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Multinuclear NMR and MRI reveal an early metabolic response to mTOR inhibition in sarcoma

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Abstract

Biomarkers predicting rapalog responses in sarcomas where PI3K and mTOR are often hyperactivated could improve the suitable recruitment of responsive patients to clinical trials. PI3K/mTOR pathway activation drives energy production by regulating anaerobic glycolysis in cancer cells, suggesting a route toward a monitoring strategy. In this study, we took a multi-modality approach to evaluate the phenotypic effects and metabolic changes which occur with inhibition of the PI3K/mTOR pathway. Its central role in regulating glycolysis in human sarcomas was evaluated by short- and long-term rapamycin treatment in sarcoma cell lines. We observed an overall decrease in lactate production in vitro followed by cell growth inhibition. In vivo we observed a similar quantitative reduction in lactate production as monitored by hyperpolarized MRI, also followed by tumor size changes. This non-invasive imaging method could distinguish reduced cell proliferation from induction of cell death. Our results illustrate the use of hyperpolarized MRI as a sensitive technique to monitor drug-induced perturbation of the PI3K/mTOR pathway in sarcomas.

Keywords

Sarcoma; cancer metabolism; rapamycin; treatment response; hyperpolarized MRI

INTRODUCTION

Sarcomas are rare heterogeneous mesenchymal malignancies arising from different tissues (11,000 cases per year in USA) (1), accounting for 21% of all pediatric solid malignant tumors and 38% of soft tissue tumor at 65+ years old (2). More than 50 subtypes of sarcoma differing by origin, localization and grade of invasiveness have been identified. Because of

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this heterogeneity, sarcomas are considered multiple malignancies rather than a single tumor type, though targeting such a vast array of malignancies poses a challenge both in approaching a treatment strategy and assessing response.

Deregulation of several elements in the phosphatidylinositol-3-kinase/Akt/mammalian target of rapamycin (PI3K/AKT/mTOR) pathway has been identified in many types of sarcoma. An analysis performed using the c-bioportal database (TCGA dataset) shows that the PI3K/AKT/mTOR pathway is altered in 74% of sarcoma patients (n=265 patients, Supplementary Figure S1) (3, 4). Given the frequency of alterations in this pathway, multiple clinical trials have been initiated to target mTOR in the setting of sarcoma, though with limited success. The SUCCEED trial being one of the largest, aimed at utilizing Ridaforolimus as a maintenance therapy, and ultimately was deemed unsuccessful. A major critique of this study and many others has been the heterogeneity of the treatment response, making it difficult to assess tumor dependence on the targeted pathway and predict possible therapeutic benefit. This coupled with the challenge of treating these tumors further exemplifies the need for approaches to stratify patients non-invasively as well as possibly predict treatment response outcomes (5).

The role of the PI3K/mTOR pathway is well established in cancer and directly controls protein and lipids synthesis, autophagy and glucose metabolism (6). mTOR is a protein kinase composed of two distinct multiprotein complexes, mTORC1 and mTORC2, that act as master regulators of cellular metabolic homeostasis (7–9). Rapamycin, an FDA approved drug, specifically targets mTORC1 and was used in this study as a tool compound to evaluate its effect on tumor metabolism in vivo. Inhibition of mTORC1 leads to the down-regulation of the phosphorylation status of the ribosomal protein S6 (S6) with a subsequent reduction of energy (ATP) and cofactors (NADPH), both essential for glucose metabolism and for other biosynthetic processes. The metabolic reprogramming of cancer cells is essential to sustain the energy needs for cell survival and proliferation. A key metabolic hallmark of carcinogenesis is the shift from oxidative phosphorylation (OXPHOS) to aerobic glycolysis (Warburg effect) with an increase in glucose consumption and an elevated rate of lactate production (10, 11). This has been probed in vitro most routinely by mass spectrometry methods and in vivo using ^1H and ^{13}C magnetic resonance spectroscopy (MRS). While ^1H and ^{13}C MRS can assess the metabolic status of a specific tissue at a steady state it is severely limited by both scan time and sensitivity (12).

Currently, visualization and quantification of glycolytic metabolic flux can be achieved using hyperpolarized MRI (HP MRI). This technique overcomes the sensitivity issue of conventional MRS techniques by dramatically increasing the spin population, beyond the Boltzmann distribution for a given magnetic field strength, of a target molecule prior to its introduction into the system of interest. In this work we take advantage of dissolution dynamic nuclear polarization (dDNP) as a methodology to hyperpolarize our molecules of interest (13). Using dDNP, many substrates have been hyperpolarized and a strong enhancement of MR signal can be obtained ($>10^4$ fold increase) at various magnetic field strengths, permitting the evaluation of metabolism non-invasively (14). Moreover, imaging of glycolytic metabolism with hyperpolarized $[1-^{13}\text{C}]\text{pyruvate}$ has shown considerable potential in preclinical oncology studies, particularly for the assessment of treatment

response (15–17), though early response and the delineation of reduction in cell proliferation versus cell death remains a challenge. In this work, we utilize these approaches, along with molecular biology approaches to characterize in vitro and in vivo early metabolic responses to mTOR inhibition in sarcomas, demonstrating the potential to utilize HP lactate as a marker to separate eventual cell death from reduced proliferation.

MATERIAL AND METHODS

Chemicals

Unless otherwise indicated, all chemicals and solvents were purchased from Sigma Aldrich (St. Louis, MO, USA), including 99% enriched [1,6-¹³C]glucose.

Cell lines

Gastrointestinal Sarcoma Tumor (GIST-T1), DDLS, JJ012 and CS1 cell lines were cultured under standard conditions in DMEM supplemented with 10% FBS and penicillin streptomycin. GIST-T1 and DDLS were kindly provided by laboratories at Memorial Sloan Kettering Cancer Center (Chi Lab and Koff Lab at MSKCC in 2015). JJ012 and CS1 were kindly provided by the Thompson Lab at Memorial Sloan Kettering Cancer Center in 2014. All cell lines were authenticated prior to publication by short tandem repeat (STR) DNA fingerprinting.

IncuCyte in vitro cell growth measurements

IncuCyte HD system (Essen BioScience) was used to evaluate the effect on proliferation of rapamycin on GIST-T1, DDLS, JJ012 and CS1 cells. Briefly, the day before the experiment 5×10^4 cells were plated in 24-well plates and incubated overnight to allow cell adherence. At the day of the experiment, the media was exchanged with complete media containing 50nM of rapamycin or DMSO alone (1 μ L/well) as a treatment and vehicle condition, respectively. Frames were captured at 6-hour intervals from 4 separate $950 \times 760 \mu\text{m}^2$ regions per well using a 20 $\times$ objective. Confluence was measured using the IncuCyte software. Values from all 4 regions of each well were pooled and averaged across all 4 replicates. Results were expressed graphically as fold increase of cell growth normalized to day 1 versus time.

Western blot Analysis

Cell lysate for vehicle (6% DMSO) or rapamycin (50 nM) treated cell lines was prepared as previously published (18). For western blot analysis, 20 μ L of 2 mg/ml of cell lysate were mixed with 5 μ L of 5 $\times$ sample loading buffer. Separated proteins in the gels were electrophoretically transferred onto PVDF membrane. After washing and blocking, pS6K, PFK, pPKM2, pLDH-a and β -actin antibody (1 μ g/ml) (Cell Signaling Technology), diluted in TBS-T containing 5% BSA, was added and incubated for overnight at 4°C. The bound antibodies were detected by horseradish peroxidase-conjugated anti-goat Ig secondary antibody (Santa Cruz Biotechnology) followed by ECL detection system (Thermo Scientific) according to the manufacturer's instruction.

¹H NMR data acquisition and processing

Experiments were performed on a 14.1T NMR spectrometer equipped with an autosampler and ¹H cryoprobe (Bruker Biospin, Billerica, MA). ¹H NMR spectra for each cell extract and media samples were acquired with a water presaturation recycle delay of 4 s, acquisition time of 2.67 s, 90° pulse and 512 averages (18). Resonances of each metabolites were identified and quantified using Chenomx NMR Suite 8.0 professional (Chenomx Inc. Edmonton, Canada), with 0.5 mM DSS as a known reference standard to determine the concentration of individual metabolites and 10 mM of imidazole as a pH reference (19). In order to quantify and distinguish total pool of lactate from ¹³C lactate, ¹H NMR has been used. Proton resonance line shapes are split into well-established patterns when J-coupled to a ¹³C nucleus. Comparing experiments performed with and without ¹³C decoupling during acquisition indirectly measures fractional ¹³C enrichment (18).

In vivo tumor xenograft and tumor growth measurements

The animal portion of this study was performed under a protocol approved by the Institutional Animal Care and Use Committee of Memorial Sloan Kettering Cancer Institute. GIST-T1, JJ0012 and CS1 xenografted mice were use as a tumor model for in vivo HP pyruvate MRI (n=3 mice/group). DDLS cells did not develop a xenograft tumor and therefore the in vivo analysis in this tumor model was not assessed. 5×10^6 GIST-T1, 10×10^6 JJ012 and 10×10^6 CS1 cells were trypsinized and resuspended in a 1:1 solution of complete media:Matrigel before being injected subcutaneously on the flank of immune compromised NOD. CB17-Prkdcscid mice (Jackson Laboratory, Charles River, USA). 24 h before the HP experiment, mice were treated with 15mg/kg of rapamycin for comparison to 6% DMSO (vehicle). The starting tumor volume for GIST-T1, JJ012 and CS1 was $0.23 \pm 0.05 \text{ cm}^3$ and $0.21 \pm 0.04 \text{ cm}^3$, $0.35 \pm 0.03 \text{ cm}^3$ and $0.34 \pm 0.02 \text{ cm}^3$, $0.17 \pm 0.05 \text{ cm}^3$ and $0.21 \pm 0.06 \text{ cm}^3$ for vehicle and rapamycin treated animals, respectively.

All of the animal experiments were carried out on a permanent 1T MRI system (nanoScan PET/MRI, Mediso, Budapest, Hungary). Tumor region were identified in axial anatomic images acquired using T₂-weighted fast-spin-echo (FSE) acquisition (EchoTime/Repetition Time= 88.5/200 ms). Fifteen 2 mm-slice were acquired with 50-mm FOV and 256×252 matrix to cover the whole tumor. The images were acquired at day 1, 3, 5, 7, 9, 11, 13, 15. Osirix (Pixmeo, Bernex, Switzerland) was used for data analysis. A region of interest (ROI) was manually drawn within the tumor edge and a composite image was created to determine the total tumor volume. Results were expressed graphically as fold increase of tumor growth normalized to day 1.

Hyperpolarized [¹⁻¹³C]pyruvate magnetic resonance

100 µl of 14.2 M [¹⁻¹³C]pyruvate (GE healthcare) was mixed with 15 mM of trityl radical (GE healthcare) (prepared as previously published) and polarized on Spin lab (GE healthcare) (20). The frozen sample was dissolved in 10 ml of 40 mM of TRIS buffer. The dissolution was neutralized in a receiving vial containing sodium hydroxide. 200 µl of 100 mM hyperpolarized [¹⁻¹³C]pyruvate was injected intravenously in the tail vein of a catheterized animals in 10 seconds. After 15 seconds of pyruvate distribution a 2D-CSI image was acquired. The radiofrequency coil used in this experiment was dual-tuned ¹H/¹³C

coil. Before hyperpolarized study, T₂-weighted fast-spin-echo (FSE) images were acquired for anatomical localization (EchoTime/Repetition Time= 88.5/200 ms). 2 mm-slice were acquired with 50-mm FOV and 256×252 matrix to cover the whole tumor. 2D-CSI sequence was used to acquire the HP [1-¹³C]pyruvate MRS. In vivo [1-¹³C]pyruvate spectroscopy data were processed using a custom software (Matlab R2015b, Mathworks). The peak area of HP [1-¹³C]pyruvate to [1-¹³C]lactate was used to calculate relevant ratios. The reduction of HP [1-¹³C]pyruvate was expressed using the following formula: HP [1-¹³C] lactate/(HP [1-¹³C]pyruvate+ HP [1-¹³C]lactate). Each tumor voxel was co-registered anatomically using a T₂-weighted image and a mean value was assigned to each tumor. Only voxels with 80% of tumor tissue were included to limit the influence of partial volume effects.

Tumor xenograft histology and immunohistochemistry

Tumor tissues were embedded in paraffin and 4 μm thick sections were prepared and stained with hematoxylin-eosin (H&E). After de-paraffinization tissue sections were stained with monoclonal rabbit anti-Ki67 antibody and cleaved caspase-3 to detect proliferation and cell death, respectively. Quantification of stained cells was achieved using FiJi and the ratio between stained and unstained cells was established.

All other methods are described in Supplementary Information.

RESULTS

Rapamycin slows cell growth in vitro in the sarcoma cell lines analyzed

Four cell lines have been included in this study to survey whether tumor origin or aggressiveness have a differential effect post-rapamycin treatment. In identical medium conditions, the four cell lines grew at significantly different rates with CS1 (chondrosarcoma) cells demonstrated the fastest growth, while JJ012 (chondrosarcoma) grew moderately rapidly, and GIST T1 (gastrointestinal soft-tissue sarcoma) and DDLS (liposarcoma) showed the slowest growth rates (Figure 1A). After suppression of mTORC1-dependent signaling by treatment with 50nM of rapamycin (Figure 1B), a differential effect on cell growth was observed. The cell growth arrest becomes significant at 54 for GIST T1, 96 hours for DDLS, 36 hours for JJ012 and 36 hours for CS1. (Figure 1A). mTOR plays a central role in cell growth and proliferation and as expected the fastest proliferating cells relied heavily on this pathway (CS1 and JJ012 cells) and were the most affected by PI3K/mTOR inhibition (Fig 1B), though cell death was not observed in vitro. These results confirm interdependence of the PI3K/mTOR pathway and cellular proliferation.

3 hours labeling with [1,6-¹³C]glucose in 2D cell culture shows decreased glucose consumption and inhibition of lactate production as early as 24 hours

To verify inhibition of the mTOR pathway, the phosphorylation status of S6 and 4EBP-1 was evaluated in rapamycin treated GIST-T1, DDLS, JJ012 and CS1 as compared to control DMSO treated cells. In all cell lines we observed a significant decrease in phosphorylation of S6 meaning an effective target inhibition. No significant effect was observed in the phosphorylation status of 4-EBP1 as expected for each cell type. In order to then characterize the effect of rapamycin treatment on sarcoma cell glucose metabolism, we first

interrogated multiple proteins responsible for glucose uptake and subsequent phosphorylation. The GLUT-1 transporter, typically the main uptake pathway for glucose, and hexokinase-2 (HK-2), routinely upregulated in cancer, were considered as markers for upstream glycolysis, while downstream lactate production was measured as an indicator of total glycolysis (21, 22). No changes in GLUT-1 and HK-2 expression were detected in all the four sarcoma cell lines after 24 hours rapamycin treatment (Fig 1B). Moreover, no changes in total protein and phosphorylation level of lactate dehydrogenase (LDH) were detected (Supplementary Figure S2).

In vitro metabolic analyses were performed in order to assess changes in metabolic pools sizes in all cell lines. In vitro the total metabolites pool size concentrations are highest in JJ012 and CS1 as compared to GIST-T1 and DDLS. Changes in intracellular and extracellular metabolites are also the largest in those two cell lines with rapamycin treatment. Several metabolic pathways, such as glutaminolysis, and amino acid uptake are affected by rapamycin treatment (Supplementary Figure S3AB). However, in all cell lines analyzed, the largest difference was a decrease in glycolytic flux from glucose to lactate after mTOR inhibition.

Figure 1C shows the changes in glucose consumption after 24 hours of treatment with rapamycin. We observed a significant decrease in glucose consumption of 35.8%, 35.0%, 58.5% and 54.5% for GIST-T1, DDLS, JJ012 and CS1, respectively ($p < 0.05$). Importantly, this change in glucose consumption was observed before any demonstration of significant growth arrest (Figure 1A). With treatment, glucose consumption significantly decreased in all cell lines with a corresponding decrease in both total intracellular and extracellular pool size of lactate in all the 4 cell lines (intracellular: 49%, 48%, 56%, 49% and extracellular: 54%, 60%, 51%, 47% for GIST-T1, DDLS, JJ012 and CS1 respectively, $p\text{-value} < 0.05$) (Figure 2A).

To determine whether the changes in lactate production were specifically derived from glucose, we traced [1,6- ^{13}C]glucose labeling to lactate in culture. The same degree of decrease was observed in ^{13}C labeled lactate derived from 3 hours of incubation with [1,6- ^{13}C]glucose (Figure 2B). After 24 hours of rapamycin treatment, we did not observe a significant change in either the extracellular or intracellular fractional enrichment of lactate. These data demonstrate that the inhibition of mTOR pathway modulates lactate production, which derives predominantly from glycolysis.

HP [1- ^{13}C]pyruvate MRS shows a decrease in glycolytic flux at 24 hours of rapamycin, prior to anatomic response

In order to assess tumor metabolism non-invasively in vivo, hyperpolarized [1- ^{13}C] pyruvate MRS was used to monitor early changes in metabolism. DDLS cells did not efficiently xenograft and were not used for in vivo analysis. Figure 3A demonstrates a representative T_2 -weighted ^1H image with corresponding HP [1- ^{13}C] pyruvate MRS in vehicle and rapamycin treated tumor-bearing mice. Upon entering the cell, HP [1- ^{13}C]pyruvate can be rapidly reduced to [1- ^{13}C] lactate, transaminated to [1- ^{13}C] alanine and oxidized, resulting in the formation of CO_2 and later bicarbonate (HCO_3) via carbonic anhydrase (23). While at baseline GIST-T1, JJ012 and CS1 tumors exhibited similar HP [1- ^{13}C]lactate/total ^{13}C

ratios, a significant decrease in [1-¹³C] lactate production is observed at 24 hours of rapamycin treatment in all models with 36.8%, 30.9% and 47.3% observed in GIST-T1, JJ012 and CS1, respectively ($p < 0.001$ for all rapamycin tumor treated vs. vehicle, Figure 3B). When quantitatively comparing the HP lactate/total ¹³C ratio post-treatment, a significant difference was observed between GIST-T1, JJ012 and CS1 sarcomas, providing a means of separating these groups (0.46 ± 0.01 , 0.50 ± 0.04 and 0.35 ± 0.01 for rapamycin-treated GIST-T1 and JJ012 v. CS1 $p < 0.001$, Figure 3C). Moreover, in the setting of CS1 sarcomas, a decrease in HP [1-¹³C]lactate and a significant increase in both [1-¹³C]alanine and [1-¹³C]HCO₃ was observed in treated tumors ($p < 0.05$ and $p < 0.01$ for alanine and bicarbonate, respectively) (Supplementary Figure S4). This data show a clear metabolic shift from aerobic glycolysis of the DMSO-treated CS1 tumors to a partial re-establishment of oxidative phosphorylation after rapamycin treatment in vivo.

Long-term rapamycin treatment of sarcoma lines in vivo results in growth arrest and histopathologic response

Both, in vitro NMR experiments and in vivo HP pyruvate suggest an inhibition of glycolytic flux in short-term rapamycin treated cells, prior to a change in cell growth. Thus, to evaluate the long-term effect in vivo, xenograft mice were treated systemically for 15 days with 15mg/kg of rapamycin without any off target side effect reported (24–26). In all sarcoma tumor models, rapamycin markedly reduced tumor volume compared to vehicle treated animals. Remarkably, we observed differences in treatment efficacy depending on the model used. GIST-T1 showed a significant inhibition of tumor growth at 13 days after starting rapamycin treatment ($p < 0.05$). As expected, for the in vitro cell experiment, we observed a greater effect of rapamycin treatment in JJ012 and CS1 compared to GIST-T1. Tumor growth arrest was observed at 5 and 3 days of rapamycin treatment for JJ012 and CS1 tumors, respectively ($p < 0.001$, for both JJ012 and CS1) (Figure 4A). The sensitivity profile of these xenografts mirrors their glycolytic metabolic status. Moreover, it seems that the more dependent the tumor is on glycolysis, the more sensitive it is to rapamycin-induced growth inhibition. The present in vivo tumor growth data correlates with the histopathologic analysis performed at 15 days of rapamycin treatment. As expected, we observed in all rapamycin-treated tumor an anti-proliferative effect, identified by a decrease of Ki-67 staining (Figure 4B). However, only JJ012 and CS1 tumors had a significant decrease in cell proliferation ($p < 0.05$ for both) (Figure 4C). Moreover, a significant increase in cell death, detected by cleaved-caspase3, was observed only CS1 tumors (3.7 fold higher, $p < 0.001$, Figure 4BC).

DISCUSSION

Our findings indicate that rapamycin induces a rapid metabolic effect and later a biological response in short- and long-term treatment. In vitro we observed an effective inhibition of the mTOR pathway in sarcomas by rapamycin, leading to a significant change in glucose metabolism as early as 24 hours post-treatment. Interestingly, short term short-term treatment in these cells models did not effect the expression or phosphorylation of major glycolysis-involved proteins. However, a decrease in intracellular ATP and NADPH was observed in all cell lines analyzed in this study (Supplementary Figure S5) and this most likely drives the

metabolic effects observed. We were also able to detect early metabolic changes in glucose metabolism in vivo (observed as early as 24 h) using HP pyruvate. A significant decrease in glycolytic flux with treatment using HP MRI correlates with a decrease of tumor growth in long-term treatment in all sarcoma models imaged. Interestingly, a decrease in approximately 50% of HP lactate production at 24 h rapamycin treatment correlates with induction of apoptosis in long-term treatment.

To date, cumulative data demonstrate a strong interdependence between metabolic changes and cell death. Specifically, various pharmacological treatments in cancer cells have shown altered glucose metabolism observed prior cell death (27–29). Zhao et al., have demonstrated that the up regulation of glycolysis in cancer cells attenuates cell death by stabilizing the anti-apoptotic Bcl-2 family protein Mcl-1 (30). Therefore, a decrease of glycolytic flux induced by rapamycin treatment in vivo can activate the pro-apoptotic pathway as shown by the increase of caspase-3 staining in the in tumor sections of treated animals (Figure 4B). Interestingly, quantitative changes in the glycolytic state leading to a specific utilization of glucose and their direct relationship to induction of apoptosis are not well understood in vivo. This is mostly due to the lack of tools available to accurately study metabolism non-invasively. The most commonly utilized metabolic imaging approach, 2-deoxy-2-[¹⁸F]fluoro-D-glucose positron emission tomography (FDG PET), has shown use in the setting of AKT, but lacks predictive power in the setting of mTOR inhibitor therapy(31). While FDG PET has provided a window into our understanding of glucose metabolism in vivo, it is limited due to its inability to directly resolve the substrate (FDG) from its compartmentalized downstream product (e.g. FDG-6-phosphate). This makes it difficult to discern uptake kinetics via glucose transporters (predominantly GLUT1) from metabolic conversion by hexokinase. Moreover, recent work has demonstrated that directly assessing the downstream metabolic products of glycolysis using HP MRI may provide a sensitive marker for signaling dependent changes in glycolysis (32).

Further studies are needed in order to address the differential induction of cell death in rapamycin treated tumors, but this work provides evidence that the magnitude of reliance on metabolic flux, assessed in vivo, may indicate degree of treatment response to targeted inhibitors. Ultimately, the successful development of targeted therapies is dependent on effective non-invasive approaches to predict and monitor response in patients. Playing a complimentary role to the use of genomic testing to place patients in to phase I clinical trials, HP MRI assessment of mTOR dependent changes in metabolism could predict which patients would benefit from targeted treatment. In summary, this would allow for better stratification of patients into appropriate clinical trials, assessment of possible heterogeneous tumor response in current trials as well as determine potential treatment efficacy.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1. Rapamycin significantly inhibits cell growth in vitro in GIST-T1, DDLS, JJ012, CS1 sarcoma cells

A) Growth rate was determined using the IncuCyte real-time video imaging system. The graphs represent the cells growth curve plotting confluence vs. time at 6 h intervals for GIST-T1, DDLS, JJ012 and CS1 incubated with DMSO (black) and 50 nM rapamycin (red).

B) Protein expression level detected with Western blot analysis. GLUT-1, Hexokinase 2 (HK-2), phosphorylated ribosomal protein p-S6 (S6), phosphorylated 4E-binding protein 1 (p-4E-BP1), beta actin as a loading control. **C)** Glucose consumption in vehicle and rapamycin treated GIST-T1, DDLS, JJ012 and CS1 cells. Results are expressed as mean $\pm$ SD of three independent tests. p-values < 0.05 were considered as statistically significant (grey shadow).

Figure 2. Isotopic tracing of lactate production using ¹H NMR

A) Total intracellular and extracellular pool size of lactate of DMSO control (black) vs. rapamycin (red) treated GIST-T1, DDLS, JJ012 and CS1. **B)** Labeled intracellular and extracellular ¹³C lactate of vehicle (black) vs. rapamycin (red) treated GIST-T1, DDLS, JJ012 and CS1. Results are expressed as mean ± SD. p-values < 0.05 were considered significantly different from control (*).

Figure 3. Hyperpolarized lactate production was reduced in GIST T1, JJ012 and CS1 after 24 hours rapamycin treatment

A) Representative T₂-weighted axial MR image of vehicle and rapamycin treated mouse, with spectra from study performed after injection of hyperpolarized [1-¹³C]pyruvate **B)** Bar plots represent the ratio of HP lactate/(HP lactate + HP pyruvate) in GIST-T1, JJ012 and CS1 tumor treated for 24 hours with 6% DMSO (black) and 15mg/kg of rapamycin (red). **C)** Bar plots represent the ratio of HP lactate/(HP lactate + HP pyruvate) in GIST-T1, JJ012 and CS1 tumor treated for 24 hours with 15mg/kg of rapamycin. Results are expressed as mean ± SD. p-values < 0.05 were considered significantly different from control (*).

Figure 4. Long-term rapamycin treatment slows down tumor growth in GIST-T1 and induces tumor arrest in JJ012 and CS1 in vivo

A) The graphs represent the fold increase of tumor curve plotting GIST-T1, JJ012 and CS1 tumor volume vs. time. The graphs compare tumor treated with 6% DMSO (black) and 15mg/kg of rapamycin (red). Results are expressed as mean $\pm$ SD of three independent tests. p-values < 0.05 were considered as statistically significant (gray shadow). **B)** Expression of Ki-67 and cleaved caspase 3 in GIST-T1, JJ012 and CS1 tumor tissues after 15 days of vehicle (left panel) and rapamycin treatment (right panel). Images are a representative tumor sections immunostained independently for each cellular marker. **C)** Bar plots represent the mean of Ki 67 (left) and cleaved-caspase 3 expression (right) in tumor treated with 6%

DMSO (black) and 15mg/kg of rapamycin (red). Results are expressed as mean $\pm$ SD. p-values < 0.05 were considered significantly different from control (*).

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