	30-40%	WT	[18]
NDCS1	Dedif	p.Cys242Ser	Pathogenic	Deleterious	3	strong	100%	WT	[44]
L2975	Dedif	p.Cys229Serfs*7	Pathogenic	Deleterious	0	Weak	5%	*IDH2* p.Arg172Trp	[20]
L3252B	Dedif	WT				Moderate	5%	WT	[20]
MCS-170	Mes	WT				no	0%	WT	

#only found in 17% of cells

Chondrosarcoma cell line panel used to study the effect of survivin inhibition using YM155. *TP53* and *IDH* mutation status are indicated. Align GVDG and SIFT prediction tools were used to predict the functional consequence of the identified *TP53* mutation. Mutations were searched in COSMIC to determine previous identifications. P53 staining intensity and percentage are indicated. CCS= Conventional chondrosarcoma, Dedif= Dedifferentiated chondrosarcoma, Mes=Mesenchymal chondrosarcoma.

Supplementary table 3 Patient samples subjected to survivin expression analysis by Q-PCR

Sample	Grade	IDH Mutation	Amount
Tumour	Low	*IDH1*	14
		IDH2	1
		Wild type	4
	High	*IDH1*	6
		IDH2	4
		Wild type	5
Growth plate	Not applicable	N/A	3
Cartilage	Not applicable	N/A	3

Information of RNA samples used to evaluate survivin expression. Shown are grade, mutation status and amounts.

Additional supplementary materials are available online:
www.nature.com/articles/oncsis201633#Sec24

Chapter 6

A screening-based approach identifies cell cycle regulators AURKA, CHK1 and PLK1 as targetable regulators of chondrosarcoma cell survival

This chapter is based on the publication:
de Jong Y, Bennani F, van Oosterwijk JG, Alberti G, Baranski Z, Wijers-Koster P, Baelde H, Cleton- Jansen AM, van de Water B, Corver W, Danen EHJ, Bovée JVMG, A screening-based approach identifies cell cycle regulators AURKA, CHK1 and PLK1 as targetable regulators of chondrosarcoma cell survival. J Bone Oncol. 2019 Nov 17; 19:100268

Abstract

Chondrosarcomas are malignant cartilage tumors that are relatively resistant towards conventional therapeutic approaches. Kinase inhibitors have been investigated and shown successful for several different cancer types. In this study we aimed at identifying kinase inhibitors that inhibit the survival of chondrosarcoma cells and thereby serve as new potential therapeutic strategies to treat chondrosarcoma patients.

An siRNA screen targeting 779 different kinases was conducted in JJ012 chondrosarcoma cells in parallel with a compound screen consisting of 273 kinase inhibitors in JJ012, SW1353 and CH2879 chondrosarcoma cell lines. AURKA, CHK1 and PLK1 were identified as most promising targets and validated further in a more comprehensive panel of chondrosarcoma cell lines. Dose response curves were performed using tyrosine kinase inhibitors: MK-5108 (AURKA), LY2603618 (CHK1) and Volasertib (PLK1) using viability assays and cell cycle analysis. Apoptosis was measured at 24h after treatment using a caspase 3/7 assay. Finally, chondrosarcoma patient samples (N=34) were used to examine the correlation between *AURKA*, *CHK1* and *PLK1* RNA expression and documented patient survival.

Dose dependent decreases in viability were observed in chondrosarcoma cell lines after treatment with MK-5108, LY2603618 and volasertib, with cell lines showing highest sensitivity to PLK1 inhibition. In addition increased sensitivity to conventional chemotherapy was observed after CHK1 inhibition in a subset of the cell lines. Interestingly, whereas *AURKA* and *CHK1* were both expressed in chondrosarcoma patient samples, *PLK1* expression was found to be low compared to normal cartilage. Analysis of patient samples revealed that high *CHK1* RNA expression correlated with a worse overall survival.

AURKA, CHK1 and PLK1 are identified as important survival genes in chondrosarcoma cell lines. Although further research is needed to validate these findings, inhibiting CHK1 seems to be the most promising potential therapeutic target for patients with chondrosarcoma.

Introduction

Chondrosarcomas account for 20% of primary bone tumors and are characterized by malignant cartilage producing cells [1]. Depending on the morphology and the location, chondrosarcoma can be subdivided into conventional chondrosarcoma and more rare subtypes; dedifferentiated chondrosarcoma, mesenchymal chondrosarcoma, clear cell chondrosarcoma and periosteal chondrosarcoma. Conventional chondrosarcoma accounts for 85% of all chondrosarcoma cases [1] and is further classified into central chondrosarcoma, located in the medulla of the bone, and peripheral chondrosarcoma, found at the surface of the bone [2]. These two different conventional chondrosarcoma subtypes show the same histological features: however a distinct molecular background is observed [1].Conventional chondrosarcoma is classified into three different grades, which is the most important prognostic factor. Atypical cartilaginous tumors (ACT)/chondrosarcoma grade I show a low cellularity, a large amount of cartilage matrix, rarely metastasize, and have a relatively good prognosis. Grade II and grade III chondrosarcomas behave more aggressive and show a more cellular histology with reduced cartilage matrix and a poor prognosis. Patients with grade II chondrosarcomas show 64% overall survival and patients with grade III chondrosarcomas show a very poor 10 years overall survival of only 29% [1, 2]. As chondrosarcomas are resistant to conventional radio- and chemotherapy, the only treatment option to date for patients with chondrosarcoma is surgical removal. This is a major problem especially for patients with tumors in inoperable locations and patients with metastatic disease [2].

Protein kinases are important for cellular processes and are often found deregulated in cancer [3]. Kinome profiling in chondrosarcoma cell cultures previously revealed that the AKT, Src and Ras/Raf/MEK pathways were most active in chondrosarcoma [4]. In addition, Src kinases were shown to be important for chemoresistance, as shown by sensitization for doxorubicin upon inhibition with dasatinib, as well as a high expression of Src kinase family members in chondrosarcoma patient tissues [5]. Using phospho-RTK arrays the phosphorylation status of 42 RTKs was investigated in chondrosarcoma cell lines, which led to the observation of a heterogeneous RTK activation pattern in these cells. P-S6 activation was found in 69% of conventional chondrosarcoma and 44% of dedifferentiated chondrosarcoma indicating that the downstream PI3K/mTOR pathway might be an important therapeutic target [6]. Furthermore, we previously showed a role for

mTORC1 and C2 as an important regulator of chondrosarcoma metabolism [7].

To further unravel the role of kinases in chondrosarcoma we chose a screening-based approach using siRNAs targeting 779 different kinases and kinase related genes. In addition a compound screen was performed consisting of 273 compounds targeting kinases implicated in survival pathways often deregulated in cancer. By comparing the hits we aim at identifying kinase regulated pathways that are important for chondrosarcoma survival.

Material and methods

Cell culture

Conventional chondrosarcoma cell lines SW1353 (ATCC), JJ012 [8], CH2879 [9], CH3573 [10] and L835, and dedifferentiated chondrosarcoma cell lines L3252B, L2975 [11] and NDCS1 [12] were cultured in RPMI-1640 (Gibco, Invitrogen Life-Technologies, Scotland, UK) supplemented with 10 or 20 % Fetal Calf Serum (Gibco, Invitrogen Life-Technologies, Scotland, UK). Mesenchymal chondrosarcoma cell line MCS170 [13] was cultured in IMDM medium (Gibco, Invitrogen Life-Technologies, Scotland, UK) supplemented with 15% fetal Calf Serum. All cell lines were cultured at 37ºC in a humidified incubator (5% CO_2). Identity of cell lines was confirmed using the Cell ID Gene Print 10 system (Promega Benelux BV, Leiden, The Netherlands) before and after completion of the experiments. Mycoplasma tests were performed on a regular basis.

Compounds

MK-5108 (S2770), LY2603618 (S2626), Volasertib (S2235) and ABT-737 (S1002) (positive control apoptosis assay) were purchased from Selleckchem and dissolved in DMSO to a working stock of 10 mM according to the manufacturer's instructions. Z-VAD-FMK was obtained from BD biosciences (550377). Doxorubicin and Cisplatin were obtained in a solution of 0.9% NaCl from the inhouse pharmacy of the Leiden University Medical Centre.

siRNA screen

To identify critical genes for chondrosarcoma cell survival, a focused targeted siRNA screen was performed on the JJ012 central chondrosarcoma cell line targeting kinases and kinase related genes (Dharmacon, GE life sciences, Landsmeer, the Netherlands, G-003505). Hits were selected for further validation, when both duplicates showed a reduction in cell numbers of 80% or more. A secondary screen including 35 most promising hits was performed in JJ012 and CH2879 chondrosarcoma cell lines (figure 1A). Reverse transfection was performed using SMARTpools of 4 different siRNAs targeting the same gene in a final concentration of 50 nM DharmaFECT 3 (Thermo Fisher Scientific Inc. Waltham, MA USA, T-2003) was used as a transfection reagent according to the manufacturer's instructions. Deconvolution confirmation screens, including 9 hits identified in both cell lines were performed on JJ012 and CH2879 cells where each of the four individual siRNAs was transfected separately. A gene was considered as a hit when three out of four individual siRNAs mimicked the SMARTpool in both cell lines or when one cell line showed at least three out of four the other at least two out of four siRNAs mimicking the SMARTpool. Mock (no siRNA), *GFP,* and *GAPDH* siRNAs were used as a negative control and *KIF11* siRNA as a positive control. Transfection was performed using 7000 cells/well for JJ012 and 10000 cells/well for CH2879 cells in µ-clear 96 well black clear bottom plates (Corning B.V. Life Sciences, Amsterdam, the Netherlands). 24 hours after transfection the medium was replaced with medium containing either 1 µM doxorubicin, 5 µM cisplatin or PBS and after five days cells were fixed with formalin and stained with Hoechst. Imaging was performed using a BD-pathway microscope. To quantify the amount of nuclei the total Hoechst area was determined using Image Pro analyzer software and normalized to mock treated cells as described previously [14].

Compound screen

A compound screen was performed in JJ012, SW1353 and CH2879 cells using a kinase library from Selleckchem (2014, L1200) containing 273 compounds targeting different pathways. SW1353 and JJ012 were plated at an optimal density of 5000 cells/well and CH2879 cells were plated at a density of 7000 cells/well. The screen was performed in duplicate in µ-clear 96 well black clear bottom plates (Corning B.V. Life Sciences, Amsterdam, the Netherlands). After overnight attachment of the cells, compounds were added in a concentration of 1 µM as single treatment or in combination with 0.05 µM doxorubicin or 0.8 µM cisplatin. A high concentration of doxorubicin (5 µM) was used as a positive control. After 72 hours of

incubation cell viability was assessed using Presto Blue viability reagent (see next paragraph).

Viability assay

Optimal cell amounts for each cell line were seeded in triplicate in 96-well plates. After 24h, increasing concentrations from 0–1000nM of MK-5108 and Volasertib or 0–1250nM LY2603618 were added to the appropriate wells and cells were incubated for an additional 72h. After the incubation period, a Presto Blue assay (Thermo Fisher Scientific Inc. Waltham, MA USA, A13262) was carried out according to the manufacturer's instructions. After 1 hour, viability results were measured by fluorescence at 590 nm on a fluorometer (Victor3V, 1420 multilabel counter, Perkin-Elmer, Groningen, the Netherlands). All experiments were performed in triplicate at least 3 times.

RNA isolation and Quantitative real time PCR

RNA was isolated from fresh frozen tissue of 34 conventional chondrosarcoma primary tumor tissues and six cartilage control tissues: three growth plates, and three articular cartilage tissues (Supplementary table 1). RNA was isolated using TRIzol (Invitrogen, Carlsbad CA) followed by RNA clean up using the RNeasy mini kit (Qiagen) according to the manufacturer's instructions. All samples were handled as approved by the LUMC ethical board (B17.021). AURKA, CHK1 and PLK1 expression was normalized towards housekeeping genes PPIA and CPSF6 as previously described [15]. Primer pairs are described in supplementary table 2.

Cell cycle analysis

Optimal cell amounts for each cell line were seeded in 6-well plates and allowed to attach overnight. Cells were treated with IC_{50} concentrations of LY2603618, MK-5108 or Volasertib. After 24h, cells were stained with Solution 18 Ao-DAPI (Catalog no.910-3018, Chemometec, Denmark) according to the manufacturer's instructions and cells were counted using an automated cell analyzer (NucleoCounter NC-250, Chemometec, Denmark). Remaining cells were centrifuged for 5 minutes at 500g at 4°C. Supernatant was removed and the cells were washed with PBS (B. BraunMelsungen AG, Melsungen, Germany). Methanol fixation was carried out, after which cells were washed with PBS/Tw 0.05%. The samples were

centrifuged using the same settings and were then washed with PBA/Tw 0.05% (PBS/1.0% BSA/Tw 0.05%). After the final centrifugation of the cells, cells were stained with10 µM DAPI in PBA/Tw 0.05%. The cells were stored at 4 degrees and analysis was carried out next day using the NC-250 nucleoCounter. Results were analyzed using, Winlist 3D and ModFit LT software (Verity software house). Five biological replicates were included for each sample.

Apoptosis assay

The caspase glo 3/7 assay (Promega, Madison, WI, USA) was used to detect apoptosis, according to the manufacturer's instructions. Cells were plated in a white 96-well plate and treated the next day with IC_{50} concentrations (obtained from dose response curves) of MK-5108, LY2603618, Volasertib for 24 hours. ABT-737 and Doxorubicin were added to the cells as a positive control. Z-vad-FMK, a caspase inhibitor was used as a positive control. After treatment period, caspase glo 3/7 assay was added to cells, which were incubated for an additional 30 minutes at room temperature. Caspase activity was measured by luminescence using a luminometer (Victor3V, 1420 multilabel counter, Perkin-Elmer) according to the manufacturer's instructions. Experiments were performed three times in duplicate.

Western blotting

Protein expression of Chk1 (Cell signaling technology #2360) and p-Chk1(S345) (Cell signaling technology, #2348) was determined in JJ012, SW1353 and CH2879 in control conditions and after treatment for 2 or 24 hours with IC50 concentrations of LY2603618 (JJ012 1µM, SW1353 441 nM, CH2879 449 nM). In addition PARP cleavage (Cell signaling technology #9532) was assessed after treatment of JJ012, SW1353 and CH2879 for 2 or 24h with IC50 concentrations of MK-5108, LY2603618 or Volastertib (MK-5108; SW1353: 1 µM, JJ012: 513 nM, CH2879: 847 nM, Volasertib: SW1353: 34 nM, JJ012 11nM, CH2879: 24 nM, LY2603618 as described above). Lysates were obtained of cells grown until 70% confluence using hot-SDS buffer (1% SDS, 10 mM Tris/EDTA with complete inhibitor (Roche #11697498001) and phosSTOP (Roche #04906837001) as previously described [4]. Expression of gapdh (Cell signaling technology #5174) was determined as a loading control. A total of 10 µg was loaded on the gel for each sample and blocking was performed using 5% milk. Primary antibodies were diluted in 5% BSA (bovine serum albumin) and incubated overnight.

Blotting was performed on PVDF membranes and detection was done using enhanced chemo-luminescence (west Pico Plus chemiluminescent Substrate, Thermo Fisher Scientific, Waltham, MA, USA) followed by visualization using the ChemiDoc imaging system of Biorad.

Statistical analysis

Dose response curves and IC_{50} values were determined using Prism 7 GraphPad software. Statistically significant differences were assessed by performing a 2-way ANOVA test, correcting for multiple comparisons using Turkey's test. To assess synergy access over Bliss was calculated and percentages above 12 were considered as synergistic [16, 17]. Overall survival was determined using SPSS software by performing a Kaplan Meyer analysis and assessing significance using a Mantel Cox Log Rank test.

Results

siRNA screen identifies PLK1, AURKA, COPB2, CHEK1, and CNKSR1 as most important survival genes in chondrosarcoma cells.

A siRNA screen targeting 779 kinases and kinase related genes identified 35 genes that decreased survival of JJ012 chondrosarcoma cells more than 80% upon inhibition in both duplicate measurements (see figure 1A, B and supplementary table 3). No siRNAs were identified that could sensitize JJ012 cells to doxorubicin or cisplatin. Transfection with control *GFP* siRNAs led to a slight reduction in cell amounts compared to mock conditions in most plates, however silencing of *GAPDH* led to a large reduction in cell amounts, indicating that this cannot be used as a control for CS siRNA screens (supplementary figure 1). Knock down of *KIF11* led to a strong reduction in cell amounts, indicating successful knock down in all plates except plate 7 which was treated with doxorubicin or cisplatin. This did not influence the hit selection process. A second validation screen was performed in JJ012 and CH2879 cell lines including the 35 most promising siRNAs to select genes important for both cell lines. This reduced the number of candidates to eight that were found in both JJ012 and CH2879 cells (Figure 1C). The remaining 19 targets, from the first JJ012 screen, could not be reproduced/identified in JJ012 and were absent as well in CH2879 cells. The eight hits identified in both JJ012 and CH2879 were selected for deconvolution and *PLK1*, *AURKA*, *CHK1*, *COPB2* and *CNKSR1* were confirmed (Figure 1D).

Compound screening identifies cell cycle regulators as most promising targets in chondrosarcoma cell lines.

In parallel with the siRNA screen a compound screen including 273 compounds targeting kinases and kinase related pathways was performed in three different chondrosarcoma cell lines. The top 50 compounds, that showed the highest reduction in cell numbers as compared to DMSO treated controls, of each cell line were compared and 34 compounds were found to be effective in all three cell lines (Figure 1E), while in total 48 compounds were shared between at least 2 cell lines. The percentage of compounds targeting a specific pathway as compared to the total of 48 hit compounds are represented in figure 1F. Inhibitors of cell cycle regulators Aurora kinases (13/16), Polo like kinases (5/5) and Cyclin dependent kinases (4/12)represent a substantial portion (figure 1F), confirming the findings of the siRNA screen. Also, inhibitors of the Pi3K and mTOR pathway (12 / 25) are one of the major hits. Furthermore, inhibitors of cMET (2/13), ALK (1/2), SRC (1/3), SYK (1/5), JAK (1/13), IKK (1/2) and CHK (1/3) are also represented (see supplementary table 4 for a list of selected compounds). Similar to the siRNA screen, no pathways were identified that upon inhibition clearly sensitized for either doxorubicin or cisplatin (supplementary table 5). Based on the overlap between the siRNA and the kinase inhibitor screen we chose to continue with PLK1, AURKA and CHK1 as most promising targets. In addition, previous studies already showed that mTOR, CDK and Src are important therapeutic targets for patients or subsets of patients with chondrosarcoma [4, 18, 19].

Inhibition of AURKA, CHK1 and PLK1 in chondrosarcoma cells results in a dose dependent decrease in viability, which is not related to RNA expression levels

Dose dependent decreases in viability were observed when different chondrosarcoma cell lines were treated with inhibitors for AURKA (MK-5108), CHK1 (LY2603618) or PLK1 (Volasertib) (Figure 2A). Lowest IC_{50} values were obtained when cell lines were treated with PLK1 inhibitor volasertib, however treatment with CHK1 inhibitor LY2603618 also led to low IC_{50} values (see Table 1). These values have previously been shown as clinically achievable in human plasma in phase I and phase II trials [20-22]. L835, L3252 and MCS170, which are slower growing*TP53* wildtype cell lines showed higher IC_{50} values for all compounds compared to the other faster growing cell lines. RNA expression analysis showed a variable expression

pattern of *AURKA*, *CHK1* and *PLK1* across the cell lines, with highest expression of all three in CH2879 (Figure 2B). Only few cell lines responded to inhibition of AURKA, which was not correlated to the level of *AURKA* expression. Likewise, no correlation was observed between sensitivity to CHK1 inhibition and *CHK1* expression levels. Interestingly, of the three lines showing low *PLK1* expression, L835 and MCS-170 showed a poor response to PLK1 inhibition, whereas L2975 exhibited a response, which although *PLK1* expression was lower, still resembled the response patterns of high *PLK1* expressors, but never reached 0% cell viability. Combination treatment with doxorubicin or cisplatin and MK-5108 and LY2603618 was performed in three conventional chondrosarcoma cell lines and results showed a synergistic effect between doxorubicin and cisplatin with the CHK1 inhibitor LY2603618 in JJ012 and SW1353 cell lines as shown by more than 12% increase in Excess over Bliss score (Figure 2C). This shows that chondrosarcoma cells are more sensitive towards treatment with chemotherapy when CHK1 is inhibited. Increased phosphorylation of CHK1 on Ser345 was observed after treatment with LY2603618 in JJ012, SW1353 and CH2879, indicating that inhibiting CHK1 under these conditions leads to activation of ATM/ATR (Figure 2D). No basic CHK1 phosphorylation was observed on Ser345.

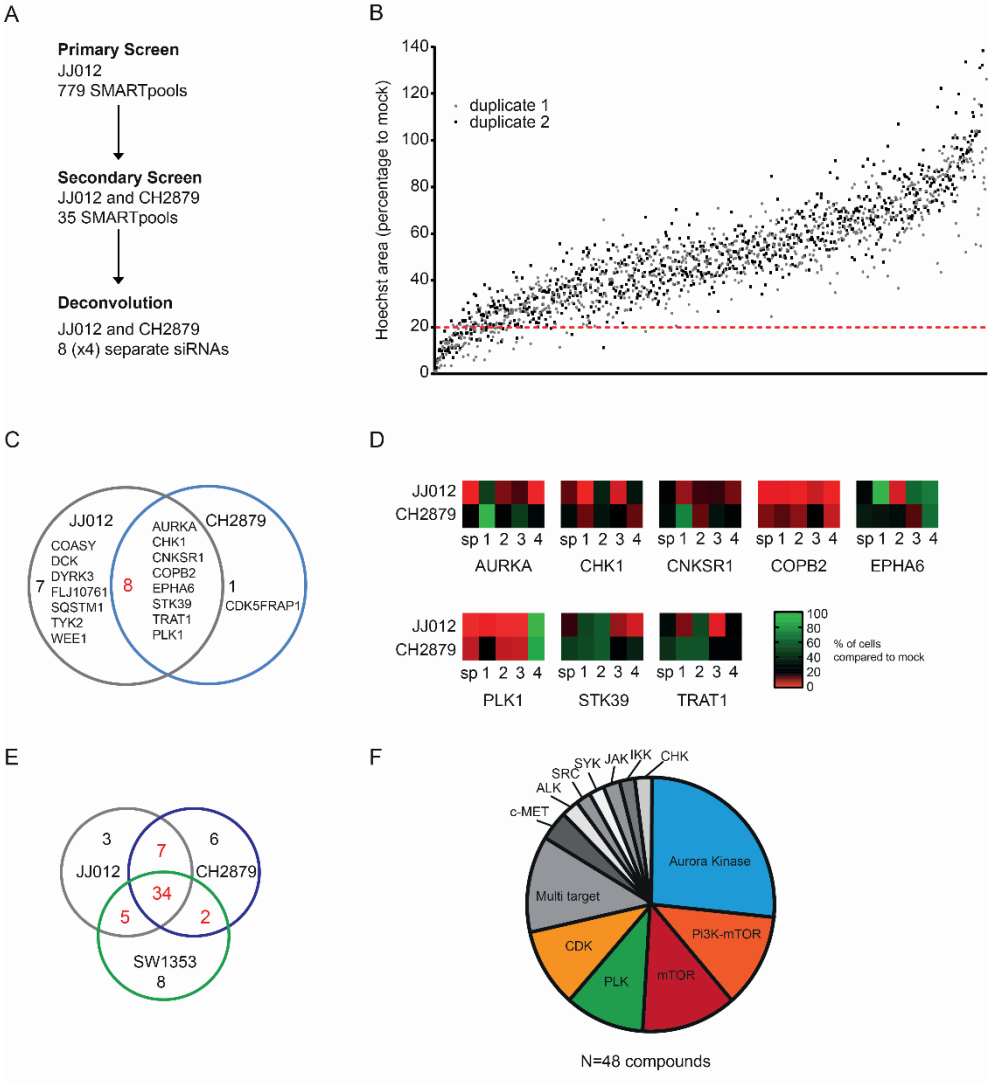


Figure 1. SiRNA screen and compound screen identify PLK1, AURKA and Chk1 as potentially important kinases for survival of chondrosarcoma cells.
A. Set-up of siRNA screen. Primary screening was performed on 779 SMARTpools targeting kinases and kinase related genes. The secondary screen was performed in JJ012 and CH2879 cells and consisted of 35 SMARTpool siRNAs identified in the primary screen (decreased cell proliferation below 20% compared to mock conditions). Deconvolution consisted of 4 separate siRNAs and the SMARTpool targeting 9 different genes. B. Hoechst area as a percentage to mock for JJ012 cells. Each dot represents one SMARTpool targeting one Kinase or kinase related gene. Duplicates are shown for each gene and only when both screens showed a percentage below 20% it was considered as a hit. C. Kinases that showed cell killing in both JJ012 and CH2879 were selected for deconvolution (AURKA, CHK1, CNKSR1,

COPB2, EPHA6, IRAK3, STK39, TRAT1, PLK1). D. Deconvolution results in JJ012 and CH2879 cells showing that AURKA, CHK1, COPB2, CNKSR1 and PLK1 are important for cell survival in both cell lines. E. Compound screen results in JJ012, CH2879 and SW1353 showing 35 hits in common in the top 50 compounds in each cell line. In addition, 8 compounds were found in JJ012 and CH2879, 6 in JJ012 and SW1353 and 2 in CH2879 and SW1353. F. Compounds that were identified in all three or two out of three cell lines were selected and showed that Aurora kinase, Pi3K-mTOR, mTOR, PLK, CDK and multi-target comprised the largest groups. In addition, compounds targeting c-MET, ALK, SRC, SYK, JAK, IKK and CHK were identified.

Table 1. IC_{50} values and 95% confidence intervals for nine different chondrosarcoma cell lines treated with inhibitors for AURKA (MK-5108), CHK1 (LY2603618) or PLK1(Volasertib).

	MK-5108	**Ly2603618**	**Volasertib**
JJ012	513.4 (464.2-570.1)	>1000	10.98 (10.31-11.73)
SW1353	>1000	441.2 (386.6-502.8)	33.67 (31.76-35.66)
CH2879	847.6 (723.3-1049)	449.3 (401-504.6)	24.28 (20.69-28.18)
CH3573	>1000	815.2 (730.2-918.4)	8.382 (7.083-9.809)
L835	>1000	>1000	>1000
L2975	520.8 (440.5-631)	826.1 (781.3-875.8)	11.33 (9.109-13.92)
Ndcs1	>1000	442.6 (420.5-465.8)	3.166 (2.54-4.003)
L3252	>1000	>1000	252.4 (187.1-363.9)
MCS170	>1000	>1000	276.1 (181.2-482)

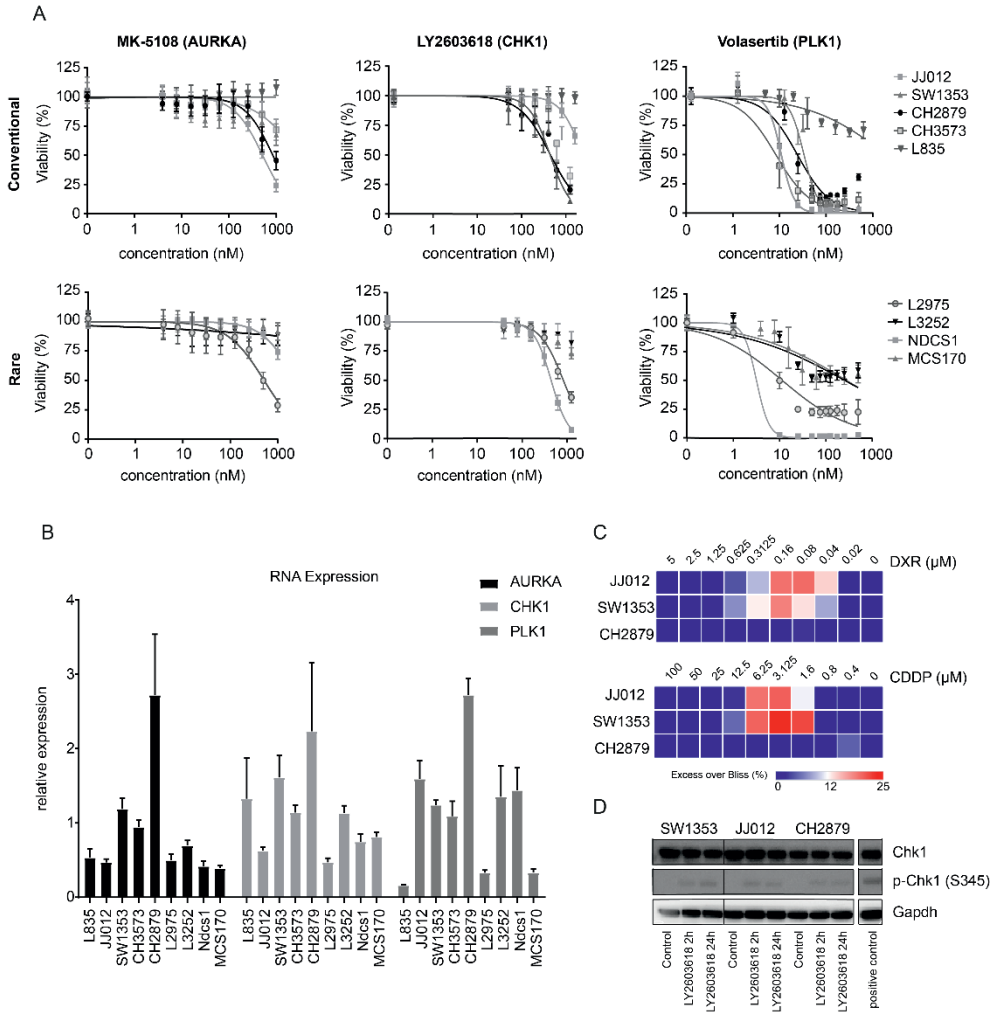


Figure 2. Chondrosarcoma cell lines are sensitive for compounds targeting AURKA, CHK1 and PLK1.

A. Dose response curves showing viability measured after 72h using presto blue viability reagent for 9 chondrosarcoma cell lines targeting AURKA (MK-5108), CHK1 (LY2603618) or PLK1 (volasertib). The top three panels represent the conventional chondrosarcoma cell lines and the bottom panel the rare chondrosarcoma cell lines including three dedifferentiated cell lines (L2975, L3252 and NDCS1) and one mesenchymal chondrosarcoma cell line (MCS-170). Highest sensitivity is observed after inhibition with Volasertib. Experiments were performed in triplicate at least three times. B. RNA expression in chondrosarcoma cell lines for *AURKA*, *CHK1* and *PLK1*. No correlation between expression and sensitivity for the different inhibitors is observed. C. Excess over Bliss percentages of combination treatment of 100 nM LY2603618 and doxorubicin (DXR) or cisplatin (CDDP) showing that JJ012 and SW1353 can be sensitized to conventional chemotherapy after Chk1 inhibition. D.

Western blot showing Chk1 and P-chk1 (S345) expression after treatment for 2 or 24h with IC50 concentrations of LY2603618. Gapdh expression is assessed as a loading control. Hela cells treated with Hydroxyurea have been used as a positive control for p-Chk1 expression.

Cell cycle analysis reveals a block in G2 after AURKA inhibition

Cell cycle analysis was carried out after 24 hours of treatment with MK-5108, LY2603618 or Volasertib and showed that inhibition of AURKA with MK-5108 caused a block in G2/M in both JJ012 ($p<0.0001$) and CH2879 ($p<0.0001$) cell lines (Figure 3A, Supplementary figure 2). Treatment with CHK1 inhibitor LY2603618 did result in an increase in S-phase ($p=0.0003$) and debris ($p=0.0004$) in JJ012, but not in CH2879.Thus, while inhibiting AURKA clearly showed a G2/M arrest in both cell lines, only JJ012 showed a clear S-phase arrest after CHK1 inhibition. To assess whether this cell cycle arrest led to apoptotic cell death, caspase 3/7 dependent apoptosis was evaluated after 24 hours of treatment with MK-5108, LY2603618 or Volasertib (Figure 3B). Results were variable, but small increases were observed after treatment with MK-5108 or Volasertib in SW1353. PARP cleavage was assessed to determine general cell death and showed a small amount of PARP cleavage, especially in MK-5108 treated JJ012 and CH2879 cells, but no clear differences were observed (Figure 3C). These results indicate that even though the cell cycle inhibitors successfully caused cell cycle arrest in both cell lines, this did not directly lead to an increase in cell death after 24hrs.

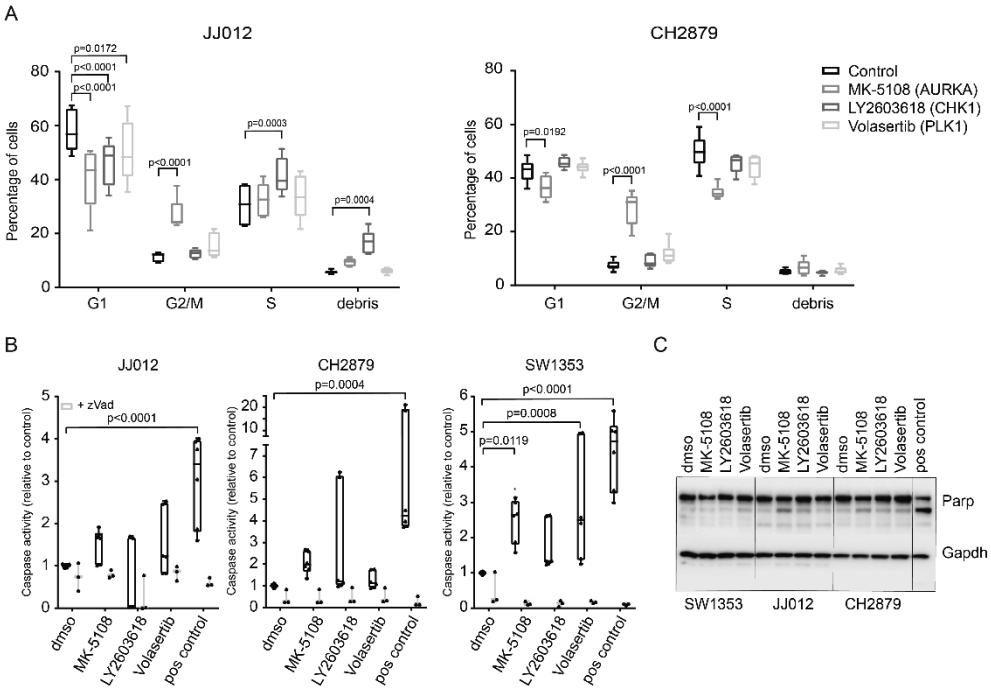


Figure 3. Cell cycle and apoptosis analysis after AURKA, CHK1 or PLK1 inhibition. A. Cell cycle analysis after 24 hours of treatment with MK-5108, LY2603618 or Volasertib in JJ012 or CH2879 cells. Both cell lines show a decrease in G1 and an increase in G2 phase after treatment with MK-5108. In addition, CH2879 cells show a decrease in S phase after treatment with MK-5108. JJ012 cells show a decrease in G1 after inhibition with either MK-5108, LY2603618 or Volasertib and an increase in S-phase and debris after LY2603618 treatment. B. Apoptosis induction measured using the caspase-glo 3/7 kit in JJ012, CH2879 and SW1353 after treatment for 24h with IC_{50} concentrations of MK5108, LY2603618 or Volasertib. Z-vad was added as a control. Only the positive control showed significant caspase induction in all cell lines. SW1353 showed significant upregulated caspase activity after treatment with MK-5108 and Volasertib compared to dmso treated controls. For both cell cycle and apoptosis experiments mean values are shown of three experiments performed in duplicate. P-values were calculated using a 2way ANOVA test, correcting for multiple comparisons using Tukeys test. C PARP cleavage assessed after 24h of treatment using IC50 concentrations of MK-5108, LY2603618 and Volasertib. As a positive control CH2879 cells treated with the combination of ABT-737 and doxorubicin has been taken along

High CHK1 RNA expression in chondrosarcoma tissue samples is correlated with a worse overall survival

Expression of *AURKA*, *CHK1* and *PLK1* was investigated in a panel of chondrosarcoma tissue samples and revealed that both *AURKA* and *CHK1* showed higher RNA expression compared to cartilage in a subset of chondrosarcoma patients (Figure 4A). *PLK1* expression was lower compared to normal cartilage in all samples tested, but high in chondrosarcoma cell lines. Most samples that showed higher *AURKA* or *CHK1* expression compared to normal cartilage were in the high-grade group, but this was not significantly different. Samples were divided into high and low expression based on expression compared to normal cartilage (>1=high expression, <1=low expression) and survival analysis was performed (Figure 4B). No significant difference in survival was observed between low or high expression of *AURKA* (p=0.131), but high *CHK1* expression was significantly correlated with a worse overall survival compared to low *CHK1* expression (P=0.018). These results show that *CHK1* expression is correlated towards a poor prognosis in chondrosarcoma patients.

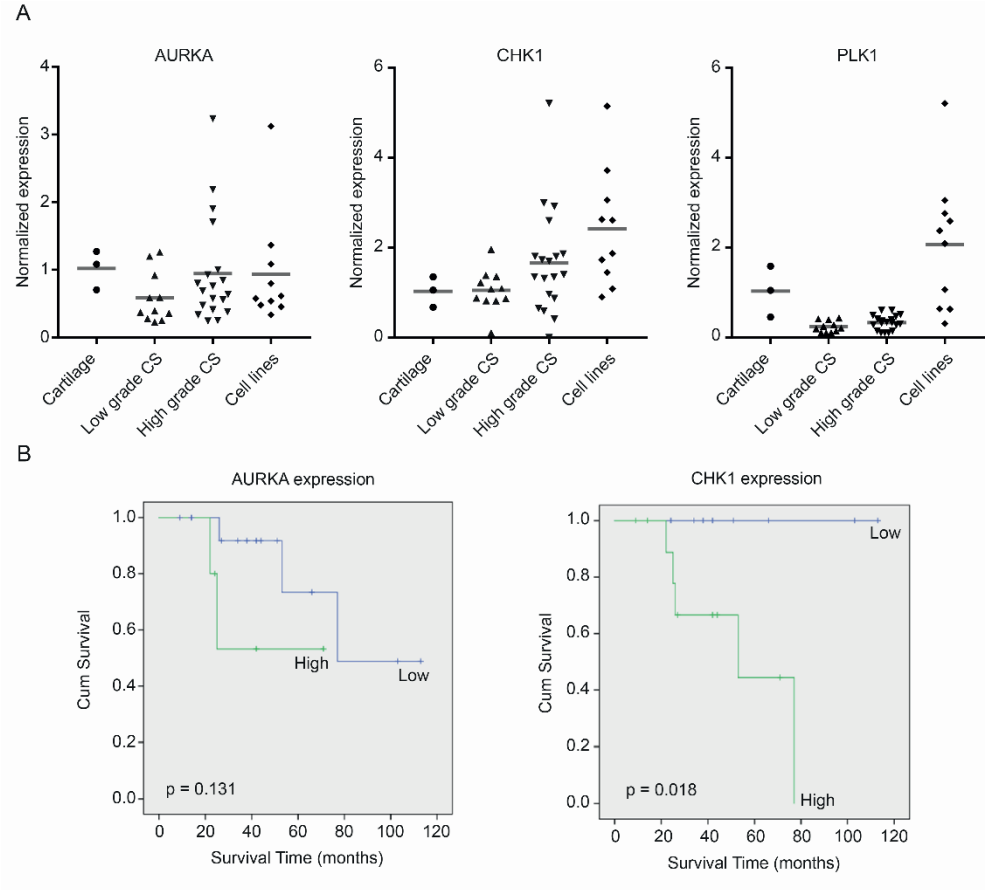


Figure 4. Gene expression analysis shows that high expression of *CHK1* is correlated to a worse survival in chondrosarcoma patients.
A.RNA expression of *AURKA*, *CHK1* and *PLK1* in chondrosarcoma patient samples and chondrosarcoma cell lines compared to expression in normal articular cartilage samples. Each dot represents one sample. B. Kaplan Meyer analysis of patient samples with low and high expression of *AURKA* and *CHK1*. High *CHK1* expression shows a significant correlation with a worse overall survival in chondrosarcoma patients. Samples with <1 expression were considered as 'low' expression and samples >1 expression were considered as high expression. P values were calculated using a mantel cox log rank test.

Discussion

Chondrosarcoma patients suffer from limited treatment options due to relative chemo- and radio resistance. In this study we sought to identify new targetable pathways in chondrosarcoma cells by performing a kinase focused siRNA and compound screen. By comparing the hits that were identified in both screens we identified AURKA, PLK1 and CHK1 as important survival regulators in chondrosarcoma cells. AURKA, PLK1 and CHK1 are all involved in cell cycle regulation and often deregulated in cancer cells [23].

Previous studies in chondrosarcoma already revealed that Src kinases could be a potential therapeutic target in chondrosarcoma [5]. In addition mTOR was shown to be active in a large portion of chondrosarcoma patient samples, as well as an important regulator of chondrosarcoma metabolism [6, 7]. These hits were also confirmed in our compound screen data.

Cell cycle progression is tightly regulated and controlled by cyclin dependent kinases (CDKs). Activity of CDKs is induced by mitogenic signals but can be inhibited by cell cycle checkpoints in response to DNA damage. Proteins that function in regulating the cell cycle are often deregulated in cancer and can function as possible therapeutic targets as mono therapy or combination with chemotherapy [24]. In this study we show a possible role for targeting the cell cycle in chondrosarcoma.

AURKA and PLK1 are involved in G2 to M phase progression and are essential during mitosis and cytokinesis. AURKA phosphorylates PLK1 to activate Cyclin B-CDK1 complexes leading to progression from G2 to M-phase. During mitosis AURKA and PLK1 form several complexes with other proteins that regulate the maturation and separation of centrosomes and the assembly of the bipolar spindle. Furthermore PLK1 is a member of the chromosomal passenger complex (CPC) that regulates chromosome dynamics and cohesion, kinetochore microtubule attachments, spindle assembly checkpoint and cytokinesis [25]. Overexpression of both AURKA and PLK1 has been shown in a variety of different tumors [26, 27].

A subset of chondrosarcoma cell lines showed a dose dependent decrease in viability after inhibiting AURKA using MK-5108, which was not related towards *AURKA* RNA expression. A clear block in G2/M phase of the cell cycle was observed after 24 hours of treatment with MK-5108. We did not find any correlation between expression of *AURKA* and survival, in contrast to a study from 2012 by Liang et al. in which they showed a correlation

between high AURKA expression and a worse overall survival in chondrosarcoma patients [28]. A clinical study including 6 chondrosarcoma patients investigating the efficacy of Aurora Kinase A inhibition using Alisertib showed a partial response in one patient with dedifferentiated chondrosarcoma [29]. However a phase III study in lymphoma was discontinued because of lack of response compared to the other study arm [30]. Different studies testing combination strategies show different adverse effects and maximum tolerated dose, which indicates that results might vary between tumor types and patients [21, 31-33]. Recently AURKA inhibitors have been identified as synthetic lethal with defective RB1 [34]. In chondrosarcoma 33% of tumors show a defect in the RB1 pathway [35], meaning that in these tumors treatment with AURKA inhibitors might be good treatment option.

Chondrosarcoma cell lines were sensitive for inhibition of PLK1 using Volasertib, but no clear cell cycle effects were observed after 24 hours of treatment. This is surprising because PLK1, just like AURKA, is important for entry in M-phase, although multiple other non-cell cycle related functions have been proposed as well, for example PLK1 can regulate mTORC1 activity [36, 37]. This can influence the effects that we detect on the cell cycle. Expression of PLK1 in chondrosarcoma tissue samples was low, indicating that targeting PLK1 might not be a good therapeutic strategy for chondrosarcoma patients.

CHK1 is activated by ATM or ATR after the occurrence of DNA damage. Its activation will cause the cell to halt cell proliferation in S or G2 and allows the cell to repair DNA damage. When CHK1 is inhibited DNA damage will accumulate and the cell will die during mitosis. Like AURKA and PLK1, CHK1 is also overexpressed in different types of cancer. Like AURKA and PLK1, CHK1 is also overexpressed in different types of cancer. Using LY2603618 CHK1 was inhibited in a panel of chondrosarcoma cell lines, which resulted in an increased phosphorylation of CHK1 at position S345, which is in agreement with previous reports [38]. Possibly single CHK1 inhibition already leads to activation of the DNA damage response, and IC_{50} concentrations are not high enough to completely prevent phosphorylation by ATM/ATR. A subset of chondrosarcoma cell lines was responding to the treatment, and an increase in S phase was observed in JJ012 cells, but not in CH2879 cells treated with LY2603618. In addition, JJ012 and SW1353 could be sensitized for chemotherapy using CHK1 inhibition. Previous studies already showed the importance of CHK1 in Ewing, Osteo- and soft tissue sarcomas [39-41] and its use in single and combination treatment in

pre-clinical models. In addition, we also found a correlation between overall survival and *CHK1* RNA expression in chondrosarcoma patient tissues, indicating that more aggressive chondrosarcomas show higher expression of *CHK1*. Second generation CHK1 inhibitors, showing less toxicity compared to first generation inhibitors are currently tested in the clinic in combination with chemotherapy in advanced cancers [42-44].

Cells with defective P53 protein function have been shown to be more sensitive to inhibitors for CHK1 [45] as well as AURKA and PLK1 compared to cells with intact P53 [46, 47]. Inactivation of P53 will lead to a compromised G1 checkpoint, which makes mutated cells more dependent on the G2 checkpoint to be able to repair DNA damage. In our study we do observe a difference in sensitivity between *TP53* wildtype and *TP53* mutant chondrosarcoma cell lines; cell lines with intact P53 are less sensitive to inhibition of AURKA, CHK1 or PLK1. However, these cells also grow slower compared to the other cell lines, which could confound the observed difference in response. Mutations in *TP53* have been observed in 20% of chondrosarcomas [35], indicating that inhibitors of cell cycle regulators might be of interest especially for these patients.

In conclusion, we performed a kinase focused siRNA and compound screen and identified cell cycle regulators AURKA, CHK1 and PLK1 as interesting targetable proteins for follow up studies. RNA expression analysis revealed expression of *AURKA* and *CHK1* in a subset of chondrosarcoma patients, while *PLK1* expression was minimal, compared to normal cartilage tissue. In addition high *CHK1* expression was correlated towards a decrease in survival time. Also inhibition of CHK1 could sensitize a subset of chondrosarcoma cells towards chemotherapy. Future studies should determine the role of cell cycle proteins in chondrosarcoma, however based on our results CHK1 seems to be a promising therapeutic candidate for patients with (*TP53* mutated) chondrosarcoma, especially in combination strategies.

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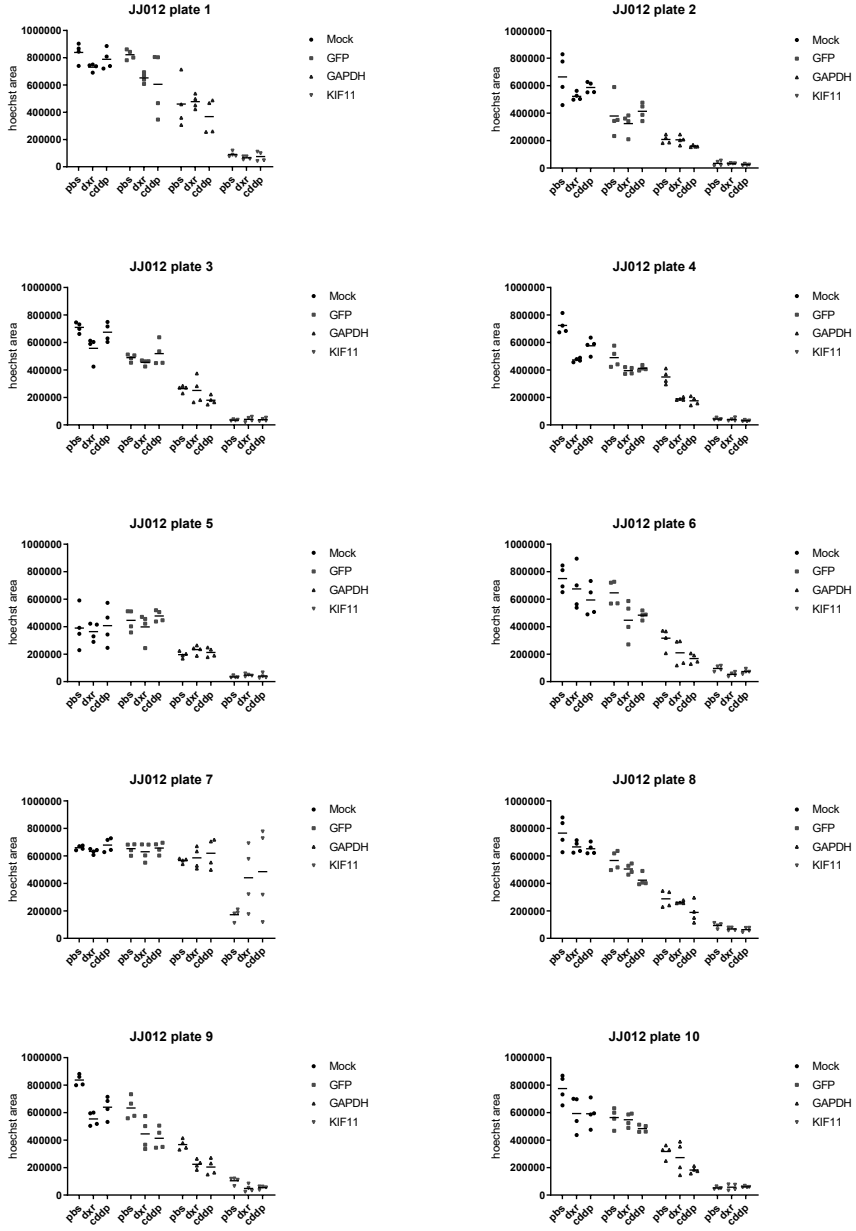
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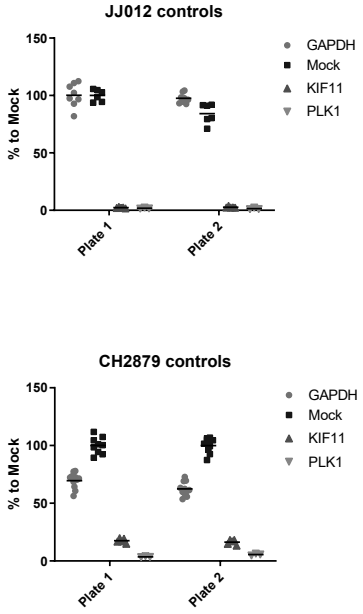
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Supplementary material

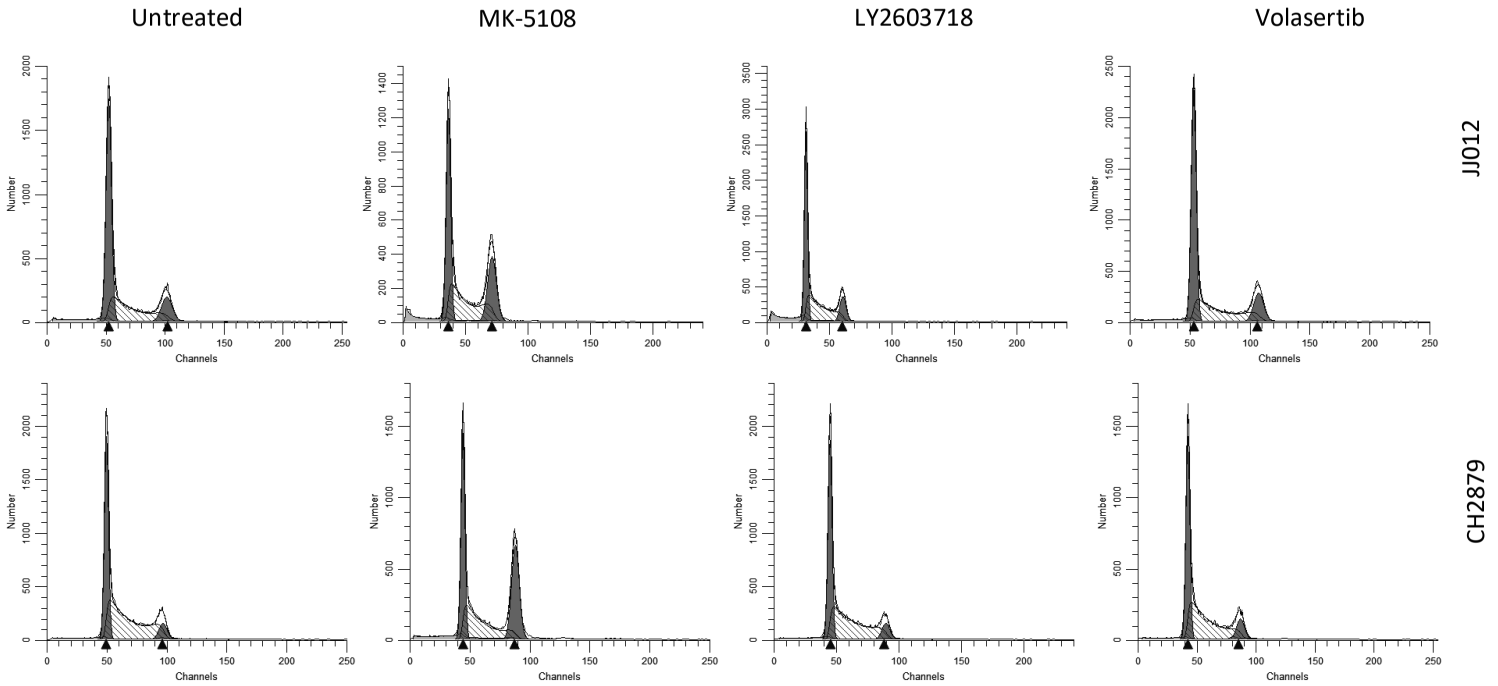


Controls primary screen



Controls secondary screen

Supplementary figure 1. Controls primary and secondary siRNA screens



Supplementary figure 2. Representative images of cell cycle profiles after 24h of treatment with IC50 concentrations of MK-5108, LY2603718 or Volasertib

Supplementary table 1. Patient and control samples included in RNA expression analysis.

Sample	Grade	Amount
Conventional Central chondrosarcoma	Low (grade 1)	11
	High (grade 2+3)	19
Growth plate	N/A	3
Cartilage	N/A	3

Supplementary table 2. Sequences of primer pairs used for RNA expression analysis.

PLK1 F	AGAGACCTACCTCCGGATCAA
PLK1 R	GCAGCTCGTTAATGGTTGGG
CHK1 F	CTTACTGCAATGCTCGCTG
CHK1 R	GAGCTAGAGGAGCAGAATCG
AURKA F	CTCCAGTCACAAGCCGGTTC
AURKA R	GCCAGTTCCTCCTCAGGATTAT

Supplementary table 5. IC_{50} values and 95% confidence intervals for nine different chondrosarcoma cell lines treated with inhibitors for AURKA (MK-5108), CHK1 (LY2603618) or PLK1(Volasertib).

	MK-5108 (AURKA)	**LY2603618 (CHK1)**	**Volasertib (PLK1)**
JJ012	513.4 (464.2-570.1)	>1000	10.98 (10.31-11.73)
SW1353	>1000	441.2 (386.6-502.8)	33.67 (31.76-35.66)
CH2879	847.6 (723.3-1049)	449.3 (401-504.6)	24.28 (20.69-28.18)
CH3573	>1000	815.2 (730.2-918.4)	8.382 (7.083-9.809)
L835	>1000	>1000	>1000
L2975	520.8 (440.5-631)	826.1 (781.3-875.8)	11.33 (9.109-13.92)
Ndcs1	>1000	442.6 (420.5-465.8)	3.166 (2.54-4.003)
L3252	>1000	>1000	252.4 (187.1-363.9)
MCS170	>1000	>1000	276.1 (181.2-482)

Additional supplementary materials are available online:
https://www.sciencedirect.com/science/article/pii/S2212137419301423?via%3Dihub#ecom0001

Chapter 7

Exploration of the chondrosarcoma metabolome; the mTOR pathway as an important pro-survival pathway

This chapter is based on the publication:
de Jong Y*, Addie RD*, Alberti G, Cleton-Jansen AM, Corver W, Morreau H, Kruisselbrink AB, Que I, Baelde H, Bovée JVMG, Exploration of the chondrosarcoma metabolome; mTOR pathway as an important pro-survival pathway. J Bone Oncol. 2019 Jan 29;15:100222.

Abstract

Chondrosarcomas are malignant cartilage-producing tumors showing mutations and changes in gene expression in metabolism related genes. In this study we aimed to explore the metabolome and identify targetable metabolic vulnerabilities in chondrosarcoma.

A custom-designed metabolic compound screen containing 39 compounds targeting different metabolic pathways was performed in chondrosarcoma cell lines JJ012, SW1353 and CH2879. Based on the anti-proliferative activity, six compounds were selected for validation using real-time metabolic profiling. Two selected compounds (rapamycin and sapanisertib) were further explored for their effect on viability, apoptosis and metabolic dependency, in normoxia and hypoxia. In vivo efficacy of sapanisertib was tested in a chondrosarcoma orthotopic xenograft mouse model.

Inhibitors of glutamine, glutathione, NAD synthesis and mTOR were effective in chondrosarcoma cells. Of the six compounds that were validated on the metabolic level, mTOR inhibitors rapamycin and sapanisertib showed the most consistent decrease in oxidative and glycolytic parameters. Chondrosarcoma cells were sensitive to mTORC1 inhibition using rapamycin. Inhibition of mTORC1 and mTORC2 using sapanisertib resulted in a dose-dependent decrease in viability in all chondrosarcoma cell lines. In addition, induction of apoptosis was observed in CH2879 after 24h. Treatment of chondrosarcoma xenografts with sapanisertib slowed down tumor growth compared to control mice.

mTOR inhibition leads to a reduction of oxidative and glycolytic metabolism and decreased proliferation in chondrosarcoma cell lines. Although further research is needed, these findings suggest that mTOR inhibition might be a potential therapeutic option for patients with chondrosarcoma.

Introduction

Chondrosarcomas, a group of malignant cartilage producing tumors, can be divided into different subtypes of which conventional chondrosarcoma is the most frequent (85%) followed by dedifferentiated chondrosarcoma (10%). The remaining 5% of chondrosarcomas consist of the rare subtypes mesenchymal chondrosarcoma, clear cell chondrosarcoma and periosteal chondrosarcoma. Conventional central chondrosarcomas are found in the medulla of the bone and histological grading is the most important prognostic factor. Atypical cartilaginous tumors (ACT) / chondrosarcomas grade I show a ten years survival rate of 83%, Grade II chondrosarcomas 64% and grade III chondrosarcomas only 29% [1]. Chondrosarcomas are treated by surgery, since these tumors show limited response to conventional chemo- and radiotherapy [2, 3]. This means that patients with inoperable disease do not have any treatment options with curative intent, emphasizing the need to develop novel targeted therapies.

Increasing evidence has shown the importance of metabolic processes and their relation to the activation of oncogenes or inactivation of tumor suppressor genes in cancer cells [4]. Therefore, targeting the cancer metabolism has been explored and has resulted in several potential therapeutic targets that are currently tested in the clinic [5]. In chondrosarcoma cells metabolic processes are deregulated as well. cDNA microarrays showed an up-regulation of glycolysis and down regulation of oxidative phosphorylation related genes in high grade central chondrosarcomas compared to low grade central chondrosarcomas [6]. In addition, hypoxia related genes HIF1A and its downstream target carbonic anhydrase (CA) IX were found to be up-regulated in high grade compared to low grade chondrosarcomas, and high expression was correlated with a shorter metastasis free survival [7]. Moreover, activation of the mTOR pathway, which plays a central role in a variety of different metabolic processes, was suggested in 69% of conventional and 44% of dedifferentiated chondrosarcoma based on immunohistochemistry [8]. Next to differences in expression levels also mutations in genes involved in metabolism are found in chondrosarcoma. Mutations in *isocitrate dehydrogenase 1* and *2* (*IDH1* and *IDH2*) are found in ~50% of central and dedifferentiated chondrosarcomas [9-12]. *IDH1* and *IDH2* are enzymes that convert isocitrate to alpha ketoglutarate (αKG) in the Krebs cycle. Mutations in *IDH1* or *IDH2* genes lead to the production of high levels of the oncometabolite D2-hydroxyglutarate (D2HG) as well as changes in the cellular metabolome through changes in levels of amino acids, glutathione

metabolites, choline derivatives and TCA intermediates [13-15]. *TP53* mutations have been identified in ~20% of chondrosarcomas especially of higher histological grade [16-18]. P53 is a tumour suppressor protein with important functions in controlling cell proliferation and apoptosis as well as being a regulator of several metabolic processes including glycolysis and mitochondrial metabolism [19].

To explore the metabolic changes that play a role in chondrosarcoma we performed a metabolic compound screen including, amongst others, compounds targeting glycolysis, glutamine metabolism, glutathione, HIF1a, mTOR and fatty acid metabolism. Compounds that targeted metabolic pathways most important for survival of chondrosarcoma cells were selected for further analysis on metabolic level using the Seahorse XFe analyzer. This led to the identification of mTOR as most promising metabolic compound which was further explored in vitro and in vivo in an orthotopic xenograft mouse model.

Methods

Cell culture

Conventional central chondrosarcoma cell lines JJ012 (*IDH1* mutant, R132G) [20] CH2879 (*IDH* wildtype) [21] and SW1353 (*IDH2* mutant, R172S) (ATCC) were cultured in RPMI 1640 medium (Thermo Fisher Scientific) supplemented with 10% heat-inactivated Fetal Bovine Serum (FBS) (F7524, Sigma Aldrich, Saint Louis, Missouri, USA). *TP53* mutations are present in all cell lines, although CH2879 shows a pathogenic *TP53* mutation in only part of the cells, as determined previously [22]. Cell lines were cultured at a temperature of 37ºC in a humidified incubator in normoxic conditions (5% CO_2). Identity of cell lines was confirmed using the Cell ID GenePrint 10 system (Promega Benelux BV, Leiden, The Netherlands) before and after completion of the experiments. Mycoplasma tests were performed on a regular basis.

Compounds

A detailed list of all compounds included in the metabolic compound screen is available in supplementary table 1. mTOR inhibitor rapamycin (S1039, Selleckchem), BH3 mimetic ABT-737 (S1002, Selleckchem) and general caspase inhibitor Z-vad-FMK (550377 BD biosciences) were dissolved in

DMSO according to the manufacturer's instructions. Chemicals for the Seahorse experiments oligomycin A (11342), trifluoromethoxy carbonylcyanide phenylhydrazone (FCCP, 15218), antimycin A (19433), rotenone (13995), UK5099 (16980), Etomoxir (11969) and BPTES (19284) were all purchased from Cayman Chemical (Massachusetts, USA) and dissolved in DMSO according to the manufacturer's instructions. Doxorubicin and cisplatin in a 0.9% NaCl solution were obtained from the in-house hospital pharmacy.

Metabolic Compound Screen

39 compounds targeting different metabolic pathways were selected (supplementary table 1), and concentrations were chosen based on literature. In addition, possible synergistic effects with rapamycin and doxorubicin were investigated. Chondrosarcoma cell lines were seeded 3000/well (JJ012 and SW1353) or 5000/well (CH2879) in 96 well plates. Cells were cultured overnight to attach and then treated with four different concentrations of compound or control for 72 hours. Cells were simultaneously treated with either doxorubicin, rapamycin or PBS. Combination treatments were carried out with concentrations that did not induce any toxicity on its own (>90% of cell viability): doxorubicin 10 nM, 2 nM and 1 nM for CH2879, SW1353 and JJ012 cell lines respectively. Rapamycin was added in a 2 pM concentration for all cell lines. As a positive control 5 μM of doxorubicin was included. After 72h incubation, presto blue viability assays (Life-Technologies, Scotland, UK) were carried out according to the manufacturer's instructions and fluorescence was measured at 590 nM using a fluorometer (Victor3V, 1420 multilabel counter, Perkin Elmer, Netherlands). This was followed by fixation of the cell with phosphate buffered 4% paraformaldehyde and nuclei counting after staining with Hoechst using the Cellomics Array Scan High content system (Thermofisher Scientific). Compounds were selected for further metabolic characterization based on their ability to decrease viability more than 50% for the two highest concentrations in at least one of the three cell lines.

Metabolic Characterization

A Seahorse XFe 96 analyzer (Seahorse Bioscience, Agilent) was used to measure both oxygen consumption rate (OCR) and the extracellular acidification rate (ECAR) in chondrosarcoma cell lines JJ012, SW1353 and CH2879 after 24 hours of treatment. 30 hours prior to the assay cells were

plated in custom Seahorse 96 well plates in optimized densities being 15000, 13000 and 30000 for JJ012, SW1353 and CH2879, respectively. After 6 hours cells were treated with the selected compound for 24 hours. Concentrations were chosen based on the results of the compound screen (AOA 1mM, BSO 100 µM, bardoxylone methyl 0.5 µM, CB-839 500 nM, sapanisertib 100 nM, lovastatin 5 µM and rapamycin 10 nM). Before the measurement, cells were incubated for 1 hour in glucose-free RPMI-1640 supplemented with 5% FBS. A customized assay was performed to measure both oxidative as well as glycolytic characteristics in a single run. During the assay, sequential injections of 10 mM glucose (Sigma-Aldrich), 2.0 µM oligomycin A, 2 µM FCCP and 0.5 µM 1:1 rotenone : antimycin A established the metabolic profile. Data was normalized to cell numbers measured in each individual well using the Cellomics platform after Hoechst staining and data represented as the average +/- SD of duplicate or triplicate measurements of treated cells and 5-7 replicates for controls. Cell line characteristics were determined by calculating the basal and maximal respiration as well as the glycolysis and glycolytic capacity. Basal respiration, the oxidative rate of cells at rest, was calculated by subtracting the final measurement value (non-mitochondrial oxygen consumption) from the value after glucose injection. Maximal respiration, the oxidative respiration rate of cells treated with 2 µM FCCP, was calculated by subtracting the non-mitochondrial oxygen consumption from the highest value after FCCP injection. Glycolysis was determined from ECAR values, subtracting the final value (non-glycolytic acidification) from the value after glucose injection. The glycolytic capacity was calculated by subtracting the non-glycolytic acidification from the highest ECAR value after oligomycin injection.

Metabolic Flexibility after mTOR Inhibition

To measure the metabolic fuel dependency, the OCR and ECAR were measured after using the MitoFlex assay on the Seahorse XFe 96 analyzer according to the manufacturer's instructions. Briefly, cells were prepared for analysis as described above and treated for 24 hours with mTOR inhibitor rapamycin or sapanisertib and during the assay with 3 µM BPTES, 4 µM Etomoxir and 2 µM UK5099. During the assay 10 mM glucose was injected followed by alternating a single inhibitor and a mixture of the remaining two inhibitors to measure metabolic dependency. Data was normalized to cell numbers measured in each individual well using the Cellomics platform and data represented as the average +/- SD of 6 replicate measurements.

Metabolic dependency was calculated by taking the last OCR value before injection (baseline OCR) and calculating the drop in OCR after injection of a single inhibitor by subtracting the lowest value after the single inhibitor injection and dividing this by the difference between baseline OCR and the lowest value after the injection of the remaining two inhibitors.

Cell Viability and Proliferation Assays

Chondrosarcoma cell lines JJ012, CH2879 and SW1353 counting seeded in previously optimized densities (JJ012 and SW1353 3000cells/well, CH2879 5000cells/well) were allowed to adhere overnight before treatment with increasing concentrations of mTOR inhibitors rapamycin and sapanisertib for 72 hours. Cell viability was measured using presto blue viability reagent (Life-Technologies, Scotland, UK) and proliferation was measured using Hoechst staining as discussed previously. Experiments were performed in normoxic as well as hypoxic conditions by incubating cells in a MCO-19M O2 /CO2 incubator (Panasonic) using nitrogen to simulate a 1% O2 environment and in combination with doxorubicin or cisplatin chemotherapeutic agents. Experiments were performed at least three times in triplicate.

Apoptosis assay

Quantification of apoptosis induction was performed using the caspase-glo 3/7 assay from Promega (Madison, USA) as described previously [23]. Briefly, JJ012, SW1353 and CH2879 cells were plated in white walled 96 well plates (Corning B.V. Life Sciences, Amsterdam, the Netherlands) and after overnight adherence incubated with either rapamycin, sapanisertib, or DMSO or pan-caspase inhibitor z-vad as control conditions. After 24 hours incubation, substrate was added in a 1:1 dilution in culture medium and incubated at room temperature (30 min). Cells treated with a combination of ABT-737 and doxorubicin were taken along as a positive control. Luminescence was measured using a luminometer (Victor3V, 1420 multilabel counter, Perkin Elmer, Netherlands). Experiments were performed three times in duplicate.

Western blot

Protein expression of Hif1α (Clone D2U3T, Cell signaling), PS6 (Clone DS7.2.2E, Cell signaling) and P-akt (ser473) (Clone D9E, Cell signaling) was

determined after 24 hours of treatment with rapamycin, or sapanisertib in normoxic as well as hypoxic conditions. To prevent reversal of hypoxic conditions, lysates were obtained immediately after removal of the culture flasks from the incubator, using hot-SDS buffer (1% SDS, 10 mM Tris/EDTA with complete inhibitor and phosSTOP) as previously described [24]. For each sample, 10 µg of protein was loaded on the gel. As loading control, α-tubulin (clone DM1A, Sigma-Aldrich Chemie B.V. Zwijndrecht, the Netherlands) expression was determined. Blocking was performed using 5% milk and primary antibodies were diluted in 5% bovine serum albumin (BSA) solution and incubated overnight. Proteins were blotted on a PVDF membrane and detected using enhanced chemo luminescence (PierceTM ECL Western Blotting Substrate, Thermo Fisher Scientific, Waltham, MA, USA) followed by exposure and development of the film (ECL hyperfilm, Amersham, GE Healthcare, Chicago, IL, USA).

Mouse Experiments

Animal procedures (AVD116002016574) were approved by the Central Committee of animal experiments (CCD, The Hague, The Netherlands) conform the European legislation (EU 2010/63/EU) and performed under permission of the Leiden University animal experimental committee. Athymic mice (BALB/c nu/nu 6 weeks old) were acquired from Jackson (Janvier-labs, France), and housed at the animal facility of the Leiden University Medical Center. A total of 12 female mice were used, using 6 mice per group. Orthotopic injection of the luciferase expressing CH2879 LUC10 clone (1x105 cells in 10 µL PBS) was performed in all mice under isoflurane anesthetics as described previously [25]. In brief, two small holes (~0.35 mm each) 4–5mm apart were created in the bone cortex of the upper right tibiae using needles (25GA 5/8 0.5 x16). A reservoir for the cells was created by flushing out the bone marrow from the proximal end of the shaft. After inoculation of the CH2879 Luc10 cells the skin was sutured with wound clips. Tumor growth was analyzed by measuring the luciferase activity by Bioluminescence Imaging (BLI) 10min. after i.p. injection of 150 mg/kg D-luciferin (Synchem UG & Co Kg, 60mg/ml in PBS stock solution). Micro Computed Tomography (mCT) (Skyscan 1076 Micro CT scanner (Bruker Microct, Kontich, Belgium) was performed to determine exact location of the tumor and the bone loss at the start of the experiment, in the middle and at the end of the experiment. Weekly, mice were weighted and tumor growth (BLI) was analyzed under isoflurane anesthetics on the IVIS Spectrum Xenogen (Perkin Elmer, Hopkinton, MA) and quantified in photons/s/cm2/sr within a standardized ROI using Living Image 3.0

(Caliper LifeSciences, Hopkinton, MA). Treatment was started as soon as tumor BLI signal reaches 105 P/s/cm2 (4 out of 16 mice failed to develop tumors). The 12 mice were divided into even sized control and treatment groups. In order to investigate the effect of mTOR inhibitor sapanisertib on tumor growth, sapanisertib (MLN0128, Selleckchem) was dissolved in 30%PEG400 (Bufa) / 0.5%Tween80 (Sigma)/ 5%Propyleenglycol (Bufa) in water conform the datasheet and further diluted in PBS. Six mice were treated by oral gavage of 100 µl 2.5 mg/kg sapanisertib in PBS for 3 days a week (Monday/Wednesday/Friday) for 8 weeks, while the control group of six mice was given only 100µl dissolvent 30%PEG400/Tween80/Propyleenglycol in PBS by oral gavage 3 days a week and monitored for 8 weeks. After 8 weeks of treatment or when BLI signal reached 108 P/s/cm2 mice were sacrificed. Both tibiae were collected and cut longitudinally in half. One part was fixated overnight in formalin and decalcified and embedded in paraffin for histological assessment and the other part was snap frozen and stored in -80oC. For histological assessment of possible metastases or toxicity of the treatment, lungs, liver and kidneys were embedded in paraffin. Furthermore, whole blood cell analysis (Sysmex XP-300 Automatic Hematology Analyzer) was performed at the end of the experiment.

Immunohistochemistry

On all mouse tumors that were collected, protein expression was evaluated for P-S6 (clone D57.2.2E, Cell signaling), Ki-67 (clone D2H10 Cell Signaling), Cleaved caspase 3 (Cell signaling #9661) and LC3B (Clone D11 Cell signaling). In supplementary table 2 all the specifics of the antibodies are described. Immunohistochemistry was performed using standard laboratory methods as described previously [26]. Slides were scored using Q-Path software or by manually counting positive cells (Cleaved caspase-3). Tumors were selected, and positive cell detection was used to calculate the percentage of positive cells.

Results

Compound screen identifies glutamine, glutathione, mTOR, NAD synthesis and fat metabolism as important metabolic regulators in chondrosarcoma cells.

The custom designed compound screen indicated that chondrosarcoma cell lines were most sensitive for compounds targeting glutamine, glutathione, mTOR and NAMPT (Figure 1). In addition, cholesterol inhibitors simvastatin and lovastatin were effective in the chondrosarcoma cells, especially in the JJ012 cell line. Furthermore, JJ012 demonstrated high sensitivity towards treatment with buthionine sulfoximine (BSO), while the other two cell lines were resistant. Interestingly, no sensitivity was observed for any of the compounds targeting the glycolysis pathway. Combination treatment with low concentrations of doxorubicin or rapamycin did not suggest additive of synergistic effects (supplementary table 3). Twelve compounds fulfilled the selection criteria as they decreased viability more than 50% for the two highest concentrations in at least one of the three cell lines. When multiple compounds targeting the same pathway met the selection criteria, one was chosen for further validation. Moreover, the inhibition of NAD+ biosynthesis using GMX1778 was already previously explored within our group [27] and was not selected for further validation as we used this as a positive control to validate proper workings of the screen. This way, six compounds were selected for further study; CB-839, AOA, bardoxylone methyl, BSO, sapanisertib and Lovastatin (figure 1). In addition, even though everolimus was tested in the compound screen and has shown higher selectivity for mTORC1 compared to rapamycin, rapamycin was taken along as a second mTOR targeting compound since it is currently in clinical trials for the treatment of chondrosaromca (NCT02821507) and targets only mTORC1, while sapanisertib is targeting both mTORC1 and mTORC2.

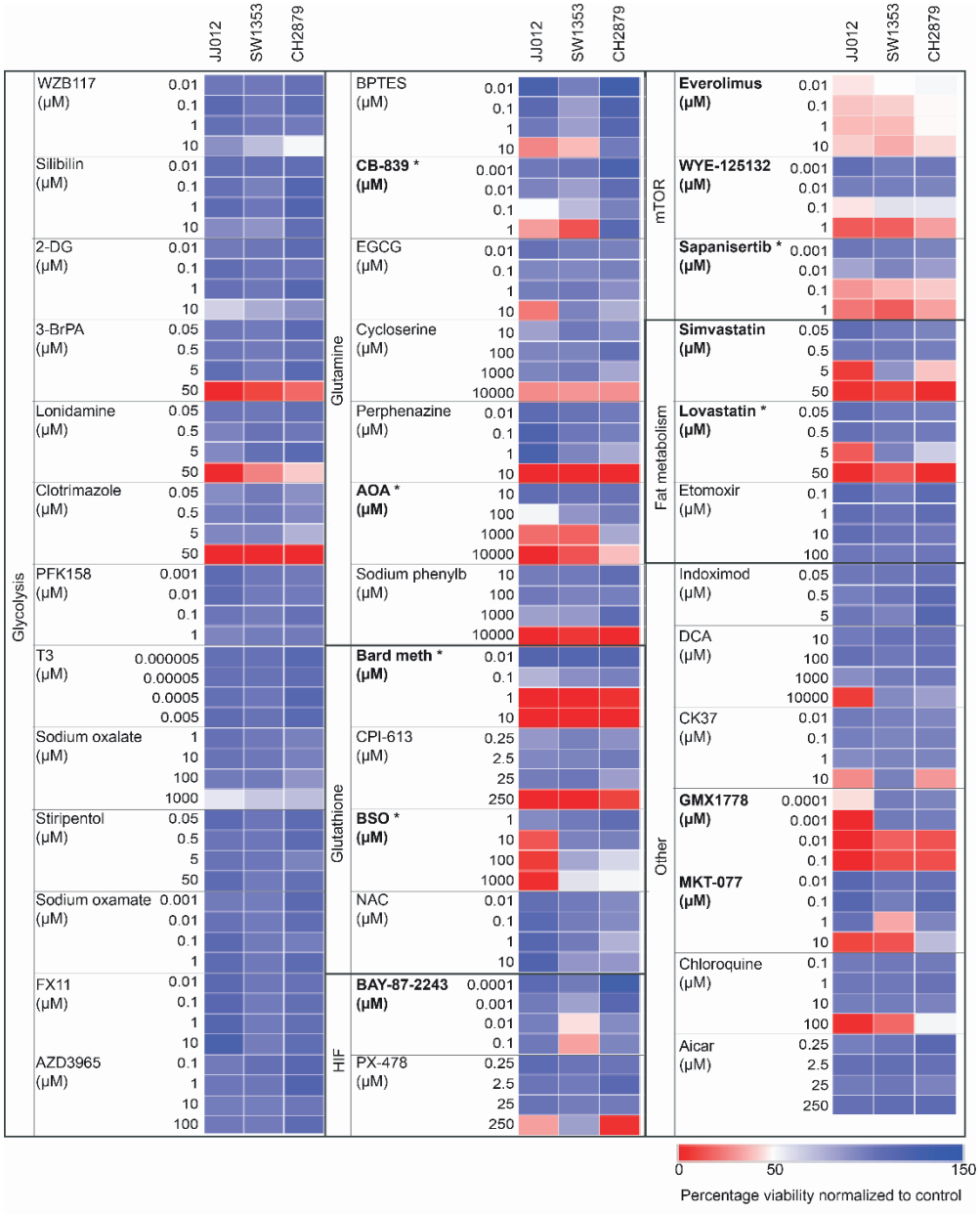


Figure 1: Viability of in vitro metabolic drug screen in three different chondrosarcoma cell lines. Four different concentrations were used per compound as indicated. Of all 39 tested compounds twelve compounds decreased cell viability more than 50% for the two highest concentrations in at least one of the three cell lines (indicated in bold). Six compounds were selected for further study; BSO, bardoxylone methyl, AOA, CB-839, sapanisertib and lovastatin (labeled with an asterisk).

Inhibition of metabolic pathways leads to effects on both oxidative and reductive metabolism

Using the Seahorse XFe96 metabolic analyzer, we evaluated the effect of the seven selected compounds on the oxidative and glycolytic respiration in JJ012, SW1353 and CH2879. The maximal respiration of all cell lines was found to decrease for all seven selected compounds (figure 2A) while the basal respiration was lowered in all but the BSO and lovastatin treated cells (figure 2B). In contrast, glycolysis and glycolytic capacity was only decreased in cells treated with AOA, bardoxylone methyl, sapanisertib and rapamycin (figure 2C-D). Combining these parameters, we found that inhibition of the aspartate aminotransferase enzyme, an important chain in the aspartate synthesis pathway, using AOA as well as the inhibition of mTOR (using rapamycin and sapanisertib) led to consistent decreases in all measured oxidative and glycolytic parameters. All compounds were confirmed to decrease viability after treatment similar to the compound screen (supplementary figure 1. A). When comparing *IDH* mutant with *IDH* wild type cells, no large differences were found. Moreover, pre-treatment of JJ012 with AGI-5198, and CH2879 with cell permeable D2-HG, did not reverse the effect of the compound on basal and maximal respiration (supplementary figure 1B). This suggests that the metabolic vulnerabilities seen are independent of the *IDH* mutation status.

Changes in metabolic fuel dependency upon inhibition of mTOR.

Since aspartate amino transaminase inhibitor AOA is not suitable for clinical use due to a high incidence of side effects [28] we decided to further investigate the mechanism of mTOR inhibition in chondrosarcoma. A mitoFlex test was performed to measure the dependency on metabolic fuels glutamine, fatty acids and glucose after mTOR inhibition. All three cell lines depended mostly on glucose for energy production (Figure 2E). We found an increase in dependency on glutamine and fatty acids after sapanisertib treatment in JJ012 ($p=0.0001$ and $p=0.0016$, respectively). This effect was more pronounced with sapanisertib as compared to rapamycin. An opposite trend was however seen in CH2879 in which the dependency on fatty acid and glucose decreased ($p=0.0036$ and $p=0.0026$). SW1353 was largely unaffected except for lower glucose dependency after sapanisertib treatment ($p=0.0414$). In summary, mTOR inhibition using rapamycin or sapanisertib decreases oxidative and glycolytic metabolism and influences the underlying metabolic fuel dependency on glutamine (JJ012), fatty acids (JJ012, CH2879) and glucose (SW1353, CH2879).

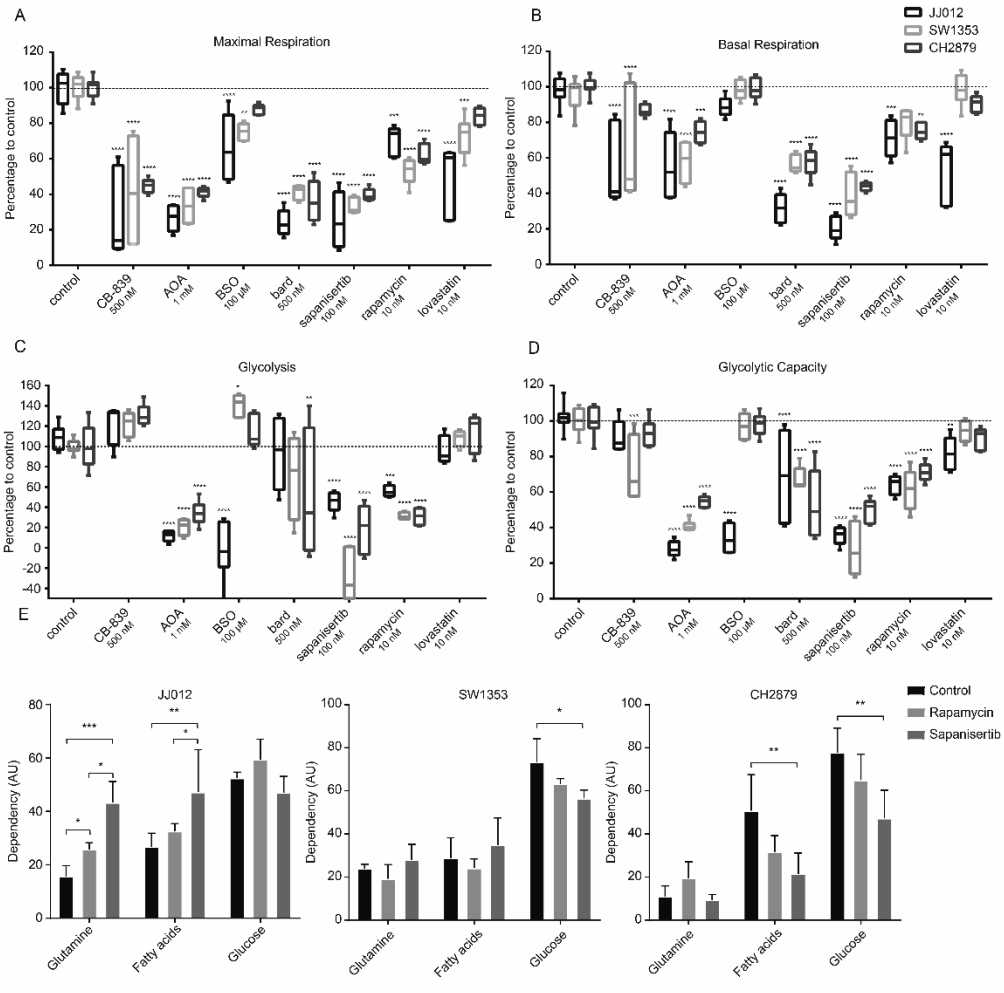


Figure 2: Real-time metabolic analysis of three chondrosarcoma cell lines after treatment with the compounds selected from the screen.
Maximal respiration (A), Basal Respiration (B), Glycolysis (C) and Glycolytic capacity (D) of all cell lines after 72h of treatment with selected compounds. Maximal and Basal respiration are most decreased in all cell lines after treatment with Bardoxylone methyl or sapanisertib (P<0.0001). Glycolysis and Glycolytic capacity are most decreased after treatment with AOA, sapanisertib and rapamycin (P<0.0001). E) MitoFLEX test shows metabolic dependency on fuels glutamine, fatty acids and glucose after treatment. JJ012 shows an increased dependency on glutamine and fatty acids and CH2879 shows an increased dependency on fatty acids and glucose after rapamycin or sapanisertib treatment. Significant changes towards control (figure A, B, C and D) or other indicated conditions (figure E) are presented by asterisks. *: P<0.05, **: P<0.01, ***: P<0.001, ****: P<0.0001. P values were calculated using Tukey's multiple comparisons test.

Inhibition of mTOR leads to a decrease in proliferation in chondrosarcoma cell lines.

Inhibition of mTORC1 with rapamycin led to a maximum reduction in viability of 50% in all three chondrosarcoma cell lines which was independent of dose; 0.5 nM led to the same reduction in viability compared to 500 nM (Figure 3A). Chondrosarcoma cell lines treated with dual mTORC1 and mTORC2 inhibitor sapanisertib showed a dose dependent decrease in viability after 72h with an IC50 of 9nM, 26nM and 27nM for JJ012, SW1353 and CH2879 respectively. However, at higher concentrations the viability remained 10-30% (Figure 3B). We therefore investigated possible synergistic effects by combination treatment with doxorubicin or cisplatin. However, combination treatment did not suggest any additive or synergistic effects (supplementary figure 2). Induction of apoptosis measured by caspase 3/7 activity was increased after 24h of treatment with sapanisertib in CH2879 but not in the other two cell lines (Figure 3C). In contrast, rapamycin treatment did not result in induction of caspase dependent apoptosis in any of the cell lines. Culturing cells under hypoxic conditions, to mimic the hypoxic chondrosarcoma microenvironment more closely [29], led to an increase in levels of Hif1α. However, hypoxia did not alter the response to mTOR inhibition as compared to normoxia (figure 3D-E). As expected, treatment with mTORC1 inhibitor rapamycin does not result in a reduction in p-Akt, while inhibition of mTORC1 and mTORC2 by sapanisertib reduces expression of p-AKT (figure 3E). Furthermore, treatment with both inhibitors led to a decrease in P-S6, but no clear differences in HIF1α expression.

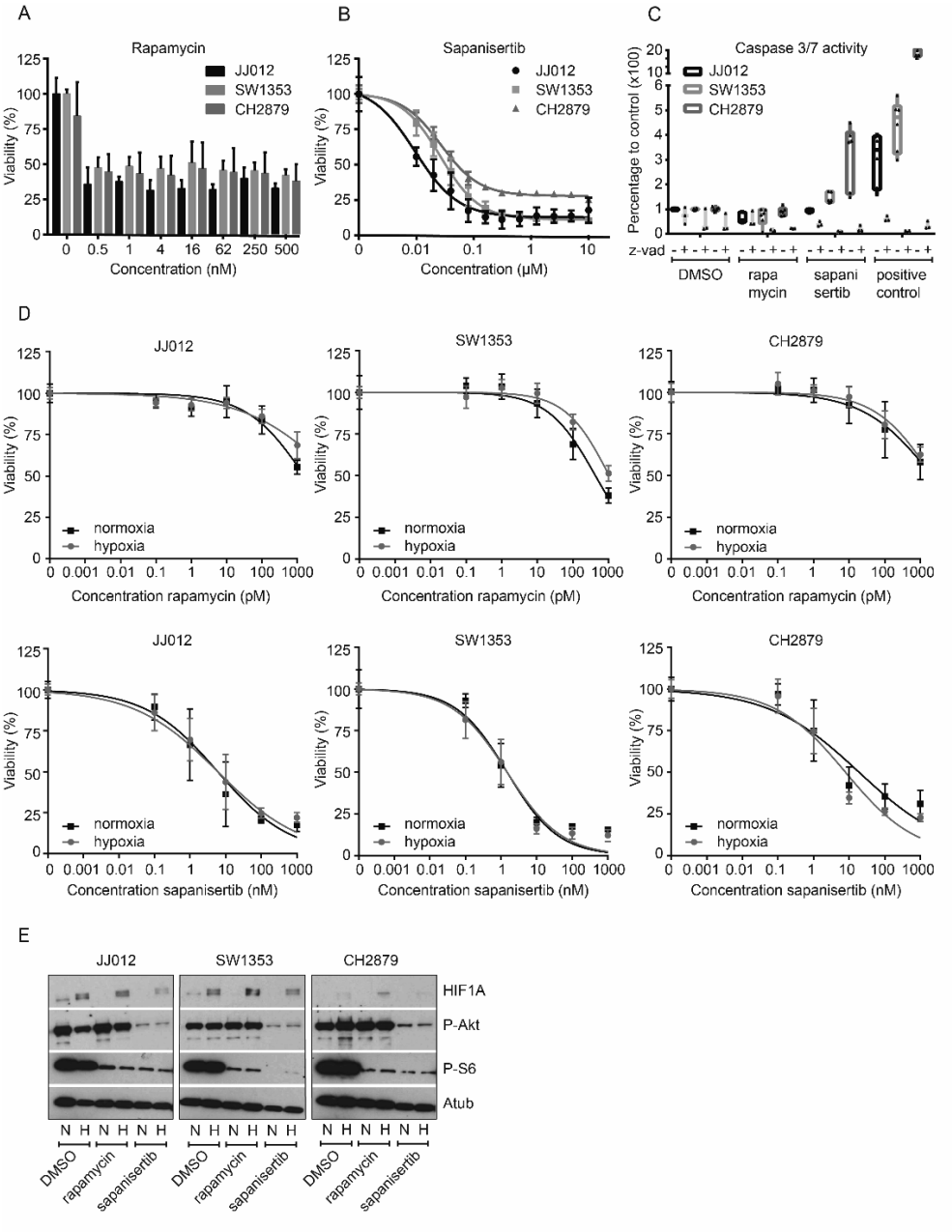


Figure 3: Inhibition of mTORC1 or mTORC1 and C2 in chondrosarcoma cell lines. A) Inhibition of mTORC1 using rapamycin for 72h led to a 50% reduction in viability in JJ012, SW1353 and CH2879 irrespective of the concentration. B) mTORC1 and mTORC2 inhibition using sapanisertib for 72 hours leads to a dose dependent decrease in viability in chondrosarcoma cell lines JJ012, SW1353 and CH2879. C) Caspase 3/7 activity in chondrosarcoma cell lines after 24h treatment with

sapanisertib or rapamycin. Only CH2879 cells treated with sapanisertib show an increase in caspase 3/7 activity. As a positive control cell lines were treated with ABT-737 and doxorubicin simultaneously. Results shown are mean and standard deviation of three individual experiments in duplicate. Points indicate individual measurements. D) No difference in response towards mTOR inhibitors sapanisertib or rapamycin between chondrosarcoma cells cultured in normoxia or hypoxia for 72h E) Western blot analysis for Hif1α, p-Akt and P-S6 of chondrosarcoma cell lines cultured in normoxia (N) or hypoxia (H) treated with either sapanisertib or rapamycin for 24 hours compared to control. A-tubulin expression is determined as a loading control.

Treatment with sapanisertib leads to a delay in tumor growth in vivo

Oral administration of sapanisertib to tumor bearing mice resulted in a delay in tumor growth compared to control conditions as measured by bioluminescence luciferase imaging (BLI) (Figure 4A, 4B). During the treatment period two control mice had to be sacrificed and excluded from the analysis. One due to an acquired infection and the other due to tumor growth outside of the bone. In the control group all mice reached a bioluminescence signal of 108 and a strong increase in percentage tumor growth within four weeks after start of treatment, while tumors of mice treated with sapanisertib showed a delay in tumor growth and a slower increase in tumor formation (figure 4B and supplementary figure 3). After 4 weeks of treatment tumor growth decreased, with tumor regrowth at week 6. No difference was observed in white blood cell counts and weight measurements between sapanisertib treated and control mice (supplementary figure 4). Furthermore, no metastases in the lungs were identified in any of the mice and no signs of toxicity of the compound were observed in liver, kidney and spleen of a selection of three treated and three control mice (data not shown). Immunohistochemical analysis of Ki-67, P-S6, cleaved caspase 3 and LC3B revealed a small increase in ki-67 positive cells in control compared to treated tumors (P=0.03571). No significant differences were observed in the other markers between treated and control tumors (see supplementary figure 5 and supplementary table 4). In addition, no morphological differences were observed in tumors of control and sapanisertib treated mice (Supplementary figure 6).

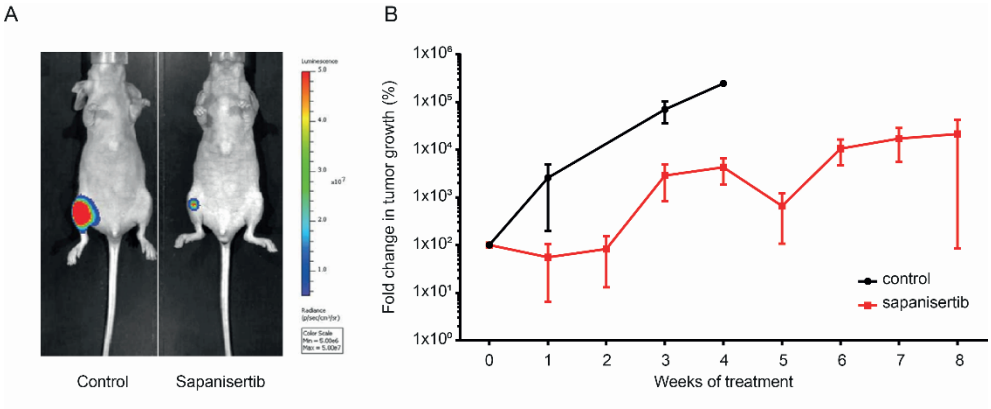


Figure 4: Inhibition of mTORC1 and mTORC2 using sapanisertib in an orthotopic chondrosarcoma xenograft mouse model leads to a delay in tumor growth.
A) Bioluminescent signal (photons/sec/cm2/sr) within standardized ROI of control mice compared to mice treated with sapanisertib after three weeks of treatment. B) Fold increase in tumor growth in control compared to mice treated with sapanisertib showing a delayed tumor fold increase in sapanisertib treated mice compared to control mice. Values represent mean with standard deviation.

Discussion

In this study, metabolic vulnerabilities of three chondrosarcoma cell lines with different mutational backgrounds were investigated by a custom-made metabolic compound screen targeting a multitude of metabolic pathways. Targeting the glutamine-, glutathione-, NAD synthesis-, and mTOR pathways decreased viability in all cell lines, of which the glutamine pathway has been previously determined to be important in chondrosarcoma with similar effects on viability of chloroquine and CB-849 [30]. Seven compounds were further investigated by metabolic profiling using the Seahorse XFe analyzer, measuring both the oxidative and glycolytic characteristics of the cells to see how the overall metabolism of these cells was affected. Compounds that caused a decrease in oxygen consumption rates showed either decreases or very minor increases in glycolytic rates, thus cells were not compensating the loss in oxidative metabolism by up regulating their glucose metabolism. This indicates that glycolysis is also affected by most treatments or cells try to compensate through other metabolic pathways. Interestingly, in the compound screen none of the cell lines were sensitive to the inhibition of glycolysis, while previous reports suggest that this can be an effective strategy in chondrosarcoma either as a single treatment or in combination with chemotherapy [31-33]. Previous studies have shown that drug resistance has an impact on the metabolism of cells, which explains the difference in sensitivity to glycolytic inhibitors that we observed in the compound screen. In our study we used previously untreated chondrosarcoma cells while all the aforementioned studies used cells that were cultured on low doses of cisplatin or doxorubicin for extended periods of time. This leads to the emergence of drug resistance and can influence the cellular metabolism. [34] Based on the Seahorse observations and the fact that we do not see any differences in viability after inhibiting the glycolysis pathway, both in the presence and absence of low concentrations of doxorubicin and cisplatin, we hypothesize that glycolysis is not essential for chondrosarcoma metabolism. High levels of reactive oxygen species (ROS) in tumors have been associated with the reprogramming of energy metabolism, which can lead to differences in activity of the glycolysis, fatty acid, pentose phosphate and serine one-carbon pathways [35, 36]. Interestingly, JJ012 was far more responsive to glutathione synthesis inhibitors BSO and AOA treatment compared to SW1353 and CH2879. Recently, our group described a metabolic vulnerability in the NADH synthesis pathway of the JJ012 cell line [27]. This might provide a rationale as to why JJ012 is more responsive, as decreased

efficiency of the electron transport chain conveys a higher ROS production as well as a dependence on NAD dependent aspartate synthesis through the GOT1 enzyme, of which AOA is an inhibitor [37, 38]. Further evidence of ROS involvement was found upon bardoxylone methyl treatment. The exact mechanism of bardoxylone methyl is under debate as its fluctuating effects on Nrf2 and NF-kβ seem to depend heavily on the available concentrations within the cell and thus might explain the high variance found in the glycolytic parameters [39].

Treatment of chondrosarcoma cell lines with rapamycin or sapanisertib showed consistent decreases in viability, coupled to a decrease in both oxidative and glycolytic metabolism, highlighting the importance of the mTOR pathway in chondrosarcoma cell lines. mTOR is the central regulator of many metabolic pathways as well as autophagy and is regulated by a multitude of stimuli related to metabolism, DNA damage, growth and hypoxia [40]. It can be found in two different complexes mTORC1 and mTORC2, with rapamycin inhibiting only the mTORC1 complex, while sapanisertib is inhibiting both mTORC1 and mTORC2. In our study we show that chondrosarcoma cell lines are responsive to both inhibitors, however treatment with the dual inhibitor sapanisertib led to the largest decrease in cell viability. The combination treatment of both inhibitors did not suggest any synergistic effects (supplementary figure 1). This can be related to the binding specifics of the drugs as the inhibition of the whole kinase domain (both the S6K and 4EBP1 by sapanisertib negates the effects of rapamycin induced via inhibition of the S6K domain only (through complex formation with the FKBP12 protein). [41, 42] This is consistent with other pre-clinical studies in multiple tumor types including sarcomas, in which sapanisertib was shown to be more potent [43]. Furthermore, this increased efficacy was also seen in the metabolic characterization where sapanisertib treated cells had lower metabolic rates compared to rapamycin. Measuring the dependency on underlying fuel pathways showed differences in glutamine, fatty acid and glucose dependency. We found increased glutamine and fatty acid dependency in JJ012 but a decreased dependency on fatty acids in CH2879. Furthermore, lower glucose dependency was observed in SW1353 and CH2879. Interestingly, all differences in dependency were more profound in sapanisertib treated cells compared to rapamycin treatment. Although the cells were treated with different concentrations of rapamycin and sapanisertib, all data was normalized to cell numbers directly after the dependency assay, eliminating the possibility of dose-related effects. Therefore, the observed changes are most likely related to the effects of the compounds as the inhibition of both mTORC complexes by sapanisertib

leads to a stronger metabolic reaction compared to the inhibition of only the single mTORC1 complex from rapamycin. However, the cellular metabolism is highly intertwined with all other cellular processes and while we observed differences in metabolic dependency upon mTOR inhibition further studies should be performed to elucidate whether this is a direct effect or from the result of changes that occur in other mTOR regulated processes upon its inhibition. mTOR is involved in many physiological processes ranging from metabolism to muscle mass and brain function. On the cellular level this is mainly regulated through protein synthesis and turnover, autophagy and lipid/glucose/nucleotide metabolism. Changes in for instance the activity of protein turnover can have a secondary effect on the metabolism, therefore measuring the activity of activators and downstream targets might provide more insight into the exact workings.

A number of studies have found chondrosarcoma to be a hypoxic tumor with increased HIF1α and VEGF signaling [44]. We found higher levels of HIF1α in cells under hypoxia but found no difference in cell viability after mTOR inhibition with rapamycin and sapanisertib in hypoxic conditions compared to normoxia. Thus, in chondrosarcoma cell lines hypoxia does not increase sensitivity to mTOR inhibition.

Sapanisertib has been found as a potent treatment option in mouse models of several tumor types, amongst them xenograft models of osteosarcoma [43, 45, 46]. Using an orthotopic xenograft mouse model of chondrosarcoma we established a delay in tumor growth upon treatment with sapanisertib. Interestingly, tumor growth was severely delayed up until 5 weeks after start of the treatment. From week 5 onwards, increased tumor growth was observed, although with a high variance, suggesting a possible resistance mechanism. This is in line with the in vitro data, where a decrease to around 10-30% viability is observed. Furthermore, rapamycin or everolimus based interventions have, in spite of favorable pre-clinical data, not progressed through phase II clinical trials in chondrosarcoma yet [47-49]. A possible solution would be to look into a combination treatment approach to circumvent resistance. A phase I study investigating the combined effect of rapamycin and cyclophosphamide in 10 chondrosarcoma patients showed stable disease in 6 patients and a partial response in one patient indicating a predominantly cytostatic effect. Currently a phase II study is on-going to confirm these results in a larger cohort (NCT02821507). Furthermore, resistance might also explain the lack of difference in expression of cleaved caspase, LC-3B, KI67 and p-S6 markers in the tumor tissue harvested at the end of the experiment, when tumors were growing again. To further

investigate the effects of sapanisertib in vivo, a larger cohort study should be set up where it would be of interest to analyze the expression of different markers such as Ki67, caspase, LC-3B and p-S6 at multiple time points during the experimental period to observe possible changes in these markers at earlier stages of treatment. Furthermore, the analysis of metabolic pathways, combinational treatment with different drugs and mTOR-related pathway analysis would provide great insight into the working and secondary resistance mechanism of sapanisertib in vivo.

Using a screening-based approach we identified glutamine, glutathione, mTOR and NAD synthesis as the most essential metabolic pathways in chondrosarcoma cells. Metabolic respiration was most affected when mTOR was inhibited using sapanisertib, a dual mTORC1 and mTORC2 inhibitor. Cell lines showed a dose dependent decrease in viability after treatment with sapanisertib; however, a plateau of 10-30% cell viability remained after treatment. Treatment of a chondrosarcoma orthotopic xenograft mouse model resulted in a decrease in tumor formation, however resistance was observed after several rounds of treatment. These results indicate that inhibition of mTORC1 and mTORC2 can be a possible therapeutic option for chondrosarcoma patients in combination strategies. Further investigation is needed to determine possible candidates for combination.

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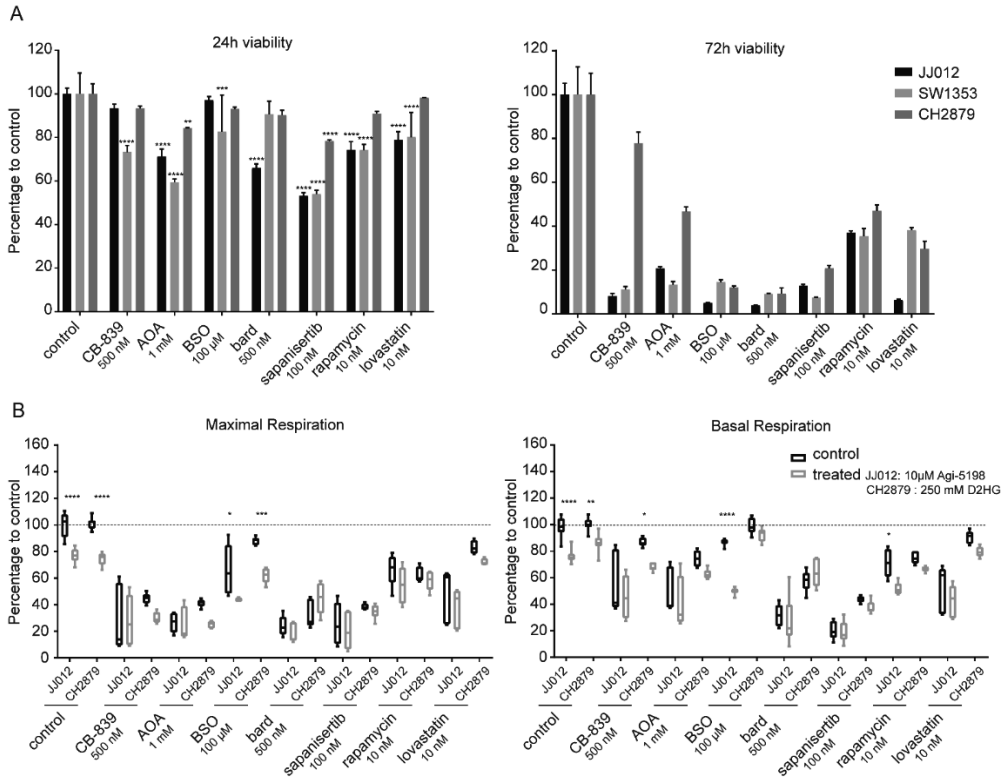
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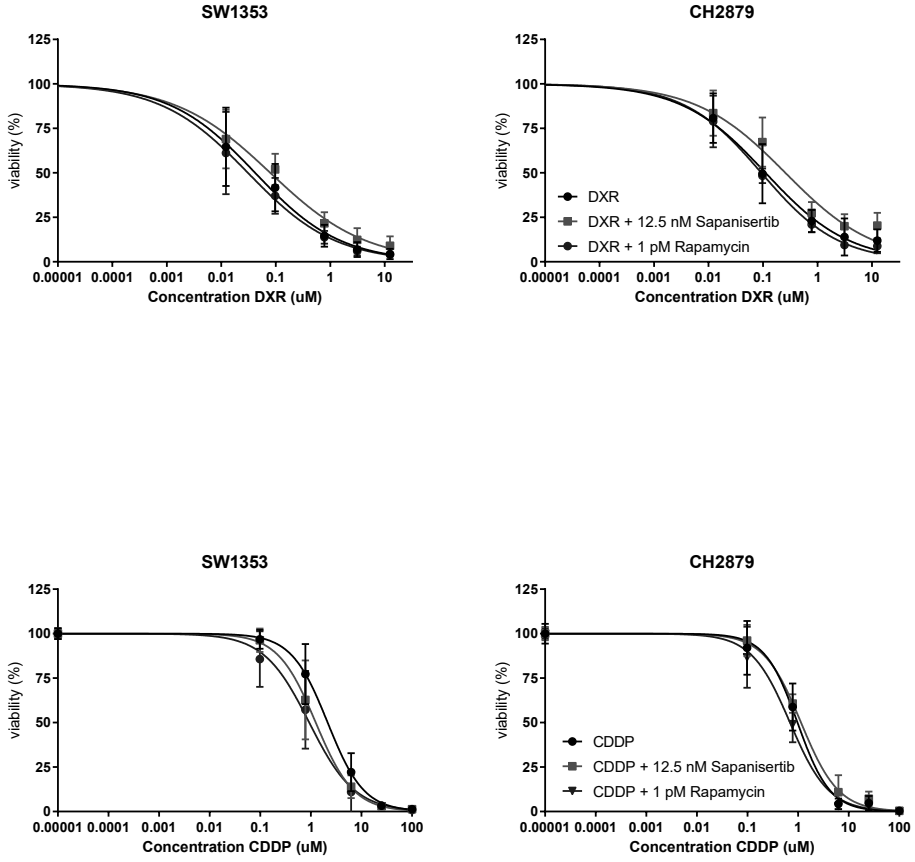
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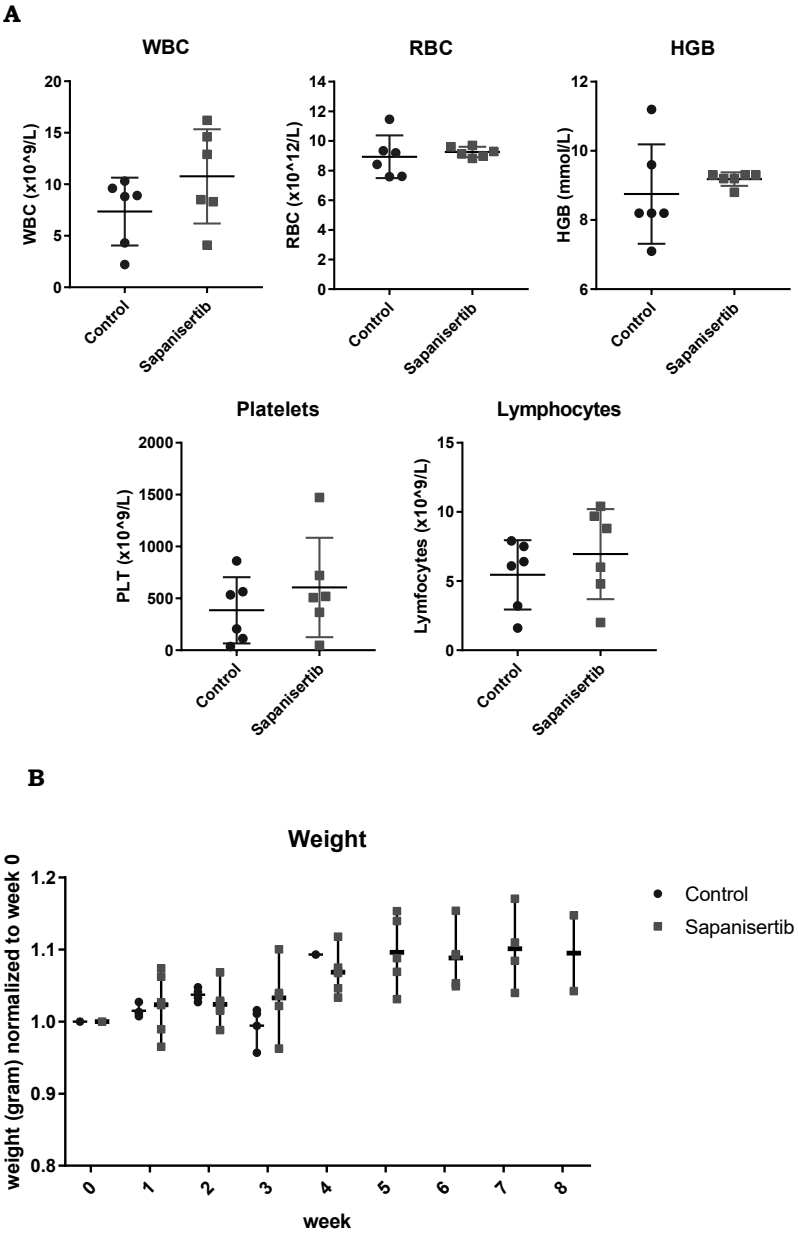
Supplementary material



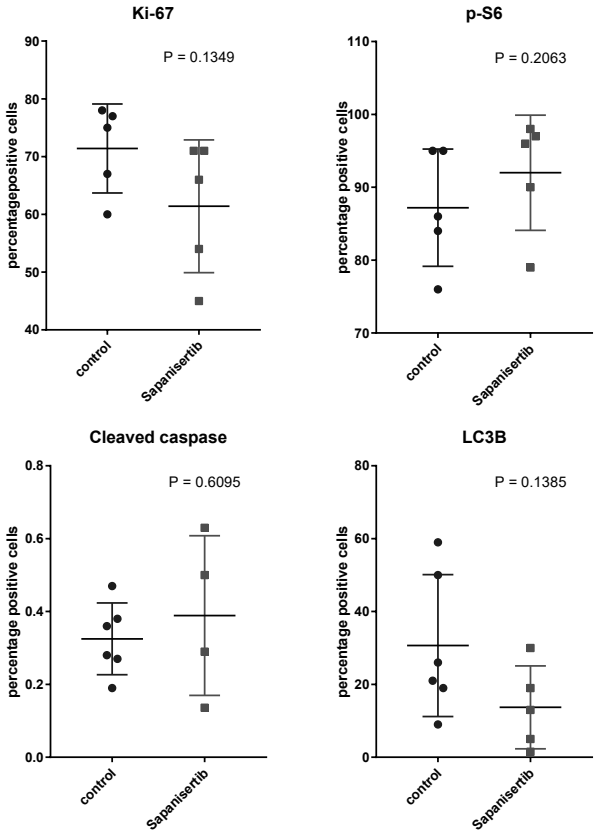
Supplementary Figure 1: A) Viability of compounds used in Seahorse metabolic characterization after 24 and 72 hours treatment. After 24h no drastic effects (>50% decrease) are observed after treatment, indicating that viable cells were still present when performing the seahorse assay. After 72h of incubation all compounds showed a similar effect as observed in the compound screen. Significant changes towards control are presented by asterisks for 24h treatment periods. *: P<0.05, **: P<0.01, ***: P<0.001, ****: P<0.0001. For 72h treatment periods all values are significantly different compared to the control (P<0.0001). P values were calculated using Tukey's multiple comparisons test. **B)** Maximal respiration and basal respiration parameters of cell lines JJ012 and CH2879 after 72-hour treatment with AGI-5198 (JJ012) or D2-HG (CH2879) normalized towards control conditions. Treatment with Agi-5198 or D2HG both resulted in a decrease in metabolic parameters compared to control. Significant changes towards control were calculated using Sidaks multiple comparison and are presented by asterisks *: P<0.05, **: P<0.01, ***: P<0.001, ****: P<0.0001.



Supplementary figure 2. No synergistic effects after combined treatment of mTOR inhibitors sapanisertib or rapamycin and doxorubicin(DXR) or cisplatin(CDDP) in chondrosarcoma cell lines SW1353 and CH2879



Supplementary figure 3. Blood count and weight of control compared to sapanisertib treated mice. **A)** No difference between WBC, RBC, HGB, Platelets and lymphocyte count in sapanisertib compared to control mice. **B)** Weight normalized towards week 0 of mice treated with sapanisertib compared to control mice. No difference is observed between the two groups. Each point indicates one mouse. WBC: White Blood Count, RBC: Red Blood Count, HGB: Hemoglobin



Supplementary figure 4. No difference is observed between percentages of positive cells for ki-67, p-S6, cleaved caspase 3 and LC3B in tumors harvested from mice treated with sapanisertib compared to control mice. Each dot represents one tumor, bars show means with standard deviation.

Supplementary table 1. Metabolic compounds

Compound	company	product number	concentration range	molecular weight	stock	dissolvent
aicar	Selleckchem	S1802	0, 1, 10, 100, 1000 uM	258.23	50 mM	dmso
AZD3965	Selleckchem	S7339	0, 0.1, 1, 10, 100 nM	515.51	10 mM	dmso
Bardoxolone methyl (CDDO-Me)	Selleckchem	S8078	0, 0.01, 0.1, 1, 10 uM	505.69	10 mM	dmso
BAY 87 2243	Selleckchem	S7309	0, 0.1, 1, 10, 100 nM	525.53	10 mM	ethanol
BPTES	Selleckchem	S7753	0, 0.01, 0.1, 1, 10 uM	524.68	10 mM	dmso
CB-839	Selleckchem	S7655	0, 1, 10, 100, 1000 nM	571.57	10 mM	dmso
clotrimazole (FB5097)	Selleckchem	S1606	0, 0.1, 1, 10, 100 uM	344.84	10 mM	dmso
CPI-613	Selleckchem	S2776	0, 1, 10, 100, 1000 uM	388.59	50 mM	dmso
cycloserine (DCS)	Selleckchem	S1998	0, 0.01, 0.1, 1, 10 mM	102.09	100 mM	water
EGCG	Selleckchem	S2250	0, 0.01, 0.1, 1, 10 uM	458.37	10 mM	dmso
Everolimus	Selleckchem	S1120	0, 0.01, 0.1, 1, 10 uM	958.22	10 mM	dmso
Indoximod (NLG-8189)	Selleckchem	S7756	0, 0.01, 0.1, 1, 10 uM	218.25	1 mM	dmso
lonidamine (AF1890)	Selleckchem	S2610	0, 1, 10, 100, 1000 uM	321.16	50 mM	dmso
PX-478	Selleckchem	S7612	0, 0.1, 1, 10, 100 uM	394.12	10 mM	dmso
Silibinin	Selleckchem	S2357	0, 0.1, 1, 10, 100 uM	482.44	10 mM	dmso

Sodium Phenylbutyrate	Selleckchem	S4125	0, 0.01, 0.1, 1, 10 mM	186.18	100 mM	water
2DG	Sigma	D8375	0, 0.01, 0.1, 1, 10 mM	164.16	100 mM	dmso
3,3',5-triiodo-L-thyronine (T3)	Sigma	T2877	0, 0.1, 1, 10, 100 nM	650.97	10mM	dmso
3-BrPA	Sigma	16490	0, 0.1, 1, 10, 100 uM	166.96	10 mM	dmso
AOA (Aminooxyacetate)	Sigma	C13408	0, 0.01, 0.1, 1, 10 mM	109.30	100 mM	water
Buthionine sulphoximine (BSO)	Sigma	B2515	0, 1, 10, 100, 1000 uM	222.31	50 mM	water
dichloroacetate (DCA)	Sigma	347795	0, 0.01, 0.1, 1, 10 mM	150.92	100 mM	water
etomoxir	Sigma	E1905	0, 1, 10, 100, 1000 uM	338.76	20 mM	dmso
MKT-077	Sigma	M5449	0, 0.01, 0.1, 1, 10 uM	432.00	10 mM	dmso
NAC	Sigma	A7250	0, 0.01, 0.1, 1, 10 mM	163.19	100 mM	rpmi
Perphenazine	Sigma	P6402	0, 0.01, 0.1, 1, 10 uM	403.97	10 mM	ethanol
sodium oxalate	Sigma	71800	0, 1, 10, 100, 1000 uM	134.00	50 mM	rpmi
sodium oxamate	Sigma	O2751	0, 1, 10, 100, 1000 nM	111.03	10 mM	water
stiripentol	Sigma	S6826	0, 0.1, 1, 10, 100 uM	234.29	10 mM	dmso
WZB117	Sigma	SML0621	0, 0.01, 0.1, 1, 10 uM	368.31	10 mM	dmso
PFK158	Axon med chem	2542	0, 1, 10, 100, 1000 nM	328.29	10 mM	dmso

FX11	Merck	217507	0, 0.01, 0.1, 1, 10 uM	352.00	10 mM	dmso
CK37	emd millipore	229103	0, 0.01, 0.1, 1, 10 uM	366.47	10 mM	dmso
WYE-125132	Selleckchem	S2661	0, 1, 10, 100, 1000 nM	519.6	10 mm	dmso
Sapanisertib	Selleckchem	S2811	0, 1, 10, 100, 1000 nM	309.33	10 mm	dmso
GMX1778	Selleckchem	S8117	0, 0.1, 1, 10, 100 nM	371.86	10 mM	dmso
chloroquine	Selleckchem	S4157	0, 0.1, 1, 10, 100 uM	515.86	50 mM	rpmi
simvastatin	sigma	S6196	0, 0.1, 1, 10, 100 uM	418,566	10 mM	dmso
Lovastatin	Selleckchem	S2061	0, 0.1, 1, 10, 100 uM	404.54	10mM	dmso

Supplementary Table 2. Conditions of Immunohistochemical staining on mouse tumor tissue

Protein	Dilution	Blocking	Antigen Retrieval	Positive control	Manufacturer and clone
P-S6	1:800	-	Citrate	Melanoma	Cell signalling Clone D57.2.2E
Cleaved caspase	1:800	-	Citrate	Tonsil	Cell signalling (Asp175) #9661
LC3B	1:40000	5% Milk	Citrate	Cerebral cortex	Cell signalling Clone D11
Ki-67	1:1600	-	Citrate	Tonsil	Cell signalling Clone D2H10

Additional supplementary materials are available online:
https://www.sciencedirect.com/science/article/pii/S2212137418302902?via%3Dihub

Chapter 8

Radiotherapy resistance in chondrosarcoma cells; a possible correlation with alterations in cell cycle related genes.

This chapter is based on the publication:
de Jong Y, Ingola M, Briaire-de Bruijn I, Kruisselbrink AB, Venneker S, Palubeckaite I, Heijs B, Cleton-Jansen AM, Haas R, Bovée JVMG, Radiotherapy resistance in chondrosarcoma cells; a possible correlation with alterations in cell cycle related genes. Clin Sarcoma Res. 2019 May 28;9:9..

Abstract

Conventional chondrosarcomas are malignant cartilage tumors considered radioresistant. Nevertheless, retrospective series show a small but significant survival benefit for patients with locally advanced disease treated with radiotherapy. And, in daily practice when considered inoperable their irradiation is an accepted indication for proton beam radiotherapy. Therefore, we investigated the sensitivity of chondrosarcoma cell lines and -tissue samples towards radiotherapy and screened for biomarkers to identify predictors of radiosensitivity.

Proliferation and clonogenic assays were performed in chondrosarcoma cell lines after γ-radiation in combination with mutant IDH1 inhibitor AGI-5198. In addition, glutathione levels were measured using mass spectrometry. Chondrosarcoma tumor explants were irradiated after which γ-H2AX foci were counted. Mutation analysis was performed using the Ion AmpliSeq™ Cancer Hotspot Panel and immunohistochemical staining's were performed for P-S6, LC-3B, P53, Bcl-2, Bcl-xl and survivin. Results were correlated with the number of γ-H2AX foci.

Chondrosarcoma cell lines were variably γ-radiation resistant. No difference in radiosensitivity, nor glutathione levels was observed after treatment with AGI-5198. Irradiated chondrosarcoma patient tissue presented a variable increase in γ-H2AX foci compared to non-radiated tissue. Samples were divided into two groups, high and low radioresistant, based on the amount of γ-H2AX foci. All four highly resistant tumors exhibited mutations in the pRb pathway, while none of the less radioresistant tumors showed mutations in these genes.

Chondrosarcoma cell lines as well as primary tumors are variably radioresistant, particularly in case of a defective Rb pathway. Whether selection for radiotherapy can be based upon an intact Rb pathway should be further investigated.

Introduction

Chondrosarcomas are a heterogeneous group of cartilage producing tumors of which the most prevalent subtype (85%) is conventional chondrosarcoma. More rare subtypes include dedifferentiated chondrosarcoma (10%), mesenchymal chondrosarcoma (2%), clear cell chondrosarcoma (2%), and periosteal chondrosarcoma (1%). Conventional chondrosarcoma can be subdivided into three histological grades, representing the most important prognostic factor. Atypical cartilaginous tumors (previously referred to as grade I) have a relatively good prognosis, exhibiting a 10 years survival of 83%. Probability of surviving 10 years is about 64% for grade II chondrosarcomas and about 29% for grade III chondrosarcomas [1-3]. Based on their location conventional chondrosarcoma can be further subdivided into central (85%) and peripheral (15%) subtypes. Rare chondrosarcoma subtypes dedifferentiated, mesenchymal and clear cell chondrosarcoma occur also centrally in the bone [4-6], while periosteal chondrosarcoma is located and originates from the periosteal surface of the bone [3]. In this study, we focused on central chondrosarcomas, as these are of highest prevalence. Central chondrosarcomas carry mutations in the genes encoding the enzymes Isocitrate dehydrogenase 1 or -2 (IDH1 or IDH2) [7-9] in approximately 50% of cases, resulting in production of the oncometabolite D-2-hydroxyglutarate (D-2HG).

The only curative treatment for patients with chondrosarcoma is radical surgery, since they are considered resistant towards conventional chemo- and radiotherapy. For this reason, linear accelerator based (conventional) radiotherapy is only given to patients with metastatic disease, incomplete resection or inoperable tumors in difficult sites like the base of skull or the sacrum [2, 10]. Several causes have been suggested for chondrosarcoma radioresistance, such as the relative hypoxic chondrosarcoma microenvironment impairing the induction of reactive oxygen species (ROS) and DNA damage, or their relatively slow dividing rate (though not applicable to high grade tumors). Additionally, knock down of anti-apoptotic Bcl-2 family members Bcl-2, Bcl-xl and XIAP has been shown to increase cell death after radiation in chondrosarcoma cell lines [11]. Furthermore, restoration of p16 activity sensitized chondrosarcoma cell lines to radiotherapy [12]. More recently, a role for *IDH* mutations has been found in predicting radiotherapy response in glioma due to altered redox responses in *IDH* mutant- compared to wild type cells thereby enhancing radiosensitivity [13].

A recent retrospective analysis suggested that a subgroup of chondrosarcoma patients with locally advanced, unresectable disease showed a favorable overall survival after conventional radiotherapy [10]. As chondrosarcoma patients are not commonly treated with radiotherapy, prognostic biomarkers for radiosensitivity were investigated using in vitro and ex vivo methods. Therefore, the aim of our study was to examine whether sensitivity to γ-radiation can be observed in central conventional chondrosarcoma cell lines by determining clonogenic survival and γ-H2AX foci induction after radiation. In addition, radiosensitivity of chondrosarcoma patient samples was determined by counting γ-H2AX foci after ex vivo radiation [14, 15]. Mutation and expression analyses were performed to investigate prognostic biomarkers for radiosensitivity which could then be used to select patients for radiotherapy.

Methods

Cell culture

Conventional chondrosarcoma cell lines JJ012 (Grade II, *IDH1* mutant) [16], SW1353 (Grade II, *IDH2* mutant) (ATCC) and CH2879 (Grade III, *IDH* wild type) [17] were cultured in RPMI-1640 (Gibco, Invitrogen Life-Technologies, Scotland, UK) supplemented with 10% Fetal Calf Serum, at 37˚C in a humidified incubator (5% CO2). Short tandem repeat analysis was performed before and after completion of experiments to confirm identity of the cell lines by using the Cell ID Gene Print 10 system (Promega Benelux BV, Leiden, The Netherlands). Mycoplasma tests were performed on a regular basis.

Compounds

The specific mutant IDH1 inhibitor AGI-5198 (14624, Cayman Chemicals, Michigan, USA) was dissolved in DMSO according to the manufacturer's instructions and stored in -20˚C. AGI-5198 was used at a concentration of 10 μM since our group previously showed that this leads to a complete inhibition of D-2HG production [18]. (2R)-Octyl-α-hydroxyglutarate (16366, Cayman Chemicals), a cell-permeable derivative of D2-HG, was freshly dissolved in PBS before use and used at a final concentration of 250 μM.

Clonogenic assays

Chondrosarcoma cell lines SW1353 and JJ012 were plated in optimal cell densities to obtain sufficient colonies after treatment. Cells were allowed to adhere overnight before treatment with a wide range (0, 1, 2, 4 or 6 Gy) of γ-radiation using a 137Cs source (YXLON, Comet technologies USA). Colony formation was assessed after 14 days of treatment by fixing and staining with 0.5% crystal violet/6% glutaraldehyde. Colonies were counted manually and the surviving fraction (SF) was calculated by normalizing towards the plating efficiency of untreated controls. The α/β ratios were calculated based on the linear quadratic model. α/β ratios describe the slope of the cell-survival curve; acute responding tissues show a higher ratio compared to late responding tissues.

Viability assay

Chondrosarcoma cells were counted using a Burker Turk counting chamber and seeded in optimized cell densities in 96 well plates. After attachment overnight cells were irradiated with increasing doses. Seventy-two hours after radiation cell viability was measured using PrestoBlue viability reagent (Invitrogen, Life-Technologies, Scotland, UK) according to the manufacturer's instructions. Fluorescence was measured at 590 nM using a Wallac plate reader (Victor3V, 1420 multilabel counter, Perkin Elmer, the Netherlands). Experiments were performed three times in triplicate.

Cell proliferation measurement

To measure cell proliferation in real time the RTCA xCELLigence system (Roche Applied Sciences, Almere, the Netherlands) was used. JJ012, SW1353 and CH2879 cells were pre-treated for 72h with either 10 μM AGI-5198 or 0.1% DMSO in T25 flasks. Thereafter, cells were plated in the presence of AGI-5198 or DMSO and left for 20 hours to attach to the wells before low-dose (2 Gy) or high dose (4 Gy) γ-radiation. After 125 hours the measurement was stopped, and the results were analyzed using RTCA software. Normalized cell index was calculated by normalizing against the amount of cells in each well after 20 hours. Experiments were performed in duplicate and repeated at least two times.

γ-H2AX staining of chondrosarcoma cell lines

Optimal cell amounts for JJ012 (550.000 cells), SW1353 (500.000 cells) and CH2879 (1000.000 cells) cell lines were cultured on alcohol sterilized APES

coated slides in a culture dish. *IDH* mutant chondrosarcoma cell lines SW1353 and JJ012 were pre-treated with 10 μM AGI-5198 or 0.1% DMSO for 72h, in order to inhibit mutant activity, and *IDH* wild type chondrosarcoma cell line CH2879 was pre-treated with 250 μM D-2HG for 24h, in order to mimic mutant activity. After overnight attachment cells were radiated to 5 Gy, in order to achieve highest response without significant damage to sample, and fixed after 2 or 24h with buffered 4% formaldehyde (VWR Chemicals) at 37°C. The procedure was performed as described previously [19]. Slides were stained with γ-H2AX antibody (JBW301, Millipore) for 60 minutes, washed, then a mixture of Alexa fluor 657 labelled secondary antibody and 0.5 μM Hoechst33342 was applied for 60 minutes. Slides were covered with ProLong Gold antifade reagent and were examined using a confocal microscope by taking tile scans of areas of interest using ZEN light software. Pictures were exported in TIF format and loaded into FoCo, a previously published format using Image J and Matlab software [20].

Mass spectrometry measurements of glutathione

Chondrosarcoma cell lines JJ012, SW1353 and CH2879 were seeded in triplicate in T25 culture flasks and allowed to adhere overnight. *IDH* mutant chondrosarcoma cell lines JJ012 and SW1353 were pre-treated with 10 μM AGI-5198 for 72h, while *IDH* wildtype cell line CH2879 was pre-treated with 250 μM D-2HG for 24h. Samples were treated with 0 or 5 Gy, in order to correlate with γ-H2AX results. Cells were harvested by scraping after one hour. For each individual replicate 1x105 cells were fixed overnight using ice-cold acetone. The supernatant was then removed and dH2O added (10 μL), followed by a freeze-thaw cycle (10 seconds dry ice-cooled MeOH, 2 minutes 37oC heating block). After 2 freeze thaw cycles glutathione was extracted with EtOH (50 μL) and the supernatant was mixed 1:1 (v:v) with matrix solution (N-(1-naphthyl) ethylenediamine dihydrochloride in 70% MeOH) for analysis. Samples were measured using a 9.4T SolariX XR Fourier transform ion cyclotron resonance (FT-ICR) mass spectrometer (Bruker Daltonics, Bremen, Germany) equipped with a CombiSource™ and a dynamically harmonized ParaCell™. The mass spectrometric analysis was performed in negative ion mode in the m/*z*-range 101.7-1000 Da, with a 1M data point transient (0.3670 second duration) and an estimated resolving power of 66000 at m/z 400. Spectra were acquired of 10 averaged scans, acquired in a random pattern covering the entire spot, where each scan consisted of 500 laser shots (laser power 70%, 1000 Hz frequency). Spectra were analyzed using DataAnalysis version 4.2 (Bruker Daltonics, Bremen,

Germany). Using a custom algorithm, intensities of the compounds of interest were extracted and exported to Excel 2016 (Microsoft) for further analysis.

Chondrosarcoma tissue samples

Chondrosarcoma patient samples (n=9, see table 1) were obtained at surgery and collected in RPMI-1640 medium supplemented with 20% FBS and 1% P/S. All samples were coded according to the Dutch code of proper secondary use of human material as accorded by the Dutch society of pathology (Federa), and as approved by the LUMC ethical board (B17.019). Samples were processed by manual dissection into approximately 2 mm fragments and incubated on a rotation shaker at 37˚C in a humidified incubator (5% CO2). The tissue was treated to 5 Gy, consistent with the cell line treatment, and incubated for 2 or 24 hours on a rotation shaker at 37˚C in a humidified incubator as described previously [21]. After overnight fixation with formalin, tissue was processed, embedded in paraffin and sections were cut and stained for γ-H2AX foci.

γ-H2AX staining and quantification of paraffin embedded chondrosarcoma tissue

Sections were deparaffinized and antigen retrieval was performed using DAKO antigen retrieval solution pH 9.0 by boiling the slides in a microwave for 12 minutes. After cooling, slides were washed with TBS and blocking was performed using TBS/5% normal goat serum/1% BSA and 0.2% Triton-x-100. Anti γ-H2AX antibody (Millipore 2310355) was applied in a 1:1000 dilution and incubated overnight at 4ºC. Slides were washed with TBS and incubated with secondary labelled GAM Alexa fluor 647 antibody. Mounting was performed with ProLong Gold containing DAPI (Thermofisher Scientific, Waltham, Massachusetts, USA). Positive control slides consisted of JJ012 cells radiated at 4 Gy as these displayed a consistent, high γ-H2AX signal. The cells were embedded in paraffin with the use of ShandonTM CytoblockTM Cell Block Preparation system (Thermofisher Scientific). Slides were analyzed using a confocal microscope by taking tile scans of areas of interest using ZEN light software. Pictures were exported in TIF format and loaded into FoCo, a previously published format using Image J and Matlab software [20]. The settings to quantify the foci were first optimized using the positive control slides, after which the complete series was analysed using the same settings.

Immunohistochemical staining

Protein expression of Bcl-2, Bcl-xl, Survivin, P-S6, P53 and LC-3B were evaluated in irradiated control patient samples (see supplementary table 1). Immunohistochemistry was performed according to standard laboratory methods as previously described [22]. All Slides were scored by two independent observers (JVMGB,YDJ) using a scoring system assessing staining intensity (0= no, 1=weak, 2=moderate, 3=strong) as well as percentage of staining (0=no, 1=1-24%, 2=25-49%, 3=50-74%, 4=75-100%) [23] This scoring system was used for all the different proteins.

Mutation analysis

Frozen tissue of chondrosarcoma patients was collected at surgery and DNA was isolated using the wizard genomic DNA purification kit (Promega, Madison, WI, USA) according to the manufacturer's instructions. Samples were subjected to next generation sequencing using the in-house developed Ion AmpliSeq™ Cancer Hotspot Panel v2 (Life Technologies, Thermo Fisher Scientific, USA, catalog number 4475346) as described previously [24, 25]. The following genes and regions were analysed on an Ion Torrent PGM/Proton for all samples (exons between brackets): *ARAF (7,10); CTNNB1 (1,2,4,7,8,12,15); KRAS (2-4); NRAS (2-4); HRAS (2-3); BRAF (11,15); EGFR (3,7,15,18-21); GNAQ (5); GNAS (8-9); H3F3A (2); H3F3B (2); IDH1 (4); IDH2 (4); KIT (2,9-18); MYD88 (3b,5), MUTYH (7,13); PDGFRA (12,14,15,18,23); PIK3CA (2,5,6-10,14,18,21); POLE (9,11,13,14); RET (10-12,15,16); TP53 (1-11).* In addition hotspot regions in the following genes were also included: *ABL1; AKT1; ALK; APC; ATM; CARD11; CD79A; CD79B; CDH1; CDKN2A; CSF1R; CTNNB1; ERBB2; ERBB4; EZH2; FBXW7; FGFR1; FGFR2; FGFR3; FLT3; GNA11; HNF1A; JAK2; JAK3; KDR; MET; MLH1; MPL; NOTCH1; NPM1; PTEN; PTPN11; RB1; SMAD4; SMARCB1; SMO; SRC; STK11; VHL*. Libraries were prepared with 10 ng of genomic DNA, and each sample was uniquely barcoded. Ion Proton chips were prepared using the Ion Chef System. The unaligned bam files generated by the Proton sequencer were mapped against the human reference genome (GRCh37/hg19) using the TMAP 5.0.7 software with default parameters (https://github.com/iontorrent/TS). The Torrent Variant Caller (TVC)-5.0.2 was used for variant calling and variant interpretation was done using Geneticist Assistant (http://softgenetics.com/GeneticistAssistant_2.php) as described. Chromosomal gains and deletions were assessed by calculating the median

base coverage per amplicon, which was normalized using the median value of all amplicons in that sample.

Results

Chondrosarcoma cell lines are variably resistant to γ-radiation

Clonogenic assay SF2 values (surviving fraction of cells after 2 Gy radiation) suggested that JJ012 cells (SF2 0.55) were more radiosensitive compared to SW1353 (SF2 0.88) (Figure 1A). JJ012 cells showed a high α/β ratio of 38.47, in contrast to SW1353 with a very low α/β ratio of -0.75, suggesting that SW1353 cells may benefit from hypofractionation. CH2879 cells were unable to form colonies and were therefore not included in this assay. In addition, dose response curves were made to assess viability after 72h. JJ012 cells were most sensitive followed by the CH2879 and SW1353 cells (Figure 1B). An X-CELLigence assay was performed to determine the effect on proliferation in real time after 2 or 4 Gy of radiation. All cell lines showed a reduction in proliferation after radiation. However, CH2879 cells showed a similar response when irradiated to 2 or 4 Gy, while JJ012 and SW1353 showed a dose response relationship (Figure 1C). The amount of double strand breaks was determined by quantifying γ-H2AX foci 2 and 24 hours after radiation treatment. JJ012, SW1353 and CH2879 cells exhibited an increase in foci after 2 hours, which was decreased again after 24 hours in SW1353 and CH2879 cells (Figure 1D-E). Conversely JJ012 cells still showed a substantial significant amount of foci compared to non-radiated cells, indicating this cell line is less proficient in repairing double strand breaks and more sensitive to radiation.

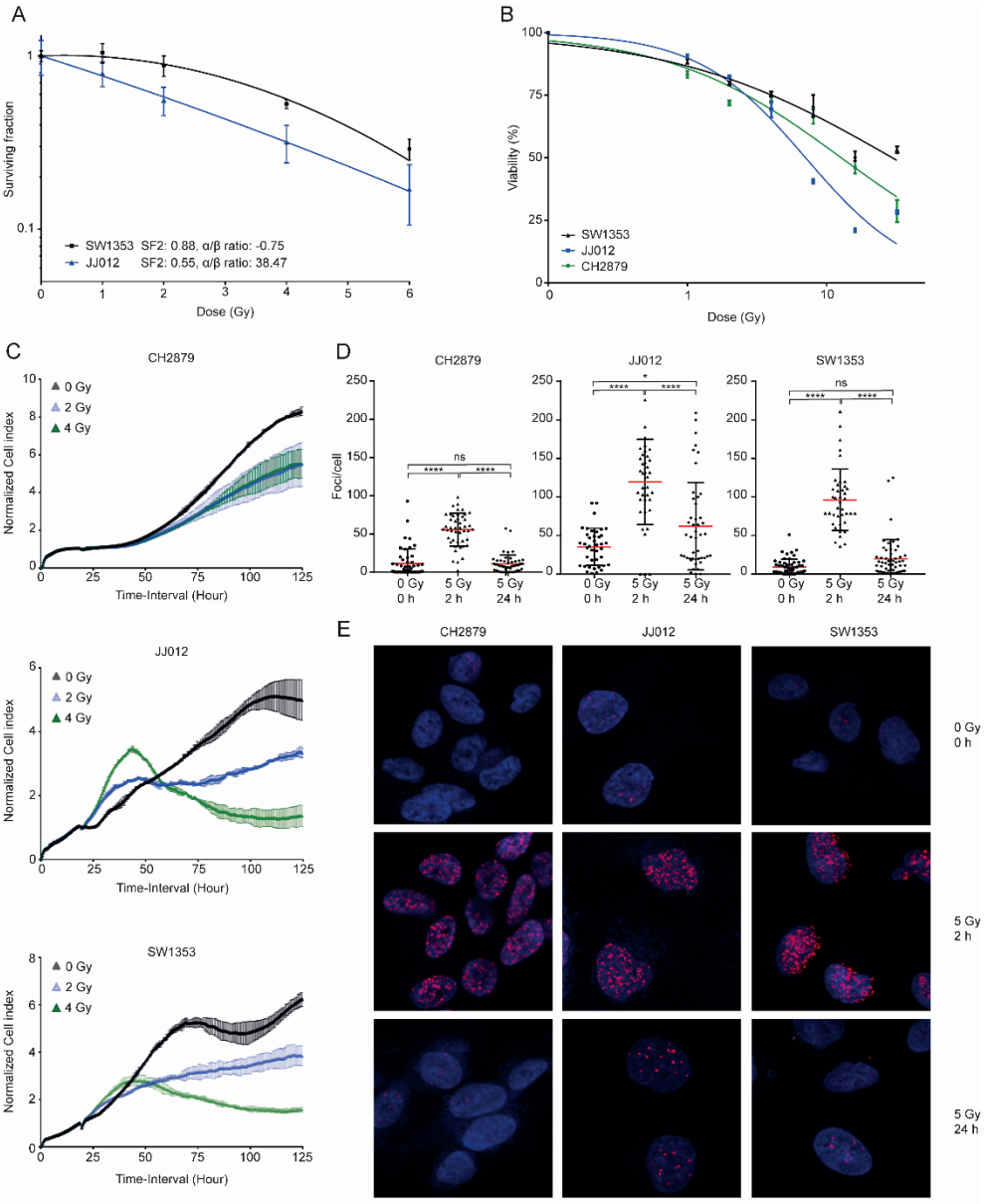


Figure 1. Chondrosarcoma cell lines exhibit variable γ-radioresistance.
A) Colony forming assay of SW1353 and JJ012 chondrosarcoma cells. B) Viability of JJ012, SW1353 and CH2879 cells, 72h after treatment with increasing doses of γ-radiation measured using presto blue viability reagent. C) Normalized cell index of CH2879, JJ012 and SW1353 cells irradiated to 0 (black), 2 (blue) or 4 Gy (green) radiation. D,E) γH2AX foci/cell in CH2879, JJ012 and SW1353 cells 2 and 24h after 5 Gy radiation.**** indicates P values <0.0001, * indicates P values <0.05

γ-radiosensitivity of chondrosarcoma cell lines is not correlated with IDH mutation status

Inhibition of D-2HG production by JJ012 cells using the mutant IDH1 inhibitor AGI-5198 did not show any difference in radiosensitivity (Supplementary figure 1A). In addition no differences were observed in proliferation capacity and foci formation between cells treated with AGI-5198 and radiation or cells treated with radiation only (Supplementary figure 1B, C). Since previous reports [13] suggest that *IDH* mutant cells have a reduced capability of producing GSH, GSH levels were measured 1 hour after radiation in combination with AGI-5198, for the mutant cell lines or D-2HG (oncometabolite resulting from an *IDH* mutation) for the wild type cell line. The most radioresistant SW1353 cells displayed the highest baseline levels of GSH, followed by CH2879 and JJ012, however no differences were observed between different treatment conditions, indicating that GSH levels are not influenced by D-2HG inhibition (Supplementary figure 1D). These results suggest that *IDH* and D-2HG do not play a role in chondrosarcoma radiosensitivity.

Chondrosarcoma samples that are more resistant towards radiotherapy have an increased incidence of mutations in cell cycle regulators

Chondrosarcoma patient samples showed an increase in γ-H2AX foci after 5 Gy radiation treatment. In table 1, 2 and supplementary figure 2 quantified results are shown for nine chondrosarcoma explant tissue samples analyzed 2 or 24 hours after treatment with 5 gy of radiation. Radiation response was heterogeneous across the samples; the largest difference between control and radiated samples was 11,2 foci (sample L5213, figure 2A,B), while the smallest difference was 0,9 foci. Samples analyzed 24 hours after radiation treatment in general showed a lower amount of foci compared to samples analyzed 2 hours after radiation treatment. Samples that showed a difference of 4 or more foci were subjected to the less radioresistant group, while samples that showed <4 foci difference were designated as radioresistant for further analysis. This cutoff value was taken for both the 2h and 24h groups and both time points were analyzed together since sample size impeded separate analyses. The division between more or less radioresistant was made prior to mutation analysis. No correlation was observed between histological grade or *IDH* mutation status and the amount of γ-H2AX foci, consistent with the results obtained in the chondrosarcoma cell lines (table 1). Mutation analysis was performed on 50 known cancer related genes and expression of Bcl-2, Bcl-

xl, survivin, P-S6, LC3B and P53, previously identified to play a role in chondrosarcoma [26-30], was determined using immunohistochemistry. No significant difference was observed in protein expression of selected markers (figure 2C, supplementary figure 3), however three CDKN2A deletions (3/4) and one RB1 deletion (1/4) were found in the highly radioresistant group and none in the less radioresistant group indicating that a defective Rb pathway may be able to impair the response to γ-radiation in chondrosarcoma (Figure 2D, Table 1 and 2). Interestingly a mutation in CDKN2A was also identified in the SW1353 cell line (supplementary table 2), which is the most radioresistant cell line.

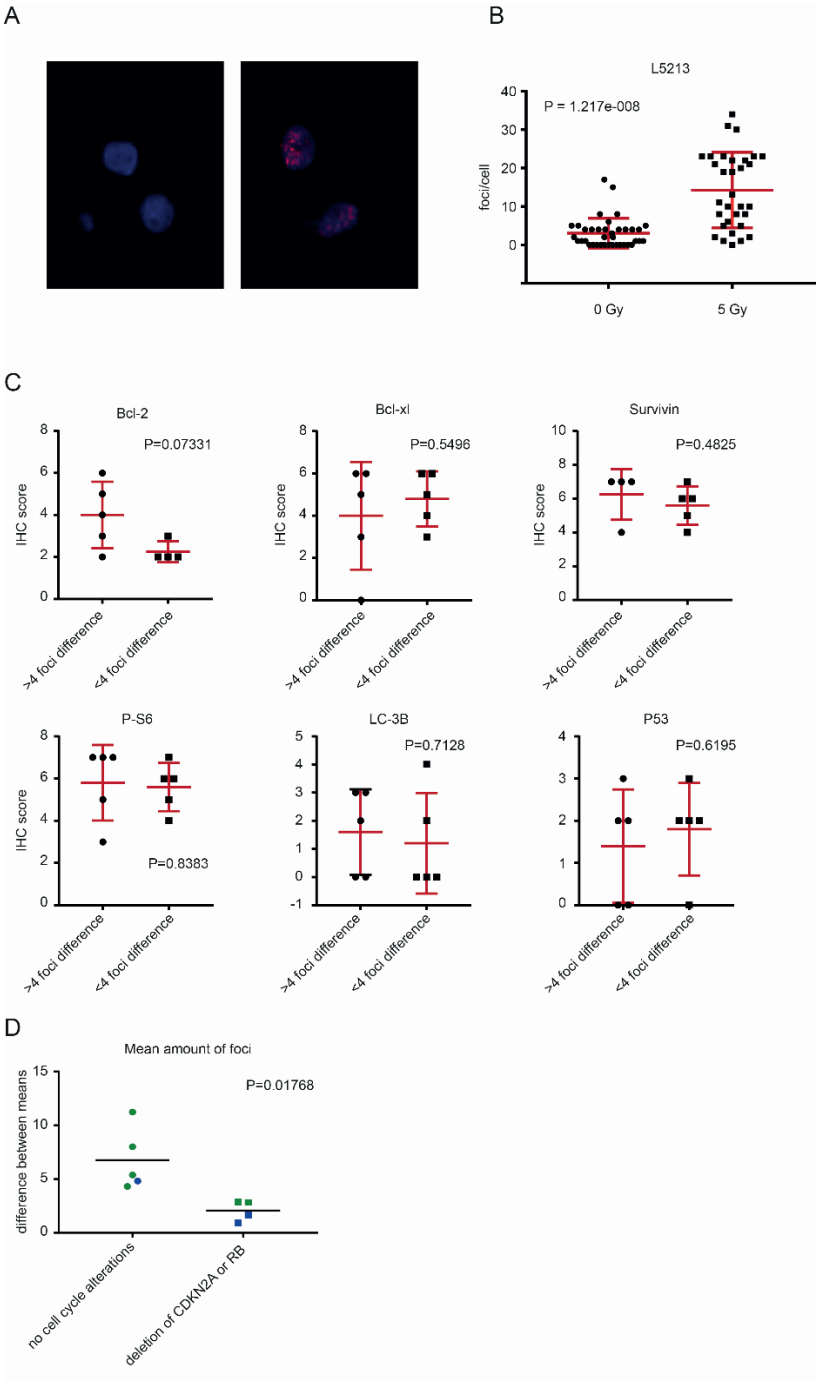


Figure 2. γ-radioresistance in chondrosarcoma tissues correlates with mutations in cell cycle related genes.

A) L5213 chondrosarcoma tissue sample showing a large induction of γH2AX foci after 5 Gy (right) compared to controls (right). B) Amount of foci/cell in sample L5213 after 5 Gy radiation compared to controls. C) Protein expression of Bcl-2, Bcl-xl, Survivin, P-S6, LC3B and P53 in highly radioresistant (<4 foci difference) compared to less radioresistant (>4 foci difference) chondrosarcomas. D) Difference between mean amount of γH2AX foci before and after radiation in chondrosarcomas with and without alterations in RB1 or CDKN2A Each dot represents one sample. Samples in green indicate 2h, while samples in blue indicate samples 24h after radiation.

Table 1. Patient samples treated with 5 gy of radiation. Foci were counted two hours after treatment

Sample	Grade	*IDH* mutation status	Other alterations	Mean +SEM basic foci level	Mean + SEM after 5gy radiation	Difference between means	P value
L5213	1	WT		3,053 ± 0,6277 n=38	14,28 ± 1,737 n=32	11,23 ± 1,733	<0.0001
L5678	3	*IDH1* R132C		0,2295 ± 0,07165 n=61	8,239 ± 1,477 n=46	8,01 ± 1,284	<0.0001
L5676	2	*IDH2* R172S		3,625 ± 0,9297 n=32	9 ± 1,493 n=34	5,375 ± 1,784	0.003712
L5541	Dediff	*IDH1* R132S		2,378 ± 0,5747 n=90	6,702 ± 1,225 n=57	4,324 ± 1,212	0.0005
L5546	2	WT	*CDKN2A* deletion	3,982 ± 0,9036 n=56	6,846 ± 1,172 n=52	2,864 ± 1,468	0.0537
L5302	3	WT	*CDKN2A* deletion	2,246 ± 0,4531 n=61	5,082 ± 0,7412 n=73	2,836 ± 0,9108	0.0023

Table 2. Patient samples treated with 5 gy of radiation. Foci were counted 24 hours after treatment

Sample	Grade	IDH mutation status	Other alterations	Mean +SEM basic foci level	Mean + SEM after 5gy radiation	Difference between means	P value
L5847	2	WT		2,644 ± 0,7399 n=59	7,467 ± 1,526 n=30	4,823 ± 1,501	0.0018
L5718	3	*IDH1* R132L	*CDKN2A* deletion	2,437 ± 0,584 n=87	4,088 ± 0,6256 n=102	1,651 ± 0,8659	0.0580
L5850	2	WT	*RB1* deletion	0,6327 ± 0,1409 n=98	1,55 ± 0,1809 n=140	0,9173 ± 0,2463	0.0002

Discussion

Chondrosarcomas are relatively radioresistant tumors and therefore, after multidisciplinary discussions, very few of these patients are offered radiotherapy [10]. In this study we investigated the sensitivity of chondrosarcoma cells and tumor explants to γ-radiation. In addition, we screened for biomarkers that could select patients that might benefit from γ-radiation. Chondrosarcoma cell lines showed a heterogeneous response to radiotherapy with relatively high SF2 values [31]. JJ012 cells presented a SF2 value of 0.55, while SW1353 were more resistant showing a SF2 value of 0.88. Compared to cell lines of other tumor types, these values are relatively high, confirming chondrosarcoma radioresistance. A previous published study by Hamdi et al. showed a SF2 value of 0.64 for the SW1353 cell line [32]. This difference might be attributed to the fact that different methods were used to perform the clonogenic assay. In our study the cells were seeded prior to radiation, while in the study of Hamdi et al. the cells were subconfluently seeded in culture flasks and subsequently radiated and seeded. In addition the differences in culture conditions (normoxic vs hypoxic) can very well influence how cells respond to radiation treatment. Quantification of γ-H2AX foci showed that SW1353 and CH2879 cells were able to repair double strand breaks within 24 hours after radiation while JJ012 cells were less capable of doing so, in line with the lower SF2 values observed in JJ012 cells.

In contrast to published studies in glioma [13, 33], we did not observe a correlation between radiosensitivity and *IDH* mutation status in chondrosarcoma. Inhibition of mutant *IDH1* using AGI-5198 did not lead to any changes in radiosensitivity, nor in altered GSH levels. Also, no difference was observed in γ-H2AX foci formation between *IDH* mutant and *IDH* wild type chondrosarcoma explants. This indicates that, unlike the observations in gliomas, *IDH1* or *IDH2* mutations in chondrosarcoma do not correlate with radiosensitivity. This is in line with our previous results in which we also did not find any correlation between *IDH1* or *-2* mutation status in sensitivity for inhibitors of glutaminolysis [34], NAD synthesis [35] or Bcl-2 family members in chondrosarcoma cells, while in other tumor types there was a clear difference in sensitivity [36-38]. This difference indicates that *IDH* mutations may have a tissue specific effect rather than a more general effect in different tumour types.

Our results suggest that deletions in cell cycle regulators CDKN2A and RB1 are associated with increased radioresistance in chondrosarcoma explant tissue. In line with this, the most resistant SW1353 cells harboured a

mutation in the splice site region of CDKN2A in addition to mutations in *TP53* and a kRAS mutation [39]. CDKN2A is a gene encoding p16(INK4A) and p14(ARF), which are two tumor suppressor proteins controlling the cell cycle. P16 inhibits CDK4 and CDK6, two inhibitors of Rb1 phosphorylation, while p14-ARF protects p53 from being broken down by inhibiting MDM2. Alterations in the pRB pathway have been described in the majority of high grade conventional chondrosarcomas [40-43]. Previous studies in chondrosarcoma cell lines (CS-7, CS-8, CS-9) showed that restoring P16 expression, and thereby increasing Rb1 phosphorylation, resulted in an increased radiosensitivity [12], in line with our results. Although we see a clear difference in the amount of foci in tumors with and without deletions in CDKN2A or RB1, this study is based on a small heterogenous group of chondrosarcomas and measurements are taken after 2 or 24 hours. In addition the threshold of 4 foci/cell is taken arbitrarily. This complicates making firm conclusions based on this data and further studies should focus on; including more patients and analyze amount of foci after 24 hours to determine the damage remaining after DNA repair. In addition when radiotherapy is included in the treatment plan, follow the response towards radiation and correlate this towards mutation status.

In contrast to our findings in chondrosarcoma, deletion of RB1 has been described to enhance radiosensitivity in breast, prostate and bladder cancer [44-47]. This observation might therefore be tissue and context specific; only a limited number of cancer types have been investigated. In addition, pRb has multiple functions, not only in the cell cycle but also in chromatin organization, transcription patterns, metabolic pathways and the proteome [48]. Several studies found that loss of pRB expression resulted in an increase in glutamine consumption and an increased glutamine incorporation into GSH. Upon RB knock down cells increased the expression of glutamine transporters and upregulated glutaminase activity, indicating that the pRB pathway can regulate glutamine metabolism and that cells with inactivated pRB are potentially more sensitive for targeted anti-glutamine treatment [49-51]. Interestingly, we recently reported that interfering with glutamine metabolism can be a therapeutic target for high grade chondrosarcoma [34]. The SW1353 chondrosarcoma cell line was particularly sensitive for inhibition of glutaminase, which is in line with the high basal GSH levels measured in this cell line. We can hypothesize that the mutation in CDKN2A observed in this cell line might contribute to the glutamine dependence, however more research is needed to further investigate this.

In addition to conventional radiotherapy, research has been focusing increasingly on proton [1H] and carbon [12C] therapy, which have several advantages compared to γ-radiation. Both these beam qualities have dose distribution advantages, causing less damage to surrounding healthy tissues, making it possible to deliver higher doses to the tumor. Chondrosarcomas of the skull base and spine are increasingly treated with proton beam radiation, showing promising results [52]. Whether chondrosarcoma radiosensitivity differs between photon (γ-) beams and proton beams is subject for further investigation.

In conclusion, this study confirms a heterogeneous radiosensitivity of chondrosarcoma cell lines and fresh explant tissue. In addition, we identified alterations in CDKN2A/RB1 as predictors of decreased double strand break formation after radiotherapy in chondrosarcoma tissue, and although further research is necessary in a larger group, this suggests that these patients might benefit less from radiation therapy.

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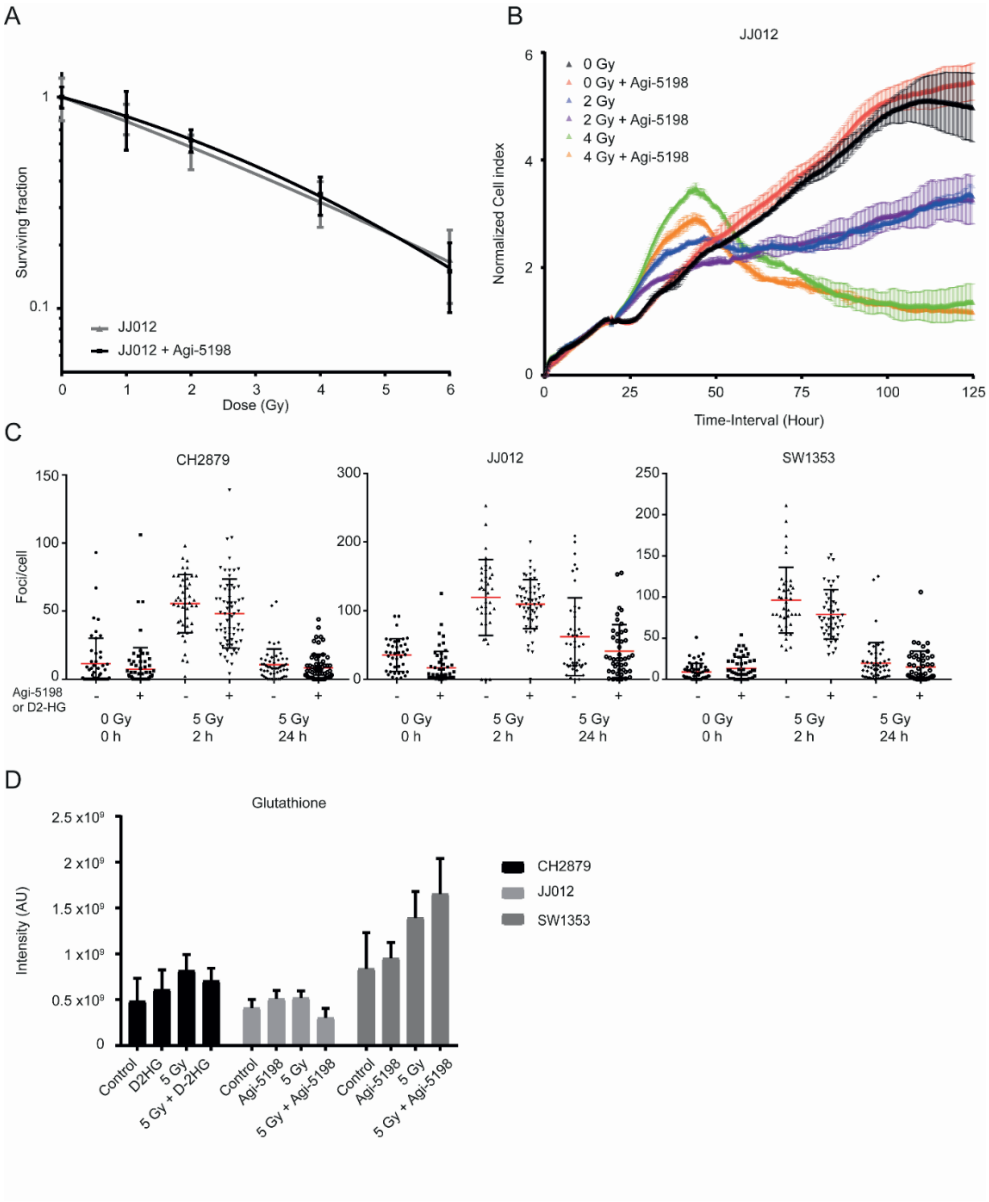
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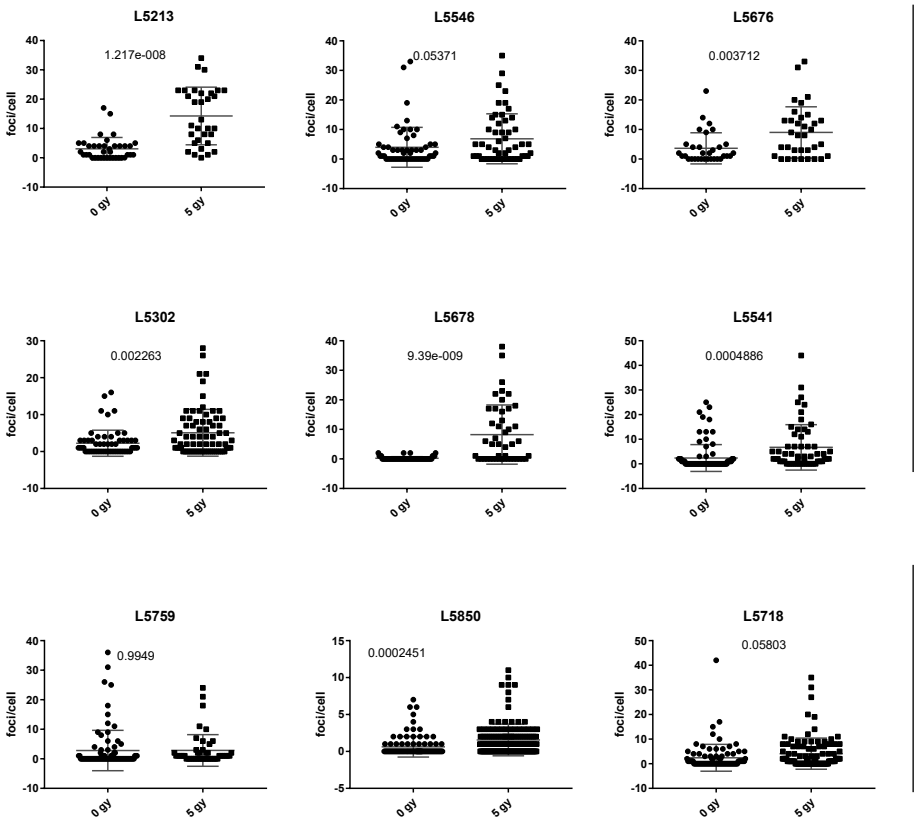
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Supplementary materials

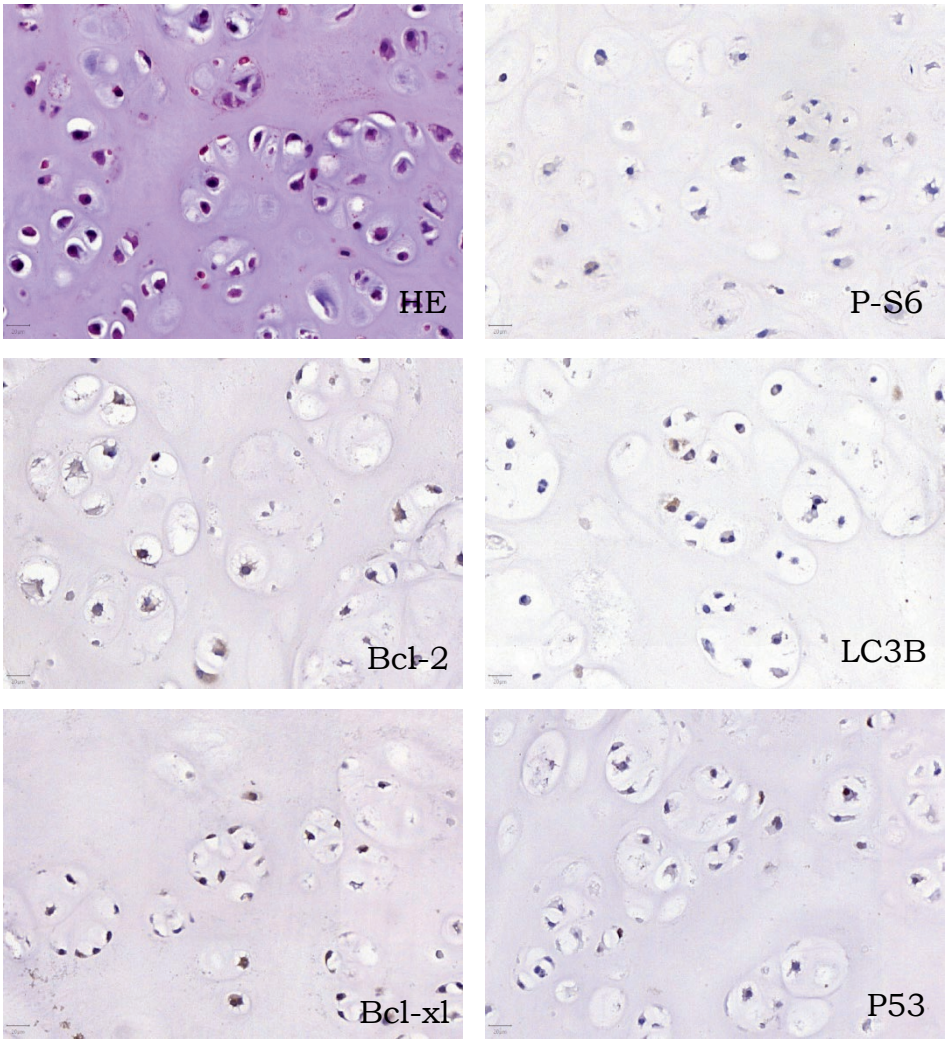


Supplementary figure 1. No relation between *IDH* mutation and radiosensitivity in chondrosarcoma cell lines A) clonogenic assay of JJ012 cells with or without 10 µM mutant IDH1 inhibitor AGI-5198.B) normalized cell index of JJ012 cells treated with 0, 2 or 4 Gy radiation in combination with 10 µM mutant IDH1 inhibitor AGI-5198 or DMSO as a control. Black indicates 0 Gy, Red 0 Gy + AGI-5198, blue 2 Gy, Purple

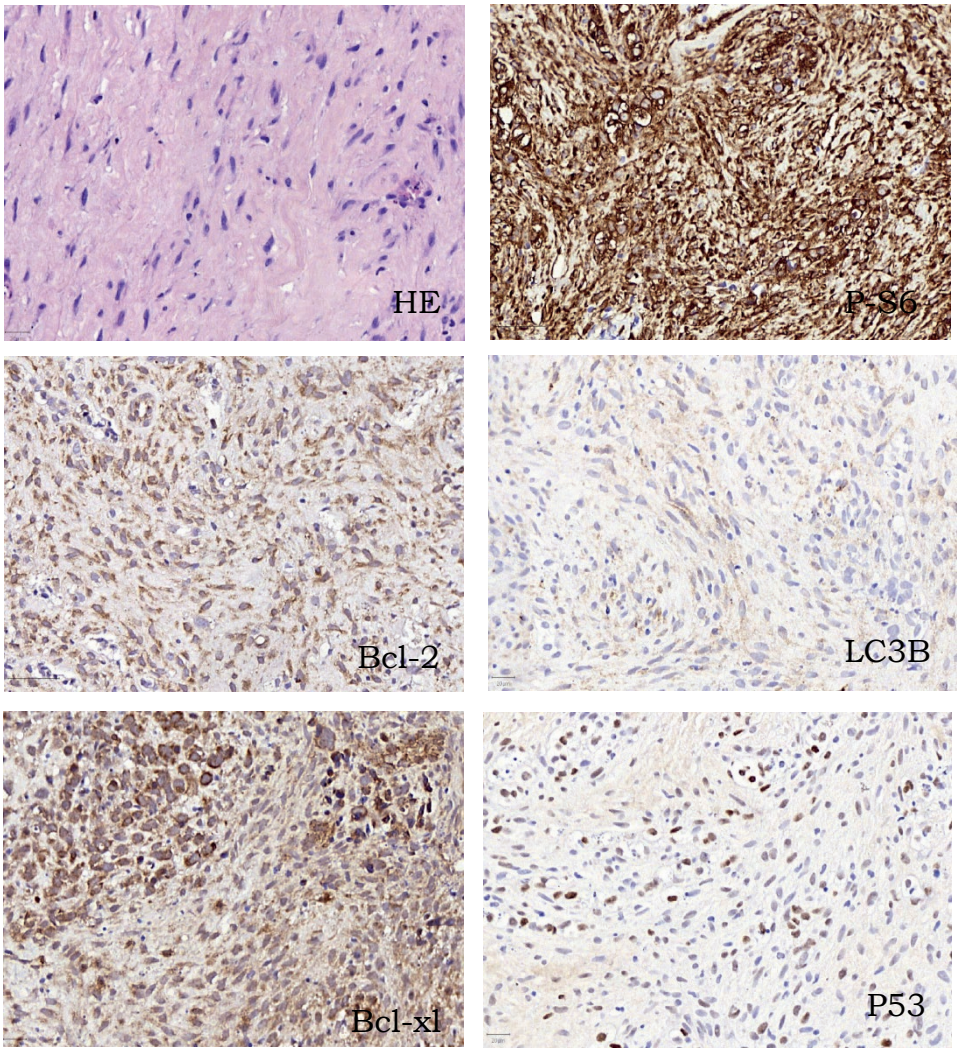
2 Gy + AGI-5198, green 4 Gy, Orange 4 Gy + AGI-5198. C) Amount of yH2ax foci in CH2879, JJ012 and SW1353 after 2 and 24h of treatment with 5 Gy radiation with and without 10 µM AGI-5198 or 250 µM D-2HG D) glutathione levels of chondrosarcoma cells treated with 10 µM AGI-5198 or 250 µM D-2HG measured 1h after radiation.



Supplementary figure 2. y-H2ax staining quantification of chondrosarcoma patient samples treated with 5 gy radiation. Significance was assessed by performing a T-test



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Supplementary figure 3. Representive images of HE and Immunohistochemical stainings of two different chondrosarcoma patient tissue samples.

Supplementary table 1. Conditions of Immunohistochemical staining on chondrosarcoma tissue

Protein	Dilution	Blocking	Antigen Retrieval	Positive control	Manufacturer and clone
Bcl-2	1:100	-	Tris-EDTA	Tonsil	Dako clone 124
Bcl-xl	1:400	5% Milk	Citrate	Prostate	Cell signalling clone 54H6
Survivin	1:100	-	Citrate	Placenta	Cell signalling clone 71G4B7
P-S6	1:800	-	Citrate	Melanoma	Cell signalling clone D57.2.2E
P53	1:800	-	Citrate	Tonsil	Dako clone DO-7
LC3B	1:40000	5% Milk	Citrate	Cerebral cortex	Cell signalling Clone D11

Supplementary table 2. Mutation analysis of chondrosarcoma cell lines. Results on *IDH* and *TP53* mutation status were published previously (de Jong et al. 2016).

	IDH1	***IDH2***	***TP53***	***CDKN2A***	***KRAS***	***PTEN***
SW1353	-	R172S	V203L	9:21971209 A-C*	G12C	-
JJ012	R132G	-	G199V	-	-	-
CH2879	-	-	S366A R273C#	-	-	R233Ter

only found in 17% of cells
* mutation found in splice site of intron

Chapter 9

Discussion and Future perspectives

Discussion

The studies presented in this thesis describe the use of compound and siRNA screens to identify new targeted treatment options for patients with chondrosarcoma. Using available chondrosarcoma cell lines as a model we investigated apoptotic proteins, kinases and metabolic regulators in a non-biased way to identify most promising hits. In addition the role of individual Bcl-2 family members Bcl-2, Bcl-xl and Bcl-w was investigated. Results reveal a role for Bcl-2 family member Bcl-xl, anti-apoptotic and cell cycle regulator Survivin, Cell cycle regulators AURKA, CHK1 and PLK1 and mTOR as important survival proteins in chondrosarcoma. Moreover treatment with Bcl-xl or CHK1 inhibitors could chemo-sensitize a subset of chondrosarcoma cell lines. Furthermore alterations in the Rb1 pathway have been identified as a marker for radioresistance in chondrosarcoma patient samples. In this chapter these findings are discussed in a broader perspective.

Bcl-xl as most important Bcl-2 family member in chondrosarcoma

The apoptosis pathway is an attractive target in the treatment of cancer, and has been studied extensively. Cancer cells can develop several mechanisms to evade apoptosis including upregulation of anti-apoptotic proteins or downregulation/inactivation of pro-apoptotic proteins or pore-forming proteins Bax and Bak [1]. The mechanism that has now been shown for all chondrosarcoma subtypes, except for periosteal chondrosarcoma, is upregulation of anti-apoptotic Bcl-2 family members [2-4]. In **chapter 3** of this thesis we confirmed this also for mesenchymal chondrosarcoma and showed that these cells can be sensitized towards conventional chemotherapy by inhibiting Bcl-2 family members. In **chapter 4** we looked at the individual Bcl-2 family members and showed that particularly Bcl-xl is important and might be most promising in targeted treatment of chondrosarcoma [5]. We showed that Bcl-2 family members Bcl-2 and Bcl-xl were highly expressed in chondrosarcoma tissue samples and showed a correlation of expression with histological grade. Selective inhibition of Bcl-2 did not result in any decrease in viability in a panel of ten chondrosarcoma cell lines, nor a decrease in tumour growth in an orthotopic rat chondrosarcoma model. However selective Bcl-xl inhibition was effective in a subset of chondrosarcoma cell lines and in vivo tumour growth was slowed down. In addition a sensitizing effect was observed with chemotherapy in a subset of chondrosarcoma cell lines, but not in vivo. These data indicate

that Bcl-xl is the dominant/most important anti-apoptotic family member in chondrosarcoma.

No correlation between protein expression levels and response rate of chondrosarcoma cell lines to specific inhibitors was observed in our study, which can complicate selection of eligible patients. The group of Letai et al developed a method called BH3 profiling to determine the dependency of a certain tumour on individual anti-apoptotic proteins. Mitochondria of cancer cells are isolated and exposed to a panel of different pro-apoptotic synthetic BH3 peptides after which the initiation of MOMP and/or apoptosis is measured. How well these mitochondria respond to the different peptides can be measured and can give an indication of the threshold to achieve apoptosis in these cells, as well as identification of the anti-apoptotic proteins they are most dependent on [6]. In addition a modified version of this method can also be used to predict cytotoxic responses of cancers to different treatment strategies [7] and already showed its predictive value in pre-clinical models of different types of hematologic malignancies [8-10]. This strategy is an attractive method to determine chondrosarcoma dependency on Bcl-2 family members and to predict responses to apoptosis inducing treatments. Further research should determine whether this method is also suitable for solid tumours.

Selective targeting of Bcl-2 family members is highly advantageous in terms of toxicities, compared to combined inhibition using for example ABT-737 or its orally equivalent ABT-263 (navitoclax). However targeting of Bcl-xl seems more problematic compared to targeting Bcl-2 or Mcl-1 due to its expression on platelets. This might be solved by careful dosing and timing of drug administration. In addition, combinations with other treatments might lower the effective dose and consequently also toxicity [11].

In our study we showed that single inhibition of Bcl-2 is ineffective in pre-clinical models, but we did not specifically look into Mcl-1 as a possible important anti-apoptotic protein in chondrosarcoma. Previous studies showed RNA as well as protein expression of Mcl-1 in chondrosarcoma cell lines and several pre-clinical studies suggest it can play a role in resistance mechanisms in solid tumours. It would be interesting to study its role in chondrosarcoma, for example using BH3 profiling [11-15].

Survivin; a pro-survival protein with a broad range of functions

As discussed in **chapter 5**, Survivin was identified as an important survival protein in chondrosarcoma cells by performing an apoptosis focussed siRNA screen [16]. Survivin has multiple functions based on its location in the cells. It's cytoplasmic function is mainly preventing apoptosis, while its nuclear function is regulating mitosis as part of the chromosomal passenger complex (CPC) [17]. Survivin was found to be highly expressed in a large panel (>200) of chondrosarcoma tissue samples of all different subtypes. Expression was found in the nucleus as well as in the cytoplasm, indicating that the apoptotic as well as its cell cycle related function is important in chondrosarcoma cells. No expression was observed in normal articular cartilage.

The exact mechanism by which Survivin inhibits apoptosis is not well understood. The current hypothesis is that Survivin has most inhibitory activity when in complex with XIAP (X-linked inhibitor of apoptosis) and HBXIP (hepatitis B X-interacting protein). Survivin is a member of the IAP family, which consists of eight different members. Only the canonical member XIAP can directly inhibit caspase activity, however interaction with other IAPs can improve its stability and increase its inhibitory effect [17]. When in complex with XIAP and HBXIP, Survivin can inhibit caspase 9, 8 and 3/7 activation [18] and increase the activity of other IAP family members acting in the extrinsic apoptosis route [19]. In addition Survivin has been shown to prevent apaf1 release from mitochondria and bind to the IAP inhibitor Smac/Diablo [20].

The function of Survivin in the cell cycle is essential for mitosis. It is a member of the CPC and ensures that chromosomes are properly aligned by targeting the CPC towards the centrosomes in prometaphase. This process is highly dependent on interaction with BUBR1 and AURKB (Aurora kinase B). In addition the CPC coordinates chromosome segregation and cytokinesis.

How Survivin is regulated is not completely understood. The main pathways that have been shown to regulate its expression are EGFR, IGF-1R or HER2/3 tyrosine kinase receptors, which will lead to activation of RAS and/or AKT and downstream signalling activation of mTOR and NF-kb. HIF-1α and STAT3 can function as a transcriptional activator of Survivin expression. Several reports suggest that wild type P53 can transcriptionally repress Survivin expression, which will lead to (amongst others) an increase

in apoptosis [21]. In addition, post-translational modifications, during different phases of mitosis, are regulating Survivin function and stability.

Since Survivin is regulated by a variety of different pathways and mechanisms it is hard to speculate which one is most important or most active in chondrosarcoma, however based on available literature and the results described in this thesis the hypothesis is that mTOR can play an important role. In addition, since chondrosarcomas are hypoxic tumours, its connection to HIF-1α would be interesting to investigate further, for example in 3D models that more closely mimic the patient situation.

A positive correlation between Survivin and P53 overexpression (suggestive for mutated *TP53*) was shown in conventional chondrosarcoma (chapter 5). In addition, chondrosarcoma cell lines with mutant *TP53* were more sensitive for Survivin inhibitor YM155, indicating that especially *TP53* mutant chondrosarcomas might be eligible for treatment with Survivin inhibitors. YM155 is an indirect inhibitor of Survivin; it inhibits the transcription of Survivin by disrupting the RNA binding protein ILF3/NF110 [22]. In addition, the binding of zinc transcription factor SP1 to the Survivin promoter is prevented by YM155 [23]. Since the SP1 transcription factor has more binding regions that activate transcription of genes involved in proliferation and cell cycle progression, there will likely be additional effects of YM155 [24]. This is also reflected in the different responses of cell lines towards YM155 treatment.

Phase I and II clinical trials show that YM155 is well tolerated, but minimal anti-tumour effects have been observed in mono- as well as combination treatment strategies [24, 25]. New Survivin inhibitors have been under development, of which the most promising one is the development of vaccines for cancer immunotherapy. Promising results have been achieved for glioblastoma multiforme and other solid tumours [26-28].

Cell cycle proteins as therapeutic targets

Besides Survivin other cell cycle regulators that might be important in chondrosarcoma have been identified using a combined siRNA screen and compound screen focussed at kinases, as described in **chapter 6** of this thesis. Hits that were identified in both screens were selected for further validation. Using this unbiased approach we identified AURKA (aurora kinase A), PLK1 (Polo like kinase 1) and CHK1 (Checkpoint kinase 1) as important kinases for chondrosarcoma cell survival [29]. These kinases are

all involved in cell cycle regulation, and are essential for proper cell division [30]. Cancer cells show aberrant cell cycle activity, which can be caused by deregulation of cell cycle proteins, or upstream cell signalling pathways [30]. This gives an opportunity to target cell cycle proteins specifically in cancer cells in mono treatment or in combination with chemotherapy [31].

PLK1 and AURKA are both involved in the progression from G2 to S phase during the cell cycle and are important for mitosis and cytokinesis [32]. CHK1 is activated in response to DNA damage by ATM or ATR, and cells will halt the cell cycle in S or G2 to repair the damage. Overexpression of these three cell cycle regulators has been shown in a variety of different tumours and correlates with a worse prognosis [33-35].

A subset of chondrosarcoma patients showed RNA expression of AURKA and CHK1, while PLK1 expression was low compared to normal cartilage. This indicates that PLK1 might not be a good target for chondrosarcoma treatment. AURKA was expressed in chondrosarcoma patient samples, and inhibitors for AURKA are already in clinical trials [30]. Monotherapy with alisertib, the most developed AURKA inhibitor was tested in different solid tumours including sarcoma, and showed a partial response in one out of six patients with dedifferentiated chondrosarcoma [36]. Although first results seemed encouraging [37], a large Phase III trial in lymphoma was discontinued due to lack of clinical response compared to the comparator arm [38]. Combination treatment studies show conflicting results, depending on the dose and tumour type, regarding adverse effects, maximum tolerated dose and efficacy [39-42]. Larger phase III randomized control studies should determine whether combination therapy is superior over standard treatment. High CHK1 RNA expression was correlated towards a worse overall survival, and inhibition of CHK1 sensitized chondrosarcoma cell lines towards conventional chemotherapy treatment. This was also previously shown in other sarcoma types [43-45]. CHK1 is part of the DNA damage pathway, and combination treatment with DNA damaging agents will lead to synthetic lethality. This means that a combination of the two treatments will lead to cell death, while treatment with only one of the two will not cause any or only minimal effect. First generation inhibitors of CHK1 were discontinued due to toxicity, however second generation inhibitors show less toxicity and are currently tested in combination with chemotherapy [34, 46, 47].

Defects in cell cycle regulators and DNA damage pathways are a general hallmark of cancer. The three cell cycle related kinases that we found in this

study are deregulated in a large portion of cancers. In chondrosarcoma specifically CHK1 might be the most interesting and most potent target to investigate further, since this was correlated to overall survival and synthetic lethality with chemotherapy was shown. A correlation was observed between *TP53* mutation status and sensitivity, which is also reported in the literature [48-50]. This indicates that the higher grade chondrosarcomas, harbouring a mutation in *TP53* (20%) [51] might benefit most from inhibitors targeting the cell cycle, as monotherapy of in combination strategies.

Metabolic vulnerabilities in chondrosarcoma; mTOR as central player

Another vulnerability investigated in chondrosarcoma was targeting metabolism. This might be a therapeutic opportunity since approximately 50% of the chondrosarcomas shows a mutation in *IDH1* or *IDH2*, which will lead to the production of oncometabolite D2HG. *IDH1* and *IDH2* have an important function in the citric acid cycle and mutations in these genes might possibly lead to a dysregulated metabolic state and cause vulnerability. In **chapter 7** the execution of a custom-made metabolic compound screen performed in three different chondrosarcoma cell lines is described. This was followed by validation of the most promising compounds on a metabolic level using the Seahorse analyser. In concordance with a recent publication of Zhang et al., we reported that cholesterol inhibitors are effective in chondrosarcoma cells, especially in the *IDH1* mutant line, and lower mitochondrial respiration levels were observed [52]. Dual mTORC1 and C2 inhibitor sapanisertib was found as the most potent inhibitor of oxidative and glycolytic metabolism, and was further validated in a panel of cell lines and in an orthotopic mouse model. Cell lines were sensitive for treatment with sapanisertib, although a plateau was reached at 10-30% viability. Mouse tumours treated with sapanisertib were growing slower compared to control tumours, however resistance was acquired after +/- 8 weeks of treatment [53].

mTOR can form two different complexes; mTORC1 and mTORC2. They both have different functions. mTORC1 is involved in many cellular processes, including mRNA translation, autophagy, amino acid signalling and lipid, glucose and nucleotide metabolism. Its activation is regulated by growth factors and intracellular and environmental stress including energy, oxygen, amino acid and DNA damage levels. The function of mTORC2 is less studied, but it is involved in cytoskeleton regulation, metabolism and cell survival.

mTORC2 is activated by Pi3K regulated mechanisms, but possibly also by metabolic signals [54-56].

Compared to Rapamycin, which is only inhibiting mTORC1, treatment with sapanisertib led to larger decreases in oxidative phosphorylation and glycolysis indicating that mTORC1 and mTORC2 both are regulating metabolism in chondrosarcoma cells. This was not dependent on *IDH1*. Previous reports suggest that D2HG, the oncometabolite produced by cells harbouring a mutation in *IDH1* or *IDH2*, inhibits mTOR signalling [57]. We did not observe any difference in metabolic response towards mTOR inhibitors between *IDH1* mutant cells in which D2HG production was inhibited, compared to control conditions. In addition all tested chondrosarcoma cell lines had a high basic level of P-S6 and P-AKT expression indicating that mTOR signalling is highly active in these cells.

One of the metabolic pathways that is regulated by mTOR is glutamine metabolism. Most cancer cells depend on glutamine for their survival [58]. Our lab previously showed that this is also the case for a subset of chondrosarcoma cells, which was again independent of *IDH* mutation status [59]. Inhibiting glutamine in chondrosarcoma cells resulted in a dose dependent decrease in viability. In addition, chondrosarcoma tissue samples showed an increased expression of GLS, the enzyme converting glutamine to glutamate, which was correlated with histological grade, but not with *IDH* mutation status [59]. Both mTORC1 and mTORC2 promote glutaminolysis and inhibiting mTOR in chondrosarcoma might be partially effective because of its effect on glutamine. Resistance to mTOR inhibition might be caused by adapting mechanisms in AKT, which will lead again to an increase in glutaminolysis [60]. Previous research in lung squamous cell carcinoma xenografts showed that combining dual mTOR inhibition with glutamine inhibition overcomes resistance to mTOR inhibitors [61]. Since we also observed resistance in our *in vivo* experiments it would be interesting to further explore this combination also in chondrosarcoma.

In future research it would be relevant to focus on identifying a metabolic signature for cancer cells to select patients eligible for treatment with certain metabolic inhibitors. This has been done by Momcilovic et al. in lung and head and neck squamous cell carcinoma and osteosarcoma. Expression of a specific set of proteins combined with PET imaging and histology predicted response towards dual mTOR inhibition and glutamine inhibitors in xenograft models [61]. In addition an 11 gene signature together with expression of GAC, a specific isoform of GLS1, was predictive of glutamine

sensitivity in breast cancer. They also showed that upon GLS1 knock down TCA and glutathione intermediates were downregulated and ROS production was increased in breast cancer cell lines. These observations indicate that the combination of glutamine inhibitors together with redox modifiers in patients that show a high prediction score might be worthwhile to investigate [62].

It is important to look into this metabolic signature on a tissue specific, and if possible patient specific level. For example in chondrosarcoma no specific metabolic profile has been found for *IDH* mutated chondrosarcomas, while gliomas harbouring an *IDH* mutation do show for example an increased sensitivity for glutaminase inhibitors [63].

Defects in CDKN2A/RB1 as a marker of radio resistance?

Chondrosarcomas are relative radioresistant tumours; conventional radiotherapy (linear accelerator based) is only given to patients with inoperable tumours, or metastatic disease [64, 65]. A recent retrospective study showed that a small group of patients might still benefit from conventional radiotherapy [65]. In **chapter 8** a study is described to identify possible markers for radioresistance or -sensitivity in chondrosarcoma. Our cell line panel showed a heterogeneous response towards γ-radiation, which was confirmed by a recent publication by Girard et al. [66]. By using an ex vivo culture system we identified that alterations in the RB1 pathway might correlate with radiotherapy resistance in chondrosarcoma [67]. Chondrosarcoma tumour explants were cultured, treated with radiotherapy and afterwards analysed for the induction of double strand breaks using a previously published γ-H2AX assay [68, 69]. Subsequently, tumour material was sequenced and mutations frequently observed in known oncogenes and tumour suppressor genes were analysed. Mutations in *IDH1* and *IDH2* were detected in approximately 50% of chondrosarcomas, but were not associated with the amount of γ-H2AX foci. This was in concordance with the fact that we did not observe a difference in radio sensitivity between *IDH1* mutant cells, in which production of D2HG was inhibited using AGI-5198, and non-treated cells. Conversely alterations in *CDKN2A* and *RB1* were associated with less γ-H2AX foci after radiation, which indicates radio resistance in these patients. In line with our results a previous study described that restoring P16 in chondrosarcoma cell lines increased radio sensitivity [70]. Other studies report an increase in radio sensitivity in tumours that display defects in the RB1 pathway [71-74], indicating a context specific effect. Alterations in one of the components of the RB1 pathway have been

described in the majority of high grade conventional chondrosarcomas, indicating that alterations in this pathway are correlating with a more malignant phenotype [75-78].

The amount of γ-H2AX foci has been shown to be a good marker for the number of double strand breaks induced by radiation and correlations with patient response and amount of foci have been observed in several studies [68, 79-81]. However there are also studies that do not find a correlation between γ-H2AX foci and radiosensitivity [82-84], indicating that this might also be tissue and context specific. The fact that we observe less γ-H2AX foci in RB1 pathway mutated tumours might be explained by an effect of the mutation on cell cycle distribution, which will lead to differences in the amount of repair and the pathway that will be used to repair the breaks. Our study describes the observation that we find more alterations in RB1 pathway genes in tumours that show less γ-H2AX foci after radiation compared to tumours that do not show alterations in RB1 pathway genes. Research more focussed on downstream repair pathways might lead to the identification of the mechanism behind this observation. In addition our panel of chondrosarcoma tissues is very small and heterogenous, and further research in a larger cohort is needed to confirm our findings. Besides its well-known role in the cell cycle RB1 is also involved in a multitude of other cellular processes, including metabolic processes [85]. Cells with inactivated RB1 showed upregulated glutaminase activity and glutamine incorporation into GSH, indicating that tumours with alterations in the RB1 pathway are more susceptible for treatment with glutamine inhibitors [86-88]. We recently published that glutamine can be a potentially therapeutic target for patients with high grade chondrosarcoma [59], although the exact mechanism remained unclear. The most glutamine sensitive cell line, SW1335, also showed the highest GSH levels, and the highest radioresistance, as measured by γ-H2AX foci counting. This cell line harbours a mutation in CDKN2A, influencing the RB1 pathway. It would be interesting to investigate whether alterations in the RB1 pathway might predict for sensitivity for glutamine inhibitors in chondrosarcoma. In addition inhibition of AURKA has been identified as synthetic lethal in cells with defective RB1 [89], suggesting this might be another therapeutic vulnerability for patients with high grade RB1 defective chondrosarcoma. Whether alterations in the RB1 pathway can also predict for sensitivity to proton or high let radiation treatment will be a subject for further investigation.

Future perspectives

In this thesis the use of two focussed siRNA screens to identify new therapeutic targets for chondrosarcoma patients is described. Since the time the siRNA screens were initiated, several technical improvements have been become available, the most important one of which is the discovery of the CRISPR technique. Using CRISPR technology genes can be knocked out very specifically, with reduced aspecific effects compared to siRNAs. In addition to the method used, other improvements are the use of isogenic cell pairs, rather than screening a panel of different cell lines, to study the role of a specific gene. This rules out cell line specific effects that are not related to the effects specific for the cancer type. Furthermore, pooled screening is also an option to detect genes that show synthetic lethality with the gene of interest. Using pooled screening, all different genes are studied at once, cultured for a prolonged period of time and the start and the end population of cells is sequenced to detect which genes are important in the specific cell line.

Another important step is creating 3D models that more closely resemble the tumour in the patient. Cell lines are easy for large screens, but not always good models to do translational research. In our study for example we found that chondrosarcoma cell lines are sensitive to certain types of chemotherapy, while patients do show resistance. In addition we do show a high expression of PLK1 in the cell lines, while expression was absent in the chondrosarcoma primary tumour tissues. This illustrates that cell lines might not be the best models to validate results. In my opinion cell lines are a good model for performing genetic and compound screens, but for validation studies, it would be better to use a 3D model, next to the cell lines, that more closely resembles the patient situation.

In the research described in this thesis, one of the most important aims is to find therapeutic options for patients with inoperable and metastatic disease. In addition, the mechanism behind resistance towards conventional chemotherapy remains to be identified, although there have been some clues from research in cell line models. In this thesis we describe a role for Bcl-xl and CHK1 as potential important proteins in chemoresistance. Since previous studies show that cell lines and 3D cultures show differential sensitivities towards chemotherapy, it will be an important next step to validate these findings in relevant chondrosarcoma 3D models.

In addition, several other potential targets have been identified, including mTOR and Survivin. A clinical trial testing mTOR inhibitors in combination

with cyclophosphamide in chondrosarcoma patients is currently ongoing. In the chondrosarcoma xenograft model after an initial delay in tumour growth, resistance towards mTORC1 and mTORC2 inhibitor sapanisertib was observed within a few weeks. Combination treatment using mTOR inhibitors with glutamine inhibitors might be an interesting strategy to overcome this resistance, and has been shown previously in a lung cancer xenograft model [61].

In conclusion, we found Bcl-2 family member Bcl-xl, cell cycle regulator CHK1 and metabolic regulator mTOR as important mediators of chondrosarcoma survival and chemoresistance. The clinical value of these findings should be further exploited in future studies. In addition, personalized approaches like BH3 and metabolic profiling to select eligible patients for specific treatments might hold great value for future exploration, since no general marker predicting sensitivity to specific inhibitors is available at the moment.

Future translational studies to identify new therapeutic targets for chondrosarcoma patients should focus on CRISPR screens and compound screens in either a large panel of chondrosarcoma cell lines, or isogenic cell pairs to study a specific genetic defect. The validation of these findings should include a 3D based model that most closely resembles the patient situation, and genomic and expression analysis in patient material with the ultimate goal to bring the findings to the clinic.

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Chapter 10

Summary

Nederlandse Samenvatting

Curriculum vitae

List of Publications

Nawoord

Summary

Chondrosarcomas are malignant cartilage producing tumours representing 20% of malignant bone tumours [1]. They predominantly develop in the bones of the pelvis, ribs and extremities and are diagnosed in a broad age range, but most commonly around the age of fifty. Chondrosarcoma can be subdivided in conventional chondrosarcoma (85%), dedifferentiated chondrosarcoma (10%) [2], mesenchymal chondrosarcoma (2%) [3], clear cell chondrosarcoma (2%) [4] and periosteal chondrosarcoma (1%) [1]. Conventional chondrosarcoma is further subdivided into central (85%) and peripheral (15%), based on its location in the medulla or next to the bone. Their histological appearance is similar however they show a different genetic background; central chondrosarcomas present with mutation in *IDH1* or *IDH2* in +/- fifty percent of the cases while peripheral chondrosarcomas show alterations in *EXT1* and *EXT2* genes. Conventional chondrosarcoma can be subdivided in three different grades based on its histological appearance (Grade I-III). Grade III chondrosarcomas are most malignant and show a 10 years survival rate of 26-29% [1, 5-7]. Dedifferentiated chondrosarcoma is a high grade tumour showing *IDH1* or *IDH2* mutations in approximately fifty percent of the cases [8]. It has a very low 10 years survival rate of only 10-28% depending on the presence of metastasis [9]. Mesenchymal chondrosarcomas are genetically characterized by a *HEY1-NCOA2* fusion, and 10 years survival rates are reported between 27 and 67% [10, 11]. Clear cell chondrosarcomas are low grade tumours with a mortality rate of 15% [12]. No recurrent initiating genetic alterations have been identified in this subtype. Periosteal chondrosarcomas are very rare and few studies have been reported investigating this subtype. *IDH1* or *IDH2* mutations have been identified in 15% of the cases [13].

Treatment of chondrosarcoma patients is mainly by surgery, since tumours are relatively resistant toward chemo-and radiotherapy. Several possible mechanisms are hypothesized why chondrosarcoma cells are therapy resistant, including upregulation of multi-drug resistance pumps and anti-apoptotic proteins, but likely up regulation of other pro-survival mechanisms and down regulation of pro-cell death mechanisms are playing a role [14-17].

The aim of this thesis was to look further into these mechanisms and find alternative targetable treatment options for chondrosarcoma patients by using a screening based approach. In addition we investigated the role of Bcl-2 family members and its use in targeting the apoptosis pathway in

chondrosarcoma. Furthermore possible mechanisms of radio-resistance were investigated in chondrosarcoma cell lines and explants. In **Chapter 2** a general overview is given about genetic alterations and pathway deregulations that have been identified in the different chondrosarcoma subtypes. This illustrates the complexity and heterogeneity of the different chondrosarcoma subtypes. **Chapter 3** describes the characterization of a new mesenchymal chondrosarcoma cell line MSC170, which is the first cell line that has been reported of this rare chondrosarcoma subtype. We confirmed the presence of the characteristic HEY1-NCAO2 fusion. Expression of Bcl-2 and especially Bcl-xl was present in this cell line and inhibiting Bcl-2 family members using ABT-737 resulted in synthetic lethality with doxorubicin as well as cisplatin as was previously shown for conventional and dedifferentiated chondrosarcoma [15, 16]. **Chapter 4** dives deeper into the role of Bcl-2 family members in chondrosarcoma and resistance towards conventional chemotherapy. We looked into single inhibition of Bcl-2 and Bcl-xl specifically in chondrosarcoma cell lines and in a Swarm Rat Chondrosarcoma (SRC) model. Interestingly we found that not Bcl-2, but Bcl-xl is most important for chondrosarcoma survival *in vitro* as well as *in vivo*. A subset of cell lines showed a synergistic response when Bcl-xl was inhibited in combination with chemotherapy, indicating that involvement of Bcl-xl in chemotherapy resistance is heterogenous across the panel of chondrosarcoma cell lines that we studied. This shows that further research, and possibly personalized approaches, are needed to assess applicability of combination treatments using Bcl-xl inhibitors in combination with chemotherapy in chondrosarcoma patients. **Chapter 5** describes the discovery of Survivin as an essential regulator in chondrosarcoma. A siRNA screen targeting 51 apoptosis related genes was performed, and identified *BIRC5*, encoding the Survivin protein as essential for chondrosarcoma survival. Survivin has multiple functions depending on its location in the cell [18]. Nuclear Survivin has a role in the cell cycle, while cytoplasmic Survivin is involved in apoptosis. We found nuclear as well as cytoplasmic Survivin expression to be correlated with an increased histological grade. In addition, nuclear Survivin expression was correlated with P53 expression. Of note, *TP53* mutant chondrosarcoma cell lines were especially sensitive for Survivin inhibition, with no caspase dependent apoptosis, but a deregulated cell cycle profile. This shows that Survivin might be a potential therapeutic option for patients with *TP53* mutated chondrosarcoma. In **Chapter 6** the discovery of cell cycle proteins as possible therapeutic targets for chondrosarcoma is described. A kinase focussed siRNA (n=779) and compound screen (n=273) have been performed

in chondrosarcoma cells, that resulted in the discovery of PLK1, CHK1 and AURKA as important targetable kinases of chondrosarcoma cell survival. Kinase inhibitors targeting these three cell cycle regulators were validated in a panel of chondrosarcoma cell lines and dose dependent decreases in viability were observed as well as increased sensitivity towards conventional chemotherapy after inhibition of CHK1. Expression of AURKA and CHK1, but not PLK1 was observed in chondrosarcoma tissue samples, raising the question of the importance of PLK1 as a therapeutic target in chondrosarcoma. A worse overall survival was observed in patients with higher CHK1 expression indicating that high CHK1 expression is correlated with a more malignant phenotype. Although more studies are needed to confirm these findings, this study points to CHK1 as the most promising targetable kinase for treatment of chondrosarcoma patients. **Chapter 7** focusses on the chondrosarcoma metabolome and its vulnerabilities. A metabolic compound screen was designed and 39 compounds targeting the most important metabolic pathways were included. This screen was executed in three different chondrosarcoma cell lines, using four different concentrations of each compound. Six compounds were selected for metabolic profiling resulting in the selection of a dual mTORC1 and mTORC2 inhibitor sapanisertib as most promising hit. Treatment with sapanisertib resulted in a reduction in glycolytic as well as oxidative metabolism and a dose dependent decrease in viability. In addition, tumour growth was slowed down in an orthotopic chondrosarcoma mouse model, indicating that inhibiting mTOR as single or combination treatment might be a potential option for chondrosarcoma treatment. **Chapter 8** shows a possible role for the RB1 pathway in radiotherapy resistance in chondrosarcoma. Chondrosarcoma cell lines showed variable resistance towards γ-radiation. Contradicting previous results [19], chondrosarcoma cell lines treated with mutant IDH1 inhibitor AGI-5198 did not show differential sensitivity towards γ-radiation, nor a difference in glutathione levels. Chondrosarcoma explants treated with γ-radiation showed a variable response pattern, as measured by γ-H2Ax foci quantification. Explants that showed less foci, which were considered more radioresistant, showed alterations in the RB1 pathway, while these alterations were absent in explants with more foci. Further investigation should be performed to determine if patients can be selected for treatment with radiotherapy based upon RB1 pathway status.

In conclusion, this thesis shows the identification of several lead targets that might be useful as targeted treatment options for chondrosarcoma patients. Future research should focus more on the therapeutic value of these targets

in a clinical setting. Novel upcoming techniques, such as CRISPR screening and 3D models that more closely resemble the patient situation, will improve the selection of targetable pathways and will lead to a better translation of pre-clinical research to clinical practise.

Nederlandse Samenvatting

Chondrosarcomen zijn kwaadaardige kraakbeenvormende tumoren, die zich voornamelijk ontwikkelen in de botten van het bekken, de ribben en de ledematen. Deze tumoren worden voornamelijk gevonden rond het vijftigste levensjaar. Chondrosarcomen zijn verantwoordelijk voor 20% van al de kwaadaardige bottumoren [1] en kunnen onderverdeeld worden in : conventioneel chondrosarcoom (85%), gededifferentieerd chondrosarcoom (10%) [2], mesenchymaal chondrosarcoom (2%) [3], helder cellig chondrosarcoom (2%) [4] en periostaal chondrosarcoom (1%) [1]. Het conventionele chondrosarcoom is verder onderverdeeld in centraal (85%) en perifeer (15%), op basis van de locatie centraal in het merg (de medulla) of aan het oppervlak van het bot. De histologische kenmerken van centrale en perifere chondrosarcomen zijn hetzelfde, maar ze vertonen een andere genetische achtergrond; centrale chondrosarcomen hebben een mutatie in *IDH1* of *IDH2* in circa vijftig procent van de gevallen, terwijl mutaties in EXT1 of EXT2 belangrijk zijn bij het ontstaan van perifere chondrosarcomen. Conventionele chondrosarcomen kunnen op basis van de histologische kenmerken worden onderverdeeld in drie verschillende graden (graad I-III). Graad III chondrosarcomen zijn het meest kwaadaardig en patiënten met deze tumor hebben een 10 jaars overleving van 26-29% [1, 5-7]. Gededifferentieerd chondrosarcoom is een hooggradige tumor die in circa vijftig procent van de gevallen een *IDH1*- of *IDH2*-mutatie laat zien [8]. Patiënten met gededifferentieerd chondrosarcoom hebben een 10-jaars overlevingskans van slechts 10-28%, afhankelijk van de aanwezigheid van metastasen [9]. Mesenchymale chondrosarcomen worden genetisch gekenmerkt door een HEY1-NCOA2 fusie en overlevingspercentages over 10 jaar worden gerapporteerd tussen 27 en 67% [10, 11]. Het heldercellig chondrosarcoom is een laaggradige tumor met een sterftecijfer van 15% [12]. Er zijn geen specifieke genetische veranderingen geïdentificeerd in dit subtype. Periostale chondrosarcomen zijn zeer zeldzaam en er zijn weinig onderzoeken gerapporteerd waarin dit subtype werd onderzocht. *IDH1* of *IDH2* mutaties zijn geïdentificeerd in 15% van de gevallen [13].

In dit proefschrift worden experimenten beschreven waarbij siRNA en compound screens zijn gebruikt om nieuwe therapeutische opties te vinden voor het behandelen van chondrosarcomen. Verder wordt de rol van apoptose eiwitten van de Bcl-2 familie beschreven en een studie waarbij gekeken is naar radioresistentie mechanismen in chondrosarcoom cellijnen en patiënt weefsel. In **Hoofdstuk 2** wordt een overzicht gegeven van genetische en andere belangrijke veranderingen die zijn gevonden in de verschillende subtypen van het chondrosarcoom. Dit illustreert de

complexiteit en heterogeniteit van de verschillende chondrosarcoom subtypen. **Hoofdstuk 3** beschrijft de karakterisatie van een nieuwe mesenchymale chondrosarcoom cellijn MCS170; de eerste cellijn beschreven van dit zeldzame chondrosarcoom subtype. We bevestigden de aanwezigheid van de karakteristieke HEY1-NCAO2 fusie en detecteerden een hoge expressie van apoptose eiwitten Bcl-2 en vooral Bcl-xl. Remmen van deze Bcl-2 familie eiwitten met ABT-737 zorgde er voor dat de cellen veel gevoeliger werden voor chemotherapie, zoals ook al eerder werd aangetoond voor het conventionele en gededifferentieerd chondrosarcoom [14, 15]. **Hoofdstuk 4** gaat dieper in op de rol van Bcl-2-familie eiwitten in het chondrosarcoom en resistentie tegen conventionele chemotherapie. We onderzochten de remming van Bcl-2 en Bcl-xl specifiek in chondrosarcoom cellijnen en in een rat chondrosarcoom model. Uit deze experimenten konden we concluderen dat niet Bcl-2, maar Bcl-xl het belangrijkst is voor de overleving van chondrosarcoom cellen zowel *in vitro* als *in vivo*. Een subset van cellijnen vertoonde een synergistische respons wanneer Bcl-xl werd geremd in combinatie met chemotherapie, wat aangeeft dat de betrokkenheid van Bcl-xl bij chemotherapieresistentie heterogeen is in dit panel van chondrosarcoom cellijnen. Dit toont aan dat verder onderzoek, en mogelijk ook een gepersonaliseerde benadering, nodig is om te beoordelen of de combinatie van Bcl-xl remmers en chemotherapie toepasbaar is bij patiënten met een chondrosarcoom. **Hoofdstuk 5** beschrijft de identificatie van survivin als een essentiële regulator in chondrosarcoom cellen. Een siRNA-screen gericht op 51 apoptose gerelateerde genen identificeerde BIRC5, coderend voor het survivin eiwit, als essentieel voor de overleving van chondrosarcoom cellijnen. Survivin heeft meerdere functies, afhankelijk van de locatie in de cel [16]. Survivin in de kern speelt een rol in de celcyclus, terwijl cytoplasmatisch survivin betrokken is bij apoptose. In onze studie tonen we aan dat expressie van survivin in zowel de kern als het cytoplasma gecorreleerd waren met een hogere histologische graad en dus met toegenomen agressiviteit van de tumor. Bovendien was survivin expressie in de kern gecorreleerd met P53 expressie. Vooral *TP53* gemuteerde chondrosarcoom cellijnen waren gevoelig voor het remmen van Survivin. Dit resulteerde niet in apoptose, maar wel verstoring van de celcyclus. Dit toont aan dat het remmen van Survivin een mogelijke therapeutische optie kan zijn voor patiënten met *TP53* gemuteerd chondrosarcoom. In **Hoofdstuk 6** wordt de ontdekking van celcyclus eiwitten als mogelijke therapeutische optie voor patiënten met een chondrosarcoom beschreven. Door het uitvoeren van een specifiek op kinases gerichte siRNA (n = 779) en drug screen (n = 273) werden PLK1, CHK1 en AURKA ontdekt als cruciale eiwitten

voor de overleving van chondrosarcoom cellen. Kinaseremmers die zich richten op deze drie eiwitten werden gevalideerd in een panel van chondrosarcoom cellijnen waarbij de vitaliteit van de cellen afnam bij toename van de dosis. Tevens werd een verhoogde gevoeligheid voor conventionele chemotherapie na CHK1 remming gezien. Expressie van AURKA en CHK1, maar niet van PLK1, werd waargenomen in chondrosarcoom tumor weefsel, wat vraagtekens zet bij het belang van het remmen van PLK1 als behandelingsmethode voor chondrosarcoom patiënten. CHK1 expressie was gecorreleerd met een slechtere algehele overleving. Hoewel verder onderzoek nodig is om deze bevindingen te valideren, lijkt CHK1 de meest veelbelovende kinase om te remmen in patiënten met een niet operabel of gemetastaseerd chondrosarcoom. **Hoofdstuk 7** richt zich op het chondrosarcoom metabolisme en hoe deze eventueel te gebruiken als therapeutische optie. Een drug screen met 39 metabole drugs, gericht tegen verschillende metabole cellulaire systemen werd uitgevoerd in chondrosarcoom cellijnen. Na een aantal selectieronden werd mTORC1 en mTORC2 remmer sapanisertib geïdentificeerd als meest veelbelovende hit. Behandeling met sapanisertib zorgde voor een vermindering van zowel het glycolytische als oxidatieve metabolisme en een afname van de vitaliteit in chondrosarcoom cellen, bij een toenemende dosis. Bovendien werd de groei van de tumor vertraagd in een chondrosarcoom muismodel, wat aangeeft dat het remmen van mTOR als enkele of gecombineerde behandeling een mogelijke optie voor chondrosarcoom behandeling zou kunnen zijn. **Hoofdstuk 8** toont een mogelijke rol voor de RB1 signaaltransductie route in de ongevoeligheid voor radiotherapie die word gezien bij chondrosarcomen. Chondrosarcoom cellijnen vertonen een wisselde gevoeligheid voor γ-straling. In tegenstelling tot eerdere resultaten [17], vertoonden chondrosarcoom cellijnen behandeld met IDH1-remmer AGI-5198 geen verlaagde gevoeligheid voor γ-straling en ook geen verschil in glutathion levels. Chondrosarcoom tumorweefsels van patiënten, *ex vivo* behandeld met γ-straling, vertoonden een variabele respons, zoals gemeten met γ-H2Ax foci kwantificatie. Onze resultaten lieten zien dat tumoren die minder foci ontwikkelden na bestraling, en dus als meer radioresistent werden beschouwd, genetische veranderingen in de RB1 signaaltransductieroute vertoonden. Tumoren die meer foci ontwikkelden na bestraling lieten deze veranderingen niet zien. Of selectie voor radiotherapie kan worden gebaseerd op een intacte RB1 signaaltransductie route moet nader worden onderzocht.

Concluderend beschrijft dit proefschrift de identificatie van verschillende eiwitten die belangrijk zijn voor de overleving en chemo- en radioresistentie

van chondrosarcoom cellen. Toekomstig onderzoek naar deze eiwitten moet uitwijzen of deze geschikt zijn als therapeutische aangrijpingspunten. Nieuwe opkomende technieken, zoals CRISPR-screening en 3D-modellen die meer op de patiënten situatie lijken, zullen de selectie van relevante targets verbeteren en leiden tot een betere vertaling van preklinisch onderzoek naar de klinische praktijk.

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Curriculum vitae

Yvonne de Jong was born on August 13, 1988 in Gouda, the Netherlands. She followed Havo (Nature and Health track) at the Goudse waarden in Gouda. Directly after her graduation in 2006 she started to study Biology and medical laboratory research at the university of applied sciences in Leiden followed by a pre-master and master Biomedical sciences at Leiden university of which she graduated in 2013. She performed several internships during her bachelor studies in diagnostics as well as in research, with her final internship being at the tumour genetics department at the Leiden University Medical Centre supervised by Dr. E.C. Robanus-Maandag, where she characterized tumours that developed in mice with a defect in Wnt signalling specifically in smooth muscle cells. During the first year of her masters she worked on improving osteo-inductive properties of titanium implants at the department of orthopaedics at Erasmus MC, with Dr. J. van der Stok as supervisor. During her second year she started with an internship at the department of pathology at the Leiden University Medical Centre working on identification of genes involved in osteosarcoma progression by studying transformed mesenchymal stem cells supervised by Dr. A.M. Cleton-Jansen. Later she switched towards chondrosarcoma research, because she was appointed as a PhD student in the Bone and soft tissue tumour group, led by Prof. Dr. J.V.M.G. Bovée. The results of this research are presented in this thesis. Currently she is working as a post-doc in the group of Dr. J.C. Dorsman, at the Oncogenetics department of the Amsterdam university medical centre, location VUMC, investigating genetic events that play a role in the initiation and progression of retinoblastoma.

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