Towards personalised medicine for cancer

From initial therapy to follow-up

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Chapter 7: Targeting glutaminolysis in chondrosarcoma in context of the IDH1/2 mutation


Abstract
Chondrosarcoma is a malignant cartilage-forming bone tumour in which mutations in isocitrate dehydrogenase 1 and 2 (IDH1 and IDH2) frequently occur. Previous studies suggest an increased dependency on glutaminolysis in IDH1/2-mutated cells, which resulted in clinical trials with the drugs CB-839, metformin and chloroquine. In this chapter, the preclinical rationale for using these drugs as a treatment for chondrosarcoma was evaluated. Expression of glutaminase was determined in 120 cartilage tumours by immunohistochemistry. Ten chondrosarcoma cell lines were treated with the metabolic compounds CB-839, metformin, phenformin (the lipophilic analogue of metformin) and chloroquine. A difference in glutaminase expression levels between the different tumour grades (P = 0.001, one-way ANOVA) was identified, with the highest expression observed in high-grade chondrosarcomas. Treatment with CB-839, metformin, phenformin and chloroquine revealed that chondrosarcoma cell lines are sensitive to glutaminolysis inhibition. Metformin and phenformin decreased mTOR activity in chondrosarcoma cells, and metformin induced autophagy, an effect which is counteracted by chloroquine. Sensitivity does not differ between IDH1/2-mutated cell lines and IDH1/2 wild-type cell lines. Targeting glutaminolysis with CB-839, metformin, phenformin and chloroquine is a potential therapeutic strategy for a subset of high-grade chondrosarcomas, irrespective of the presence or absence of an IDH1/2 mutation.

Introduction
Chondrosarcoma is the second most common primary bone malignancy in humans. It represents a heterogeneous collection of cartilage-forming tumours, which can be divided in several subtypes and histological grades.246 The most common subtype is conventional chondrosarcoma (85%), which arises centrally in the medulla of the bone. The other 15% of chondrosarcoma consist of rare subtypes, such as dedifferentiated chondrosarcoma, mesenchymal chondrosarcoma and clear cell chondrosarcoma. Conventional chondrosarcoma is histologically graded to determine treatment strategy and the patient’s prognosis. The atypical cartilaginous tumour (ACT, previously known as chondrosarcoma grade 1), accounts for 61% of chondrosarcoma cases. First-line treatment consists of curettage with local adjuvant treatment, resulting in a 5-year survival rate of 83%. Grade II (36%) and grade III (3%) chondrosarcomas have a worse 5-year survival (combined 53%) due to the occurrence of metastases.246-248 These tumours are treated with en bloc resection. Dedifferentiated chondrosarcoma is a highly malignant subtype with an overall survival rate of 7-24%.249 Mesenchymal chondrosarcoma has a 10-year survival rate between 44% and 54%. It is a rare aggressive subtype in which distant metastasis can be identified even after 20 years.250-252 Chondrosarcoma patients with inoperable disease, due to tumour location, tumour size or extensive metastatic disease benefit from a doxorubicin-based chemotherapeutic regimen, which increases the 3-year survival from 8% to 26%.253 As the overall efficacy of chemotherapy is limited, new treatment options are needed, which can be identified by further unravelling the essential driver genes and pathways of these tumours.

Potential driver mutations of central conventional and dedifferentiated chondrosarcoma are gain-of-function mutations in IDH1 and IDH2, which have been identified in 38-70% of the cases.24,254 Its occurrence in the benign precursors lesions (enchondromas), of which 52- 87% harbour an IDH1/2 mutation,22,73 further demonstrates that IDH1/2 mutations are an early event in chondrosarcoma formation. IDH1 and IDH2 are essential enzymes in cell metabolism, as they convert isocitrate to α-ketoglutarate (αKG) in respectively the cytoplasm and the mitochondria. The mutant enzyme
acquires the activity to convert αKG to D-2-hydroxyglutarate (D2HG), an oncometabolite that competitively inhibits the αKG dependent enzymes by the high structural similarities. Processes involved in chondrosarcoma progression make these cells independent of the mutant IDH1/2 enzymes, as treatment with AGI-5198, a specific IDH1 mutant inhibitor, did not influence the tumourigenic properties of these cells. Therefore, we propose to exploit the metabolic vulnerability caused by the IDH1/2 mutations as therapeutic strategy for chondrosarcoma.

IDH1/2 mutant cells need αKG for their D2HG production, which can be generated via glycolysis and glutaminolysis. It has been suggested that IDH1/2-mutated tumours depend on glutaminolysis for their αKG supply, \( ^{46,148,228} \) which lead to two clinical trials that were recently started in IDH1/2 mutated solid tumours. The first one is a phase I trial with the drug CB-839 (NCT02071862 clinicaltrials.gov), an inhibitor of glutaminase (Figure 1). The second one is a phase Ib/II trial with the drugs metformin and chloroquine, \( ^{256} \) after which the feasibility of phenformin may be explored as an alternative to metformin in case of lack of effect of metformin. \( ^{257} \) Metformin is a first-in-line drug used for the treatment of type II diabetes mellitus, which next to inhibiting gluconeogenesis in the liver: (1) activates adenosine monophosphate activated protein kinase (AMPK), thereby inhibiting the mammalian target of rapamycin (mTOR); \( ^{258} \) (2) inhibits complex 1 of the electron transport chain (ETC); \( ^{154} \) and (3) indirectly inhibits glutaminase, the enzyme that converts glutamine to glutamate, via c-Myc; (Figure 1). \( ^{259,260} \) Phenformin is a lipophilic analogue of metformin with similar working mechanisms, but in contrast to metformin it does not depend on solute carrier (SLC) 22A1-3 transport to get into cells; \( ^{154,261} \) The anti-malaria drug chloroquine, in addition to its well-known anti-autophagy potency, is able to inhibit glutamate dehydrogenase, an enzyme converting glutamate to αKG (Figure 1). \( ^{141,262} \)

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

**Material and methods**

**Immunohistochemistry on tissue microarrays.** Glutaminase monoclonal antibody (AB156776, Abcam; 1:400) was used for immunohistochemical stainings on previously generated and published formalin-fixed, paraffin-embedded tissue microarrays \( ^{263} \) as described previously. \( ^{264} \) 120 cartilage tumours could be scored, consisting of 12 enchondromas or osteochondromas, 56 ACTs, 36 grade II and 16 grade III chondrosarcoma. All samples were handled according to the Dutch code of proper secondary use of human material as accorded by the Dutch Society of Pathology (Federa). The samples were handled in a coded manner. All study methods were approved by the LUMC Ethical Board (B17.020 v2). Two independently operating observers used the following scoring procedure: intensity score (1-3; 0 = negative, 1 = weak, 2 = moderate and 3 = strong) and percentage score (0-4;
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0 = 0%, 1 = 1-24%, 2 = 25-49%, 3 = 50-74% and 4 = 75-100%) and discrepancies were discussed to reach consensus. Total score was calculated as intensity score + percentage score. Of the central cartilage lesions that could be scored, the IDH1/2 mutational status was known of 54 tumours, of which 33 harbored an IDH1 or IDH2 mutation and 21 were IDH1/2 wild-type.

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

qRT-PCR. RNA isolation of chondrosarcoma cells and the anonymised controls growth plate and articular cartilage was performed using TRIzol (Ambion Biosystems, Invitrogen) followed by a standard RNA isolation protocol and cDNA synthesis. Product size and sequence were validated using Qiaxcel (Qiagen) and sanger sequencing (Applied Biosystems 48- or 96-cappilary 3730 system, Leiden Genome Technology Centre), respectively. Standard qRT-PCR analyses were performed as described previously. Data were normalised using the delta-delta Cq method using Bio-Rad CFX Manager (Bio-Rad).

Compounds. CB-839 (s7655, Selleckchem), metformin hydrochloride (215169110, Bioconnect), phenformin hydrochloride (219590, Santa Cruz Biotechnology), Chloroquine diphosphate salt (c6628, Sigma-Aldrich) and AGI-5198 (14624, Life Technologies) were used. Stock concentrations of respectively 500 mM, 200 mM, 50 mM (in RPMI [Gibco, Invitrogen]) and 10 mM in DMSO were stored in -20°C.

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

Cell viability assay. Cells were counted with a Muse Cell Analyser using the Muse calibration kit (both Millipore) according to manufacturer's instruction. Plating was done in triplicates in densities optimised for each cell line and condition i.e. 3000-15000 cells per well for 72 hours, 200-400 cells per well for one week incubation. CB-839, metformin, phenformin and chloroquine were added after the cells adhered overnight. The metabolic drugs were incubated for 72 hours or 1 week after which cell viability was measured using the PrestoBlue Cell Viability Reagent (Promega) according to the manufacturer's instructions. Colourimetric values in the plates were subsequently measured using a Wallac 1420 VICTOR2 (Perkin Elmer). Data were analysed in Prism 5.0 (Graphpad Software). For the combination of metformin, phenformin and chloroquine with AGI-5198, cells were pretreated for 72
hours with AGI-5198 (1 µM and 10 µM) or mock. For the analyses in which the effect of FBS on CB-839 sensitivity was evaluated, the medium with or without FBS and the corresponding concentrations of the metabolic compounds were added at the same time, so after the cells were allowed to adhere overnight.

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

**Cell apoptosis.** For analysis of apoptosis, the caspase-glo 3/7 assay (Promega) was used according to manufacturer’s instructions. Cells were seeded in white walled 96-wells plates (Corning) in densities which resulted in 70% confluence after 24 hours as described previously. HT1080, JJ012, SW1353, NDCS1 and CH2879 cells were treated with 75% of their maximal inhibitory concentrations (IC75) of metformin, phenformin, chloroquine and CB-839 (based on 72-hour dose response curves). The concentration of compounds used was 10 mM metformin, 100 μM phenformin, 50 μM chloroquine and 6 μM CB-839 if the IC75 was above these concentrations. CH2879 cells treated with 1 μM doxorubicin (obtained from the in-house hospital pharmacy) and 50 μM ABT-737 (#S1002, Selleckchem) were used as positive control. For the negative control doxorubicin and ABT-737 were combined with Z-vad-FMK (#550377, BD Biosciences). After 24 hours the caspase-glo substrate was added 1:1 followed by incubation of 60 minutes at room temperature. Wells were analysed using Wallac 1420 VICTOR2 (Perkin Elmer). The experiment was performed two times in duplicate. Data was corrected for plane RPMI control and normalised to untreated control for each cell line. Viability was measured on a simultaneously treated plate after 24 hours.

**Western blot analysis.** HT1080, JJ012, SW1353, NDCS1 and CH2879 cells are treated with their IC50 values (based on 72-hour dose response curves) of metformin, phenformin, chloroquine or CB-839 and lysed after 72 hours. A maximum concentration of compounds of 10 mM metformin, 100 µM phenformin and 6 µM CB-839 was used if the IC50 was above these concentrations. Western blotting was performed for LC3B (1:1000, clone D11, #3868, Cell Signaling Technology) and phospho-S6 (1:1000, clone 2F9, #4856, Cell Signaling Technology). As a loading control, α-tubulin (1:10000, clone DM1A, Sigma-Aldrich) expression was used. Cells were lysed using hot-SDS buffer (1% SDS, 10 mM Tris/EDTA with complete inhibitor and phosSTOP). For each sample, 10 μg protein was loaded on TGX Stain-Free FastCast 12% Acrylamide Gels (Bio-Rad). Proteins were transferred to a Polyvinylidene difluoride (PVDF) membrane using Trans-Blot Turbo Transfer System (Bio-Rad) and Trans-Blot® Turbo™ Transfer kit PVDF (Bio-Rad) and detected using enhanced chemiluminescence (Pierce ECL Western Blotting Substrate Fisher Scientific), followed by exposure of 30 seconds to 5 minutes and development of the film (ECL Hyperfilm, GE Healthcare).

**Seahorse experiments.** A Seahorse XFe 96 analyser (Seahorse Bioscience, Agilent) was used to measure the oxygen consumption rate (OCR) and the extracellular acidification rate (ECAR) in chondrosarcoma cell lines JJ012, SW1353 and CH2879 after metformin treatment. 30 hours prior to the assay, cells were plated in optimised densities being 15000, 13000 and 30000 for JJ012, SW1353 and CH2879, respectively. After 6 hours cells were treated with 5 mM metformin for 24 hours. Before the measurement, cells were incubated for 1 hour in glucose-free RPMI-1640 supplemented with 5% FBS. During the assay, sequential injections of 10 mM glucose (Sigma-Aldrich), 20 μM oligomycin A, 2 μM carbonyl cyanide-4-(trifluoromethoxy)phenylhydrazone (FCCP) and 0.5 μM 1:1 rotenone:antimycin A (Cayman Chemicals) established the metabolic profile of all cell lines. Data was normalised to cell numbers measured in each individual well and data represented as the average ±SD of triplicate measurements for metformin-treated cells and 5-7 replicates for controls.
Results

Glutaminase is a potential therapeutic target in a subset of chondrosarcomas. By immunohistochemistry, a difference in glutaminase expression levels between the different tumour grades was identified (ANOVA, $P = 0.001$), and the post-hoc analysis revealed that specifically the grade II-III chondrosarcoma had higher expression levels compared to the ACTs (both $P = 0.001$; Figure 2A-E). Grouping the high-grade cartilage tumours (grade II and grade III chondrosarcomas) and the low-grade cartilage tumours (ACTs and enchondromas/osteochondromas) further demonstrated the significant difference in glutaminase expression levels between high-grade and low-grade cartilage tumours ($P < 0.0001$, independent-samples T test; Figure 2A). No difference in glutaminase expression between $IDH1/2$-mutated and $IDH1/2$ wild-type central cartilage tumours was observed (Figure 2B). Therefore, glutaminase is higher expressed in high-grade compared to low-grade cartilage tumours but does not correlate to $IDH1/2$ mutation status.

Using qRT-PCR analyses, we demonstrate that all cell lines have higher expression levels of glutaminase compared to the controls growth plate and cartilage, although expression levels are variable (Figure 3A). Inhibition of glutaminase using CB-839 in 10 chondrosarcoma cell lines revealed that HT1080 ($IDH1^{WT/R132C}$), SW1353 ($IDH2^{WT/R172S}$) and, to a lesser extent, JJ012 ($IDH1^{WT/R132C}$), were very sensitive for inhibition, with IC$_{50}$ values below 5 μM (Figure 3B and Table 1). L2975 ($IDH2^{WT/R172W}$), NDCS1 ($IDH1/2^{WT/WT}$) and CH3573 ($IDH1/2^{WT/WT}$) had IC$_{50}$ values of 10.2 μM, 13.5 μM and 17.5 μM, while the remaining four cell lines (one $IDH1^{WT/R132C}$, three $IDH1/2^{WT/WT}$) had IC$_{50}$ values above 50 μM. Interestingly, absence of FBS, and therefore lower concentrations of glucose, increased the sensitivity to metformin especially in the $IDH1/2$-mutated cell lines, while there was no clear difference in the cell lines with wild-type $IDH1/2$ (Figure 3C). In conclusion, these experiments demonstrate that a subset of chondrosarcomas is dependent on glutaminase-mediated glutaminolysis to maintain cell viability.

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

Cellular effects of glutaminolysis inhibition. To investigate the effect on apoptosis, caspase-glow 3/7 assays were performed. Chloroquine slightly increased caspase 3/7 activity in 3 out of 5 cell lines tested (Figure 5A). While the other compounds did impact cell viability after 24 hours (Figure 5B), no effect on caspase 3/7 activity was observed. Next, we evaluated the effect of the four metabolic compounds on phosphorylated S6 protein levels, as this is an indicator of mTOR activity. As shown in Figure 5C, metformin and phenformin decreased phosphorylated S6 levels in 4/5 and 3/5 cell lines respectively. However, metformin and phenformin did not affect phosphorylated S6 levels in HT1080 cells, the cell line that is most sensitive to metformin. Interestingly, metformin decreased LC3B levels in 4 out of 5 cell lines, which indicates it induces autophagy in a subset of chondrosarcoma cell lines.
As expected, chloroquine greatly increased LC3B levels. CB-839 did not affect phosphorylated S6 or LC3B protein levels. Thus, while the induction of apoptosis is very limited, metformin and phenformin decreased mTOR activity in chondrosarcoma cells, and metformin induced autophagy, an effect which is counteracted by chloroquine.

**Chondrosarcoma cells sufficiently express SLC22A1 for metformin to completely inhibit mitochondrial respiration.** Next, expression levels of SLC22A1-3 were determined by qRT-PCR analyses, as these transporters are essential for the cellular uptake of metformin and might explain

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**Figure 2. Glutaminase expression correlates to tumour grade but not to IDH1/2 mutation status.**

A: Total score (intensity + percentage) of glutaminase expression. * P = 0.001 by one-way ANOVA with the LSD post-hoc analysis. ** P < 0.0001 by independent-samples T test, grouping the high-grade and the low-grade cartilage tumours. B: No difference between IDH1/2 mutant and IDH1/2 wildtype central tumours was observed. C: ACT without expression of GLS, scored as percentage 0, intensity 0. D: Grade II chondrosarcoma with medium expression of GLS, scored as percentage 2, intensity 2. E: Grade III chondrosarcoma with high expression of GLS, percentage 4 intensity 3. Black bars represent 50 µM.
Figure 3. The glutaminase inhibitor CB-839 inhibits chondrosarcoma cell viability.

**A:** Expression levels of glutaminase in 10 chondrosarcoma cell lines and two controls. Glutaminase is higher expressed in chondrosarcoma cell lines compared to growth plate and articular cartilage. **B:** Ten chondrosarcoma cell lines were treated for 72 hours with CB-839, a glutaminase inhibitor. Sensitivity differed between the different cell lines. **C:** Indicated cell lines were treated with 0, 20 or 200 nM CB-839 in the presence or absence of FBS. In the absence of FBS, all cell lines are more sensitive for inhibition with CB-839, especially the IDH1/2 mutant cell lines.

Discussion

In this chapter, it was found that the expression of glutaminase increased in chondrosarcoma with histological grade, thereby suggesting an increased dependence on glutaminolysis. We therefore explored whether this metabolic vulnerability could be exploited as a therapeutic target for high-grade chondrosarcoma. Based on current ongoing clinical trials targeting glutaminolysis that are including chondrosarcoma patients, we interfered with glutaminolysis at different levels (Figure 1).
First, we inhibited glutaminase using the glutaminase inhibitor CB-839, and indeed, six out of ten chondrosarcoma cell lines showed IC\textsubscript{50} values below 20 μM, suggesting that chondrosarcoma cell lines are sensitive to inhibition of glutaminolysis. CB-839 did not affect apoptosis, autophagy or mTOR activity, suggesting that it likely impacts cell viability via other mechanisms.

Second, we studied the widely-used anti-diabetic drug metformin, which indirectly inhibits glutaminase via c-Myc; \textit{(Figure 1)}, inhibits complex I of the ETC and inhibits mTOR signalling. Indeed, a subset of chondrosarcoma cell lines was sensitive to metformin, especially when treated for a longer time period. mTOR signalling was previously shown to be important in chondrosarcoma\textsuperscript{276} and we confirmed that metformin decreased mTOR activity in all but one chondrosarcoma cell lines tested, which is in line with findings in other studies.\textsuperscript{258} Interestingly, mTOR activity was not inhibited in HT1080 which was the most sensitive for metformin, suggesting that mTOR inhibition alone cannot explain the impact of metformin on chondrosarcoma cell viability. Furthermore, while apoptosis was absent, metformin seemed to induce autophagy in the majority of cell lines tested, which can likely be linked to the effect of metformin on AMPK.\textsuperscript{277} In this chapter, we further demonstrate that metformin completely blocks mitochondrial respiration in chondrosarcoma cells, likely caused by its effect on complex I of the ETC. However, blocking mitochondrial respiration (and therefore oxidative glutaminolysis) alone was not sufficient to inhibit cell viability, as the concentration used did not impact cell viability within the 24-hour time frame from the Seahorse experiment. The small difference in glycolysis observed upon treatment with metformin is likely insufficient to compensate for the total block in oxygen use, suggesting that chondrosarcoma cells do not depend on oxidative glutaminolysis and glycolysis for their energy production.

\textbf{Figure 4. Chondrosarcoma cell lines are sensitive for metformin, phenformin and chloroquine, irrespective of the IDH1/2 mutation.}

\textit{A}: Chondrosarcoma cell lines were treated for 72 hours with corresponding compounds. Cell viability was measured using the PrestoBlue assay. \textit{B}: Four chondrosarcoma cell lines were treated for 7 days with the corresponding inhibitors. \textit{C}: IDH1 mutant JJ012 cells were pretreated for 72 hours with 10 µM AGI-5198, 1 µM AGI-5198 or DMSO, after which they were treated with a combination of AGI-5198 and the corresponding compounds for 72 hours. No effect of AGI-5198 was observed.
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Third, as an alternative to metformin, we used its lipophilic analogue phenformin. In contrast to metformin, phenformin does not need SLC22A1-3 transporters to get into cells. As expected, the effect of phenformin on cell viability, mTOR activity, and apoptosis was very similar to metformin. We show that all chondrosarcoma cell lines sufficiently express the SLC22A1 transporter that is necessary for metformin uptake, suggesting that there is limited rationale to move to phenformin trials for chondrosarcomas when the metformin trial demonstrates limited efficacy. In contrast to metformin, phenformin is not used in the clinic for the treatment of diabetes mellitus due to an increased risk of lactic acidosis.

Fourth, we evaluated the anti-malaria drug chloroquine, which, in addition to inhibiting glutamate dehydrogenase, is a well-known inhibitor of autophagy and thereby inhibits many other metabolic and signal transduction pathways.\textsuperscript{278,279} The chondrosarcoma cell lines were sensitive to chloroquine. Also, we confirmed that chloroquine inhibited autophagy in all chondrosarcoma cell lines tested. Moreover, a slight induction of apoptosis was seen in 3 out of 5 chondrosarcoma cell lines.

Thus, we used four different drugs to evaluate whether the increased dependence on glutaminolysis could be therapeutically exploited using repurposing of existing drugs, and confirmed that a subset of chondrosarcoma cell lines is indeed sensitive to glutaminolysis inhibition. We could not identify a
correlation between levels of glutaminase expression and sensitivity for any of these metabolic compounds in the panel of 10 chondrosarcoma cell lines. Of note, there was also no correlation of the IDH1/2 mutation status of chondrosarcoma cells with sensitivity to these compounds, or with the expression levels of glutaminase in primary tumours. We could therefore not confirm the prevailing hypothesis that IDH1/2 mutant cells rely on glutaminolysis to generate sufficient αKG for D2HG production, as it seems that also high-grade chondrosarcomas that are IDH1/2 wild-type depend on glutaminolysis. Therefore, our results indicate that there is limited preclinical rationale to select chondrosarcoma patients for treatment with these compounds based on their IDH1/2 mutation status. This is in contrast to the studies by Cuyas et al. and Molenaar et al., where, respectively, an increased sensitivity for metformin was identified in an engineered IDH1 mutant breast cancer cell line, and in an engineered IDH1 mutant colorectal cancer cell line compared to their wildtype parental cells. The differences in tumour types and the fact that these cell lines harbour an engineered instead of an endogenous IDH1/2 mutation likely explain the differences in experimental findings.

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

Figure 6. Chondrosarcoma cells sufficiently express SLC22A1 for metformin to completely inhibit mitochondrial respiration.

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