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

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Chapter 7

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

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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.

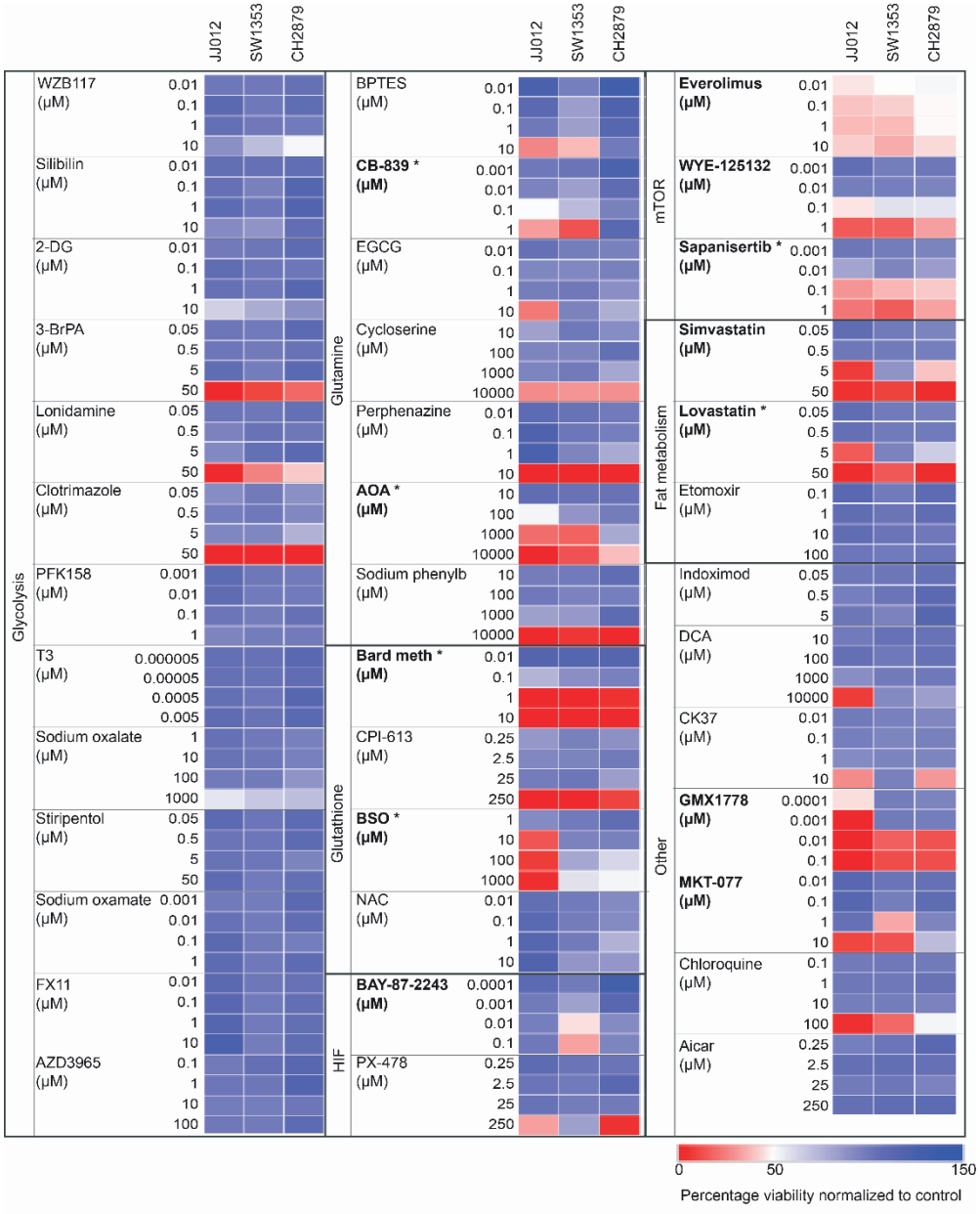

Figure 1: Viability of in vitro metabolic drug screen in three different chondrosarcoma cell lines. Four different concentrations were used per compound as indicated. Of all 39 tested compounds twelve compounds decreased cell viability more than 50% for the two highest concentrations in at least one 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).

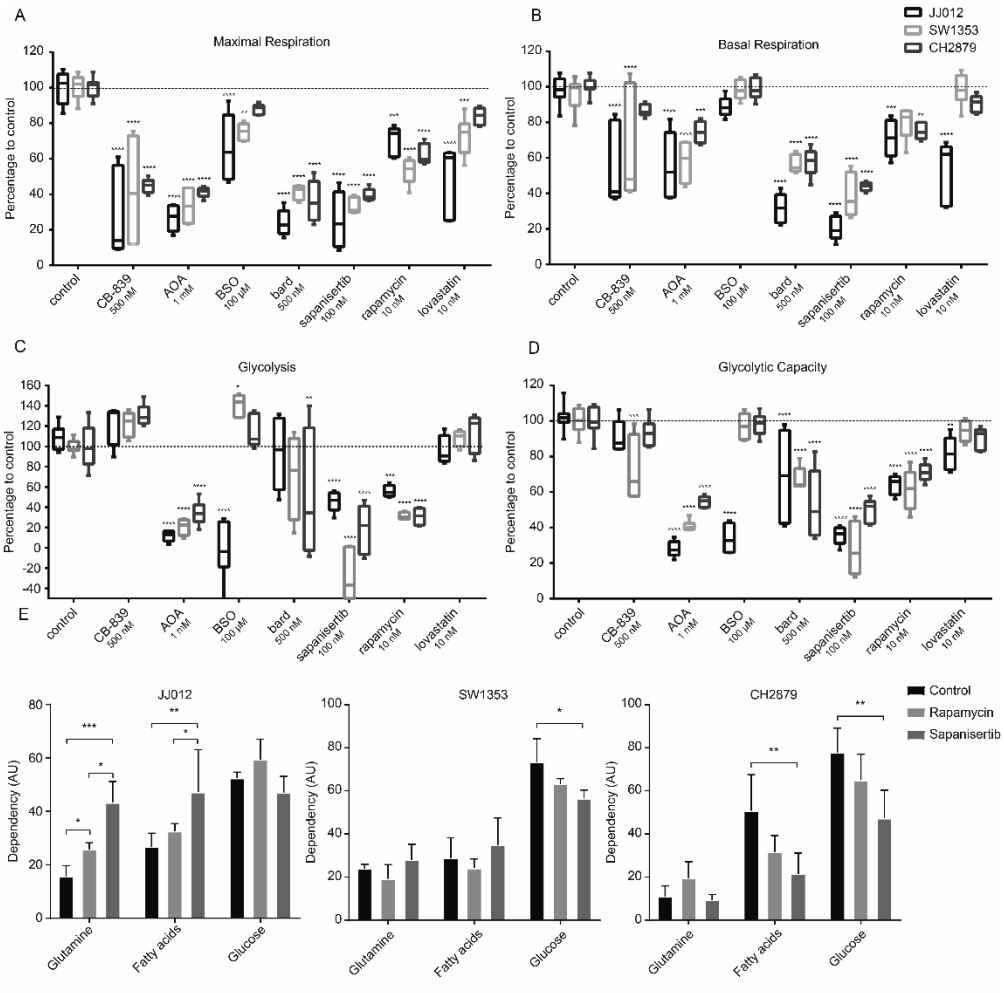

Figure 2: Real-time metabolic analysis of three chondrosarcoma cell lines after treatment with the compounds selected from the screen.
Maximal respiration (A), Basal Respiration (B), Glycolysis (C) and Glycolytic capacity (D) of all cell lines after 72h of treatment with selected compounds. Maximal and 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.

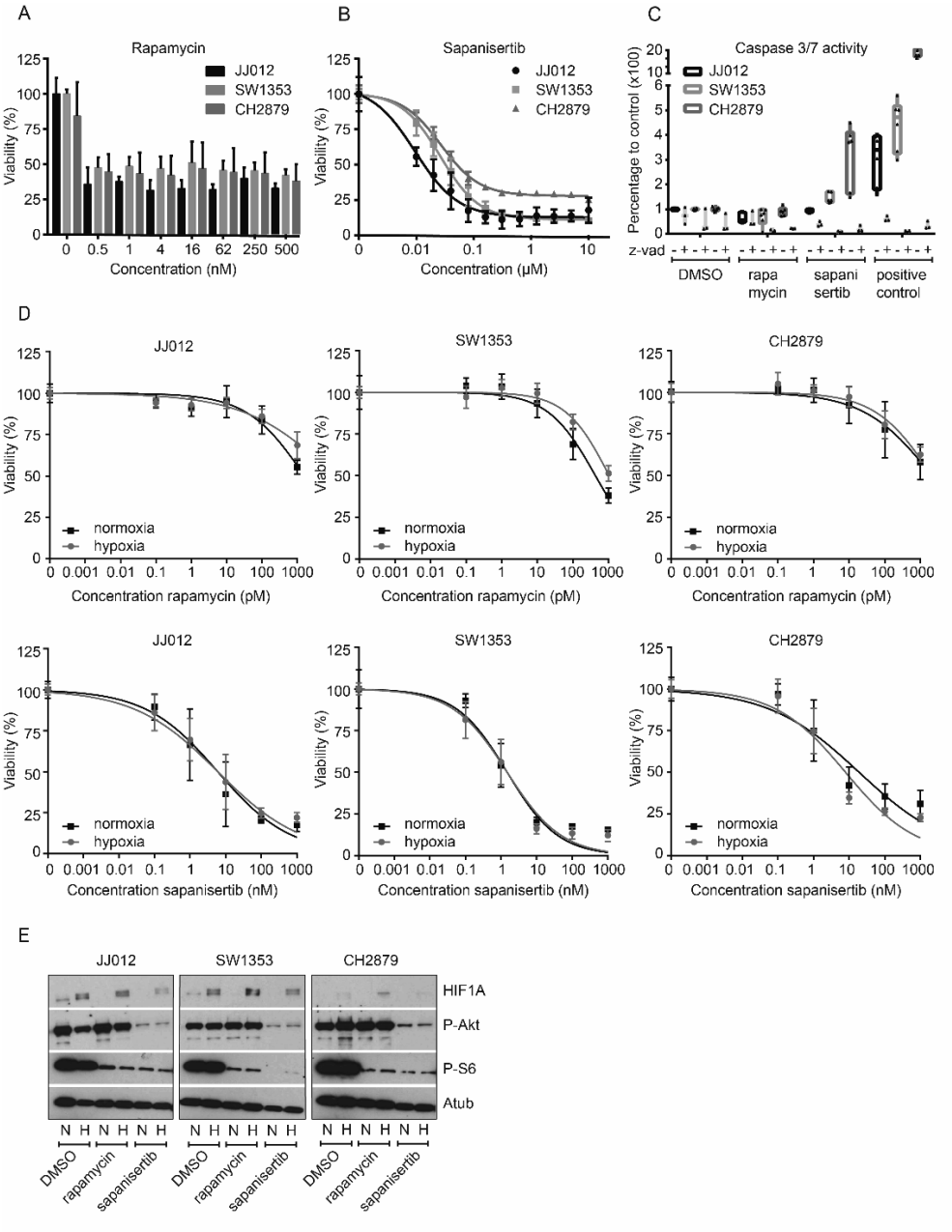

Figure 3: Inhibition of mTORC1 or mTORC1 and C2 in chondrosarcoma cell lines. A) Inhibition of mTORC1 using rapamycin for 72h led to a 50% reduction in viability in JJ012, SW1353 and CH2879 irrespective of the concentration. B) mTORC1 and mTORC2 inhibition using sapanisertib for 72 hours leads to a dose 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).

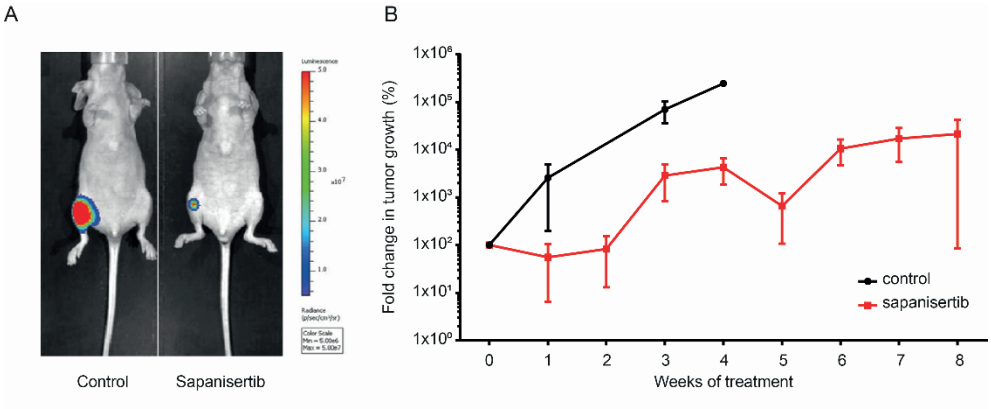

Figure 4: Inhibition of mTORC1 and mTORC2 using sapanisertib in an orthotopic chondrosarcoma xenograft mouse model leads to a delay in tumor growth.
A) Bioluminescent signal (photons/sec/cm2/sr) within standardized ROI of control mice compared to mice treated with sapanisertib after three weeks of treatment. 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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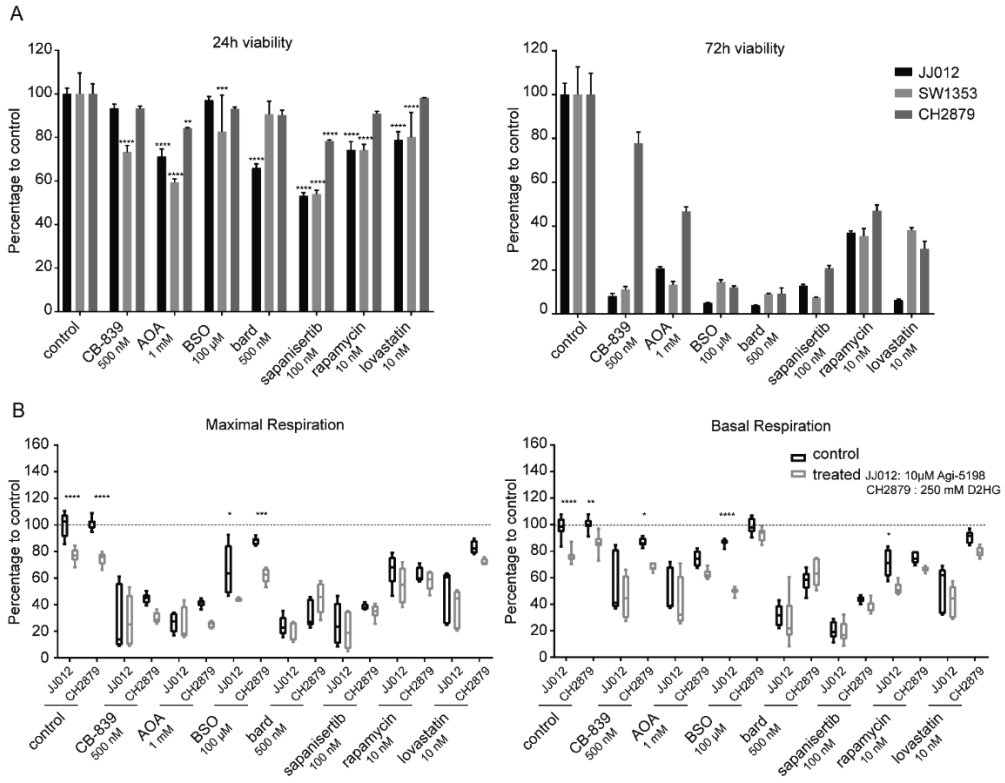
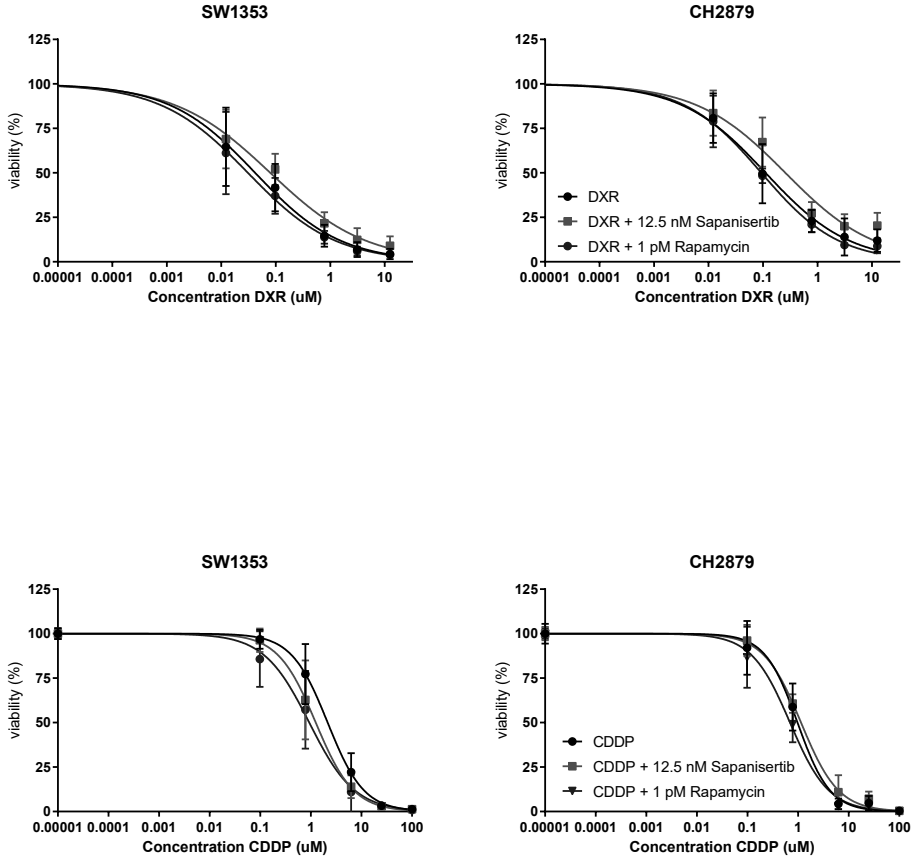
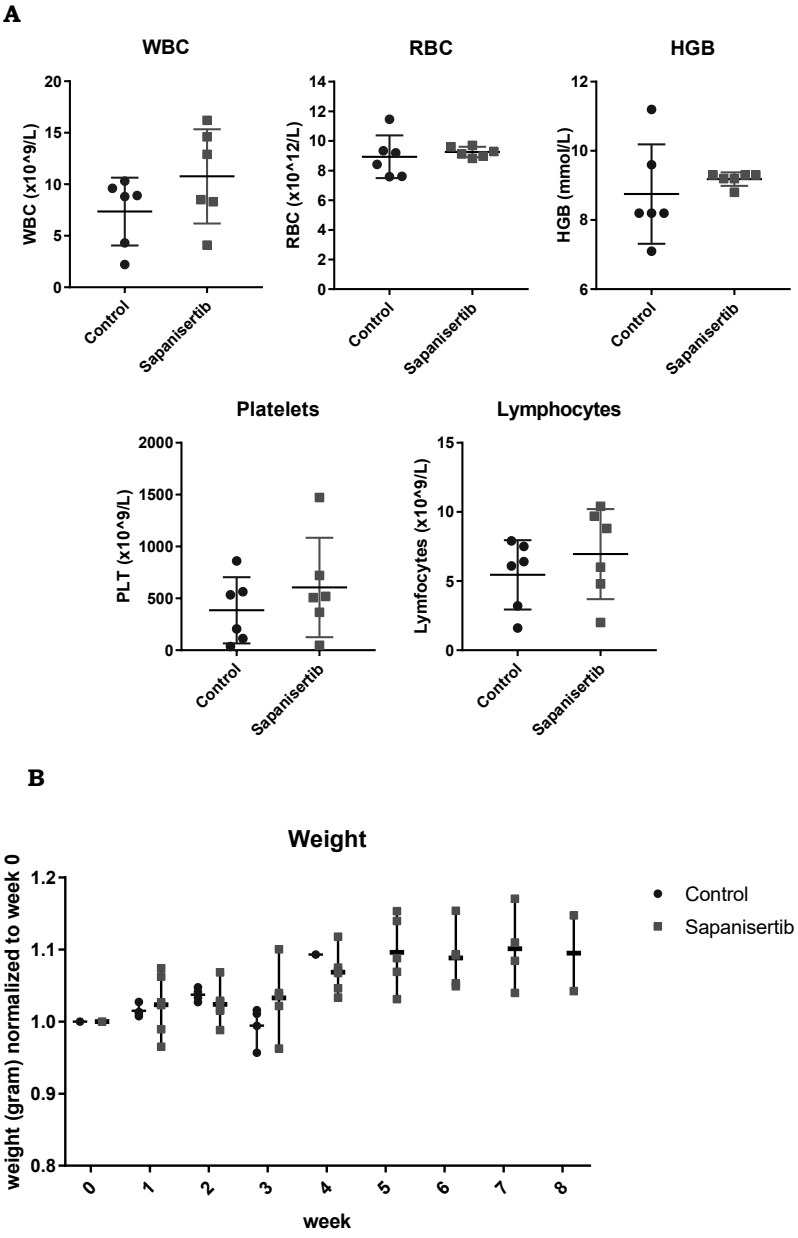
Supplementary material



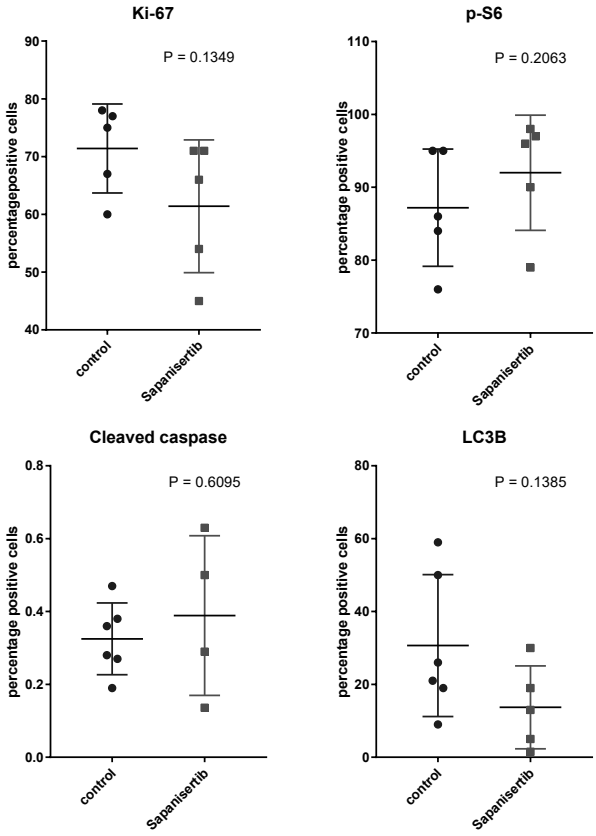
Supplementary Figure 1: A) Viability of compounds used in Seahorse metabolic characterization after 24 and 72 hours treatment. After 24h no drastic effects (>50% decrease) are observed after treatment, indicating that viable cells were still present when performing the seahorse assay. After 72h of incubation 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