Research Paper

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

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ABSTRACT

Background: 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.

Methods: 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.

Results: 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 24 h. Treatment of chondrosarcoma xenografts with sapanisertib slowed down tumor growth compared to control mice.

Conclusions: 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.

1. Background

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

Abbreviations: α-KG, α-ketoglutarate; ACT, Atypical cartilaginous tumor; BLI, Bioluminescence imaging; BSA, Bovine serum albumin; BSO, Buthionine sulfoximine; D2HG, D-2-Hydroxyglutarate; DMSO, Dimethyl sulfoxide; ECAR, Extracellular acidification rate; FBS, Fetal bovine serum; FCCP, Carbonyl cyanide-4-(trifluoromethoxy)phenylhydrazone; FLI, Fluorescence imaging; HIF, Hypoxia-inducible factor; IDH, Isocitrate dehydrogenase; mCT, Micro computed tomography; mTOR, Mammalian target of rapamycin; OCR, Oxygen consumption rate; ROS, Reactive oxygen species

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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 HIF1α and its downstream target carbonic anhydrase (CA) IX were found to be upregulated 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 metabolism 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 tumor 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, HIF1α, 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.

2. Methods

2.1. Cell culture

Conventional central chondrosarcoma cell lines JJ012 (IDH1 mutant, R132G) [20] CH2879 (IDH1 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). CH2879 is known to be wild type for both IDH1 and IDH2 while JJ012 and SW1353 are IDH1 (R132G) and IDH2 (R172S) mutant, respectively. 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 37°C in a humidified incubator in normoxic conditions (5% CO₂). 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.

2.2. 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 (S50,377 BD biosciences) were dissolved in DMSO according to the manufacturer’s instructions. Chemicals for the Seahorse experiments oligomycin A (11,342), trifluoromethoxy carbonylcyanide phenylhydrazone (FCCP, 15,218), antimycin A (19,433), rotenone (13,995), UK5099 (16,980), Etonomix (11,969) and BPTES (19,284) 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.

2.3. 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 h. 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 72 h incubation, preto 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.

2.4. 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 h of treatment. 30 h prior to the assay cells were plated in custom Seahorse 96 well plates in optimized densities being 15,000, 13,000 and 30,000 for JJ012, SW1353 and CH2879, respectively. After 6 h cells were treated with the selected compound for 24 h. Concentrations were chosen based on the results of the compound screen (AOA 1 mM, BSO 100 μM, bardoxolone 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 h 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 nonmitochondrial 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 olgolycin injection.

2.5. 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 h with mTOR inhibitor rapamycin or sapanisertib and during the assay with 3 µM BPTES, 4 µM Trametrex 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.

2.6. 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 h. 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-19 M 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.

2.7. 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 a positive control. Luminescence was measured using presto blue viability reagent (Life-Technologies, Amsterdam, The Netherlands) expression was determined in white walled 96 well plates (Corning B.V. Life Sciences, Amsterdam, The Netherlands) and proliferation was measured using Hoechst staining as described previously [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).

2.9. 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 (1 × 10⁶ 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) 4–5 mm apart were created in the bone cortex of the upper right tibiae using needles (25GA 5/8 0.5 × 16). 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) 10 min. after i.p. injection of 150 mg/kg 6-luciferin (Synchem UG & Co Kg, 60 mg/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 Xeogen (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 10⁴ 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%Propyleneglycol (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 100 µl dissolve 30%PEG400/ Tween80/Propyleneglycol in PBS by oral gavage 3 days a week and monitored for 8 weeks. After 8 weeks of treatment or when BLI signal reached 10⁴ 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 –80 °C. 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.

2.10. 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 speciﬁc 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.
Fig. 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).
3. Results

3.1. 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 (Fig. 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. No additive or synergistic effect was observed when cells were treated in combination with low concentrations of doxorubicin or rapamycin (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 (Fig. 1). In addition, rapamycin was taken along as a second mTOR targeting compound, since it is already used in the clinic and targets only mTORC1, while sapanisertib is targeting both mTORC1 and mTORC2.

3.2. 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 (Fig. 2A) while the basal respiration was lowered in all but the BSO and lovastatin treated cells (Fig. 2B). In contrast, glycolysis and glycolytic capacity was only decreased in cells treated with AOA, bardoxylone methyl, sapanisertib and rapamycin (Fig. 2C and 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 1A). 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.

3.3. 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 (Fig. 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).

3.4. 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 (Fig. 3A). Chondrosarcoma cell lines treated with dual mTORC1 and mTORC2 inhibitor sapanisertib showed a dose dependent decrease in viability after 72 h with an IC50 of 9 nM, 26 nM and 27 nM for JJ012, SW1353 and CH2879 respectively. However, at higher concentrations the viability remained 10–30% (Fig. 3B). We therefore investigated possible synergistic effects by combination treatment with doxorubicin or cisplatin. However, no additive or synergistic effects were observed (supplementary figure 2). Induction of apoptosis measured by caspase 3/7 activity was increased after 24 h of treatment with sapanisertib in CH2879 but not in the other two cell lines (Fig. 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 (Fig. 3D and 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 (Fig. 3E). Furthermore, treatment with both inhibitors led to a decrease in P-S6, but no clear differences in HIF1α expression.

3.5. 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) (Fig. 4A and B). In the control group all mice reached a bioluminescence signal of 10⁶ 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 (Fig. 4B). 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 3). 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 no significant differences between treated and control tumors. (Supplementary figure 4)

4. 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]. In all these studies, doxorubicin or cisplatin resistant chondrosarcoma cell lines were used that were cultured under low concentrations of doxorubicin / cisplatin for extended periods of time. Previous studies have shown that resistance has an impact on the metabolism of the cells, which explains the difference in sensitivity that we observed in the compound screen since we used non-resistant cells [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)

Fig. 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 72 h 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.
Fig. 3. Inhibition of mTORC1 or mTORC1 and C2 in chondrosarcoma cell lines. (A) Inhibition of mTORC1 using rapamycin for 72 h 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 h 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 24 h 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 72 h (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 h compared to control. A-tubulin expression is determined as a loading control.
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-kB 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. This is consistent with other pre-clinical studies in multiple tumor types including sarcomas, in which sapanisertib was shown to be more potent [41]. 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. This is 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.

A number of studies have found chondrosarcoma to be a hypoxic tumor with increased HIF1α and VEGF signaling [42]. 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 [41,43,44]. 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 [45-47]. 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. It would have been of interest to analyze expression of these markers at multiple time points during the experimental period to observe possible changes in these markers at earlier stages of treatment.

5. Conclusions

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.
Conflict of interest

The authors declare that there are no conflicts of interest.

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Supplementary material

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.jbonet.2019.100222.

References

[1] H.L. Evans, A.G. Ayala, M.M. Romondahl, Prognostic factors in chondrosarcoma of bone: a clinicopathologic analysis with emphasis on histologic grading, Cancer 40 (2) (1977) 818–831.
[2] A.M. van Maldegem, H Gelderblom, E. Palmerini, S.D. Dijkstra, M. Gambarotti, A. Italiano, O. Mir, A. Cio – alised histone modifications do not predict outcome and do not cause loss of 5-hydroxymethylcytosine or altered metabolic pathways for rational targeted therapy, Clin. Cancer Res. 19 (2013) 619–629.
[3] Y. de Jong, J.G. van Oosterwijk, A.B. Kruisselbrink, L.H. Brieurai-de Bruijn, G. Agrogiannis, Z. Baranski, A.H. Cleven, A.M. Cleton-Jansen, B. van de Water, E.H. Danen, J.V. Bovee, Targeting survival as a potential new target for chondrosarcoma, Oncogene 5 (2016) e222.
[4] Y. de Jong, A.M. van Maldegem, A. Marinos-Enriquez, D. de Jong, J. Suiker, L.H. Brieurai-de Bruijn, A.B. Kruisselbrink, A.M. Cleton-Jansen, K. Szuhai, H. Gelderblom, J.A. Fletcher, J.V. Bovee, Inhibition of Bcl-2 family members sensitizes mesenchymal chondrosarcoma to conventional chemotherapy: A report on a novel mesenchymal chondrosarcoma cell line, Lab Invest 96 (10) (2016) 1128–1137.
[5] Y.M. Schrags, L.H. Brieurai-de Bruijn, N.F. de Miranda, O.J. van, A.H. Taminiau, W.T. van, P.C.W. Hodgendoorn, J.V.M.G. Bovee, Kinome profiling of chondro- sarcoma reveals SRC- pathway activity and dasatinib as option for treatment, Cancer Res. 69 (15) (2009) 6216–6222.
[6] J.V. van Oosterwijk, J.R. Luijten, D. Meijer, K. Bartelmez, I. Que, M. Karperien, J.V. Bovee, An orthotopic mouse model for chondrosarcoma of bone provides an in vivo tool for drug testing, Virol. Arch. 466 (1) (2015) 101–109.
[7] Z. Baranski, T.H. Booij, A.M. Cleton-Jansen, L.S. Price, B. van de Water, J.V. Bovee, P.C.W. Hodgendoorn, E.H. Danen, Radiation- mediated checkpoint kinase control regulates proliferation and resistance to chemotherapy in conventional osteosarcoma, J. Pathol. 236 (3) (2015) 348–356.
[8] E.P. Peterse, B. van den Akker, B. Niessen, J. Oosting, J. Suiker, J. de Jong, E.H. Danen, J.V. Bovee, NAD-dependent sirtuin 6 is a viable therapeutic strategy for chondrosarcoma, Mol. Cancer Res. (2017).
[9] S. Boeuf, J.V. Bovee, L. Neirynck, F. van den Akker, J.V.M.G. Bovee, Targeting glutaminolysis in chondrosarcoma in context of the IDH1/2 mutation, Br. J. Cancer. 110 (2014) 96–103.
[10] Y.D. Song, K.F. Zhang, D. Liu, B. Xu, G. Xiao, X. He, S. Xu, J.H. Shen, X.G. Li, L. Zhang, F.J. Shi, Inhibition of EGFR-induced glucose metabolism sensitizes human chondrosarcoma cells to cisplatin, Tumour Biol. 35 (7) (2014) 7017–7024.
[11] G. Han, Y. Liu, X. Li, P. Xu, Y. Luo, Targeting glucose metabolism in chondrosarcoma cells enhances the sensitivity to doxorubicin through the inhibition of lactate dehydrogenase-A, Oncol. Rep. 31 (6) (2014) 2274–2279.
[12] C. Staubert, H. Bhuiyan, A. Lindahl, O.J. Broom, Z. Shi, S. Islam, S. Linnarsson, J. Lehtio, A. Nordstrom, Rewired metabolism in drug-resistant leukemia cells: a metabolic switch hallmarks reduced dependence on exogenous glutamine, J. Biol. Chem. 290 (13) (2015) 8384–8398.
[13] S.W. Kang, S. Lee, E.K. Lee, ROS and energy metabolism in cancer cells: alliance for fast growth, Arch. Pharm. Res. 38 (3) (2015) 333–346.
[14] E. Panieri, M.M. Santoro, ROS homeostasis and metabolism: a dangerous liaison in cancer cells, Cell Death Dis. 7 (2016) e2253.
[15] K. Birsoy, T. Wang, W.W. Chen, E. Freinkman, M. Abu-Ramal, D.M. Sabatini, An essential role of the mitochondrial electron transport chain in cell proliferation is to
enable aspartate synthesis, Cell 162 (3) (2015) 540–551.

[38] L.B. Sullivan, D.Y. Gui, A.M. Hosios, L.N. Bush, E. Freinkman, M.G. Vander Heiden, Supporting aspartate biosynthesis is an essential function of respiration in proliferating cells, Cell 162 (3) (2015) 552–563.

[39] S. Kapur, F. Picard, M. Perreault, Y. Deshaies, A. Mareille, Nitric oxide: a new player in the modulation of energy metabolism, Int. J. Obes. Relat. Metab. Disord. 24 (Suppl 4) (2000) S36–S40.

[40] R.A. Saxton, D.M. Sabatini, mTOR signaling in growth, metabolism, and disease, Cell 168(6) 960–976.

[41] E.K. Slotkin, P.P. Patwardhan, S.D. Vasudeva, E. de Stanchina, W.D. Tap, G.K. Schwartz, MLN0128, an ATP-competitive mTOR kinase inhibitor with potent in vitro and in vivo antitumor activity, as potential therapy for bone and soft-tissue sarcoma, Mol. Cancer Ther. 14 (2) (2015) 395–406.

[42] C. Lin, R. McGough, B. Aswad, J.A. Block, R. Terek, Hypoxia induces HIF-1alpha and VEGF expression in chondrosarcoma cells and chondrocytes, J Orthop Res. 22 (6) (2004) 1175–1181.

[43] S. Zhang, X. Song, B. Cao, Z. Xu, B. Fan, L. Che, J. Hu, B. Chen, M. Dong, M.G. Pilo, A. Cigliano, K. Evert, S. Ribback, F. Dombrowski, R.M. Pascale, A. Cossu, G. Vidili, A. Porezu, M.M. Simile, G.M. Pes, G. Giannelli, J. Gordan, L. Wei, M. Evert, W. Cong, D.F. Calvisi, X. Chen, Pan-mTOR inhibitor MLN0128 is effective against intrahepatic cholangiocarcinoma in mice, J. Hepatol. 67 (6) (2017) 1194–1203.

[44] H. Jiang, Z. Zeng, Dual mTORC1/2 inhibition by INK-128 results in antitumor activity in preclinical models of osteosarcoma, Biochem. Biophys. Res. Commun. 468 (1–2) (2017) 255–261.

[45] J. Song, X. Wang, J. Zhu, J. Liu, Rapamycin causes growth arrest and inhibition of invasion in human chondrosarcoma cells, J. BUON. 21 (1) (2016) 244–251.

[46] J. Perez, A.V. Decouvelaere, T. Pointecouteau, D. Pissaloux, J.P. Michot, A. Besse, J.Y. Blay, A. Dutour, Inhibition of chondrosarcoma growth by mTOR inhibitor in an in vivo syngeneic rat model, PloS One 7 (6) (2012) e32458.

[47] R. Bernstein-Molho, Y. Kollender, J. Issakov, J. Bickels, S. Dadia, G. Flusser, I. Meller, R. Sagi-Eisenberg, O. Merinmsky, Clinical activity of mTOR inhibition in combination with cyclophosphamide in the treatment of recurrent unresectable chondrosarcomas, Cancer Chemotherapy Pharmacol. 70 (6) (2012) 855–860.