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The effect of the mTOR inhibitor rapamycin on glucoCEST signal in a preclinical model of glioblastoma

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Abstract

Purpose: The mammalian target of rapamycin (mTOR) is an enzyme that regulates cell metabolism and proliferation. It is up-regulated in aggressive tumors such as glioblastoma, leading to increased glucose uptake and consumption. It has been suggested that glucose Chemical Exchange Saturation Transfer (glucoCEST) signals reflect the delivery and tumor uptake of glucose. The inhibitor rapamycin (sirolimus) has been applied as a glucose deprivation treatment and hence glucoCEST MRI could potentially be useful for monitoring the tumor responses to inhibitor treatment.

Methods: A human U87-EGFRvIII xenograft model in mice was studied. The mice were treated with an mTOR inhibitor, rapamycin. The effect of the treatment was evaluated in vivo with dynamic glucoCEST MRI.

Results: Rapamycin treatment led to significant increases ($P < 0.001$) in dynamic glucose enhanced (DGE) signal in both the tumor and contralateral brain as compared to the no-treatment group, namely a maximum enhancement of $3.7\% \pm 2.3\%$ (tumor, treatment) versus $1.9\% \pm 0.4\%$ (tumor, no-treatment), $1.7\% \pm 1.1\%$ (contralateral, treatment), and $1.0\% \pm 0.4\%$ (contralateral, no treatment). DGE-contrast remained consistently higher in treatment versus no-treatment groups for the duration of the experiment (17min). This was confirmed with area-under-curve analysis.

Conclusion: Increased glucoCEST signal was found after mTOR inhibition treatment, indicating potential for DGE MRI to study tumor response to glucose deprivation treatment.

Introduction

Altered energetics is a hallmark in cancer(1,2), and especially altered glucose metabolism (Warburg effect) is characteristic of malignant tumors(3,4). Moreover, an overexpression of glucose transporter (GLUT) proteins has been found in numerous cancers, including glioblastoma(5–7). The monitoring of altered glucose metabolism and the development of methods to control it may be keys for better treatment outcome(8). Glucose deprivation treatment has been demonstrated to be an effective tumor therapy(9–12).

A current interest for cancer therapy is the mammalian target of rapamycin (mTOR)(13,14), a protein kinase that is hyperactivated in a majority of human tumors(15). Inhibition of mTOR prevents the accumulation of the transcription factor HIF-1 α , thereby reducing the expression of key molecules involved in cellular glucose delivery, such as the cell surface expression of glucose transporter GLUT1, and the maintenance of the tumor vascularity. The latter is due to a decrease in the expression of proangiogenic mediators such as vascular endothelial growth factor (VEGF)(16). Inhibitor approaches(17) have been applied to treat patients on a stand-alone basis or in the form of a metabolic cocktail since multiple metabolic processes are altered in tumors(9,12,18). Rapamycin, also generically known as sirolimus, an inhibitor for mTOR, has shown to be useful in the treatment of certain diseases related to the mTOR signal pathway(19). It is one of the clinically approved first-generation of mTOR inhibitors, and new mTOR inhibitors are under investigation to optimize the efficacy and minimize side effects for glioblastoma (GBM) treatment. Nevertheless, rapamycin has been widely used to study the effect of mTOR inhibition in mice. The therapeutic efficacy of rapamycin in GBM by inhibiting glucose uptake(9,20–22) was previously shown by FDG-PET(23).

Recently **D**-glucose has shown potential as a biodegradable contrast agent for cancer detection by employing its hydroxyl protons as a natural MRI label that can be detected using either chemical exchange saturation transfer (CEST) MRI(24–29), or through changes in the MRI relaxation times T1 ρ (30–32), and T2(31,33,34). In analogy to dynamic contrast enhanced (DCE) with Gadolinium (Gd)-based agents(35–41), dynamic glucose enhanced (DGE) MRI(27,29,42,43), has been proposed to dynamically study perfusion parameters using dynamic glucoCEST. DGE measures the glucoCEST signal changes at temporal resolution of 5–10 s(27,29). The signal contributions from glucose can in principle originate from three different compartments: the vascular space (v), extravascular extracellular space (EES) and intracellular space (c), with the latter mainly containing phosphorylated glucose analogues. However, since concentrations of the latter are in the low micromolar range(44–46), most signal has been attributed to EES and vascular origin(24,29). In addition, the pH of the microenvironment will affect the CEST contrast for OH groups with different chemical shift (MR frequency) and exchange rate(47,48). Our hypothesis is that use of the mTOR inhibitor rapamycin will slow down cellular metabolism and **D**-glucose uptake(7,23), which would be reflected in increased vascular and EES signals for glucoCEST contrast. We applied the DGE technique to study the effect of rapamycin on healthy mice and mice

implanted with GBM tumor cells. Since rapamycin has been applied in tumor treatment, the observations in this study could also be useful for monitoring pharmacodynamics during mTOR inhibitor treatment.

Methods

Animal preparation

All procedures complied with Animal Welfare Act regulations and Public Health Service (PHS) Policy for Animal Research: Reporting of *In Vivo* Experiments guidelines. Animal experiments were approved by the Johns Hopkins University Institutional Animal Care and Use Committee. The program was accredited by the Association and Accreditation of Laboratory Animal Care (AAALAC) International. Human U87-EGFRvIII cells were grown in Dulbecco's Modified Essential Medium high glucose with L-glutamine and sodium pyruvate (Mediatech Inc.) supplemented with 10% fetal bovine serum, 1% of 10 mM MEM-non-essential amino acids and penicillin and streptomycin(20). SCID mice (female; n=4, 6–8 weeks) were anesthetized with isoflurane and human U87-EGFRvIII cells were implanted stereotactically (10^5 cells/2 μ l/mouse) into the right caudate/putamen of mice. Mice in the inhibitor group received intraperitoneal injection of rapamycin (3 mg/kg) on day 5 and 7 post tumor implantation prior to MRI experiments on day 7(23). Another group of healthy SCID mice (female; n=5, 6–8 weeks) served as healthy controls and underwent DGE imaging before and after inhibitor treatment. For the control group, the first DGE MRI experiment was performed on Day 1, the inhibitor treatment took place on Day 3 and 5, and the second DGE MRI experiment was conducted within 3 hours of the inhibitor treatment on Day 5.

MR imaging protocols

All MRI images were acquired on a horizontal bore 11.7 Tesla (T) Bruker Biospec system equipped with a 23 mm volume transceiver coil. Mice were anesthetized by 1–1.5% isoflurane and kept warm with a heating bed, and imaged before, during and after D-glucose infusion. In brief, the tail vein of the mouse was cannulated with a catheter and the dead volume minimized to 30 μ l. A tee connector was attached to a 2.8 M **D**-glucose solution (0.5 g/mL, clinical-grade Dextrose, Hospira), ProHance solution (Gadoteridol; 100 mM in saline, Bracco Diagnostics, Milan, Italy), and the tail vein. The **D**-glucose injection (0.15 mL 50% w/w glucose, 60 s) was performed with a syringe pump(24,29). No Gd contrast was given to the control group.

Anatomical images were acquired to identify the slice with maximum tumor size. DGE images were acquired over 20 minutes using a short-echo Rapid Acquisition with Relaxation Enhancement (RAREst) sequence, TR/TE = 5.0 s/3.8 ms, RARE factor of 23, a matrix of 128 $\times$ 64, partial Fourier acceleration factor of 1.4 and a FOV of 1.6 cm² (All images were normalized to an image without saturation acquired prior to injection). A single slice with 0.12 $\times$ 0.25 mm² in plane resolution and thickness of 1 mm was imaged with a slice location that showed maximum tumor size determined by the anatomical images. Saturation was achieved by a single continuous wave magnetization transfer (MT) pre-pulse of 3 s at B1 = 1.6 μ T at the hydroxyl proton frequency offset of 1.2 ppm. An S₀ image with the identical

acquisition parameters but no saturation pulse was acquired before injection. The dynamic scanning started before the glucose injection by acquiring 18 baseline scans over three minutes. Without stopping the scanning, D-glucose was infused over a one-minute period. The dynamic acquisition continued for a total scan time of 15 minutes. Full z-spectra were acquired over a frequency range from -5.2 to 5.2 ppm (0.4 ppm intervals) before and after dynamic acquisition using the same readout parameters. The scan time for acquiring a full z-spectrum was 4min 30s. The post glucose z-spectrum was collected approximately 20 min post glucose infusion.

Localized ^1H magnetic resonance spectroscopy (MRS) was acquired in the inhibitor group using a stimulated echo acquisition mode (STEAM) technique with outer volume suppression (OVS). MRS spectra were recorded prior and approximately 35 min post glucose injection, respectively. Localized shimming up to second order was performed using field mapping before each MRS acquisition. A single voxel of $2.0 \times 2.0 \times 2.0 \text{ mm}^3$ was chosen encompassing the tumor based on the actual tumor size. The sequence parameters were TR = 2500 ms, TE = 3 ms, mixing time TM = 10 ms, 2048 complex points were acquired. Signal was averaged for 256 times resulting a total acquisition time of 10 min 40 s.

DCE images were acquired after the second MRS study for mice with brain tumors. Multi-slice T1-weighted images were obtained using a nonselective saturation-recovery fast low angle shot (FLASH) with TE=1.5 ms, TR=26 ms and flip angle 90° (49), at the same spatial and temporal resolution as DGE imaging. A large flip angle was used to enhance T1 weighting and to suppress the water exchange effect as recommended in the literature(49,50). After acquiring eight pre-contrast images, a bolus injection (10 s) of 0.1 mL of 0.1 M Gadoteridol was given and 32 post-contrast images were collected over 320 s.

Data Analysis

Images were processed using custom-written Python scripts. For DGE, contrast was quantified by calculating the S images recorded at the offset of 1.2 ppm. The DGE difference images were processed as previously reported. Briefly, all images were normalized to the S_0 image to correct the proton density difference in brain. The pre-injection images were averaged to obtain the baseline image S_{base} , and the change in water signals was calculated using $\Delta S = (S - S_{base})/S_{base}$. For conventional z-spectra, the asymmetry in the magnetization transfer ratio (MTR_{asym}) was calculated using $MTR_{asym} = (S_{\omega} - S_{-\omega})/S_0$. The DCE images were processed similar to DGE experiments, i.e., baseline images were generated by averaging the eight pre-injection images (S_{base}) and the difference images were calculated using $\Delta S = S - S_{base}$.

Area-under-curve ($AUC_{initial}^{5min}$) images were calculated for DCE and DGE images by integrating images acquired over a period of the first five minutes after the start of the bolus injection. To compare the treatment effect dynamically, ROIs were drawn from the entire tumor area and in the contralateral brain area with similar size and time resolved DGE signals ($AUC_{dynamic}^{3min}$) were obtained by averaging the S signals in the whole ROIs every 3 minutes (except the last time point, which was averaged using images acquired between 15–

17 min post injection). The time resolved $AUC_{dynamic}^{3min}$ values were compared between the inhibitor treated and control animals with the same U87 tumor model (n=4) using data from previous work(29). A two-sample t test was performed between the inhibitor and no-treatment group, and was considered statistically significant for $p<0.05$ and highly significant when $p<0.001$.

All in vivo MRS experiments were processed using Bruker Topspin software. A line broadening factor of 5 Hz was applied and spectra were referenced by setting the methyl group of creatine at 3.02 ppm. The difference NMR spectra were generated by subtracting the NMR spectra acquired before and after glucose injection after alignment at the creatine frequency.

Results

Figure 1a shows the DGE dynamic images for a mouse in the healthy control group before and after inhibitor treatment (each image is an average of 6 images acquired over a period of one minute). This mouse represents the largest signal change post treatment among all mice and a pronounced signal increase can be observed across the brain. Figures 1b and c show the average signal change of 5 mice in the control group as a function of infusion time in the brain tissue and the jugular vein or the superficial temporal vein (blood vessels), respectively. Despite a large variability in DGE response between individual mice (as seen from the standard deviation), It can be seen that in both brain tissue and blood vessels, the average DGE signal started to differ around 400–500 s post infusion, at which point the signal was higher post inhibitor treatment, an increase that persisted until the end of the experimental period.

Figure 2 shows the anatomical T2w (a), post Gd T1w (b), the DGE- $AUC_{initial}^{5min}$ (c), the DCE- $AUC_{initial}^{5min}$ (d) images for a representative mouse with a brain tumor in the inhibitor treated group as well as glucose uptake curves (e) for some ROIs in the same mouse. DGE contrast at 1.2 ppm increased during D-glucose injection and, similar to the no-treatment group(29), the tumor was highlighted relative to the contralateral brain. The maximum DGE contrast was highest in the blood vessel and markedly higher in the tumor than in the contralateral brain, with signal changes of 4.8%, 2.9% and 1.1% respectively (Figure 2e). The curves in Figure 2e show that, during the whole experimental period of 1000 s (17 minutes), the glucose concentrations in the tumor and blood vessel remained higher than that pre-injection. In addition, it is clear that the tissue and tumor DGE curves reflect the shape of the D-glucose input function in blood vessels.

In Figure 3, the DGE curves of the inhibitor-treated are compared with those of the no-treatment groups published previously(29). We consistently observed a higher glucoCEST signal in both the tumor and the contralateral brain in the animals with treatment compared to those without (Figures. 3a,b). This higher signal for the treatment group persisted throughout the experiment for both the tumor and contralateral brain regions. On the other hand, the blood vessel DGE signal in the groups with and without treatment was the same within error for the first 3 min post injection. The blood glucose signal peaked immediately

(20 s) after the completion of injection and reached $4.3\% \pm 0.4\%$ and $4.9\% \pm 0.6\%$ ($N=4$) for the treatment and no-treatment groups, respectively. DGE signals in the blood vessels decayed to $2.4\% \pm 0.4\%$ and $0.7\% \pm 0.5\%$ for the treatment and no-treatment groups at the end of experiment (17 min post injection). In the tumor and contralateral brain regions, the DGE signals reached maximum values of $3.7\% \pm 2.3\%$ (tumor, treatment), $1.9\% \pm 0.4\%$ (tumor, no-treatment), $1.7\% \pm 1.1\%$ (contralateral, treatment), and $1.0\% \pm 0.4\%$ (contralateral, no treatment) at 90 s post infusion, respectively (Figure 3). While the tissue DGE signals in the no-treatment group decayed and remained stable, signals of the treatment group first decayed and then increased before leveling off. The remaining signals at 17 min post injection were $2.7\% \pm 1.1\%$ (tumor, treatment), $0.7\% \pm 0.1\%$ (tumor, no treatment), $0.9\% \pm 0.4\%$ (contralateral, treatment), and $0.3\% \pm 0.1\%$ (contralateral, no treatment).

To better compare the uptake and retention of glucose, the time dependent $AUC_{dynamic}^{3min}$ signals from the blood vessels, the tumor and the contralateral brain regions were calculated. Pair-wise comparison using two-sample t-tests were performed between the treatment and no-treatment groups and the results are shown in Figure 4. The data shows that while there is a significant difference ($p=0.0095$) in the tumor region immediately after **D**-glucose injection for the treatment and control groups, this was not the case in the contralateral brain region. The treatment versus no-treatment difference in the tumor became highly significant ($p<0.001$) from 6 min onwards, while this was from 9 min onwards for the contralateral brain region. No significant difference was found in the blood vessels immediately following (0–3 min) the glucose injection; the difference becomes significant in the period of 3–6 min ($p=0.002$) and highly significant in the later period.

The z-spectra acquired pre- and post-glucose injections from 3 mice are presented in figure 5. These z-spectra show excellent signal stability however it is difficult to pinpoint the subtle (a few percent) signal changes. Therefore the difference spectra and the MTR_{asym} were calculated. Signal in the difference spectra and the MTR_{asym} between 0–3 ppm can be attributed to the glucoCEST effect, with the difference in the asymmetry showing a profile similar to that of **D**-glucose(29). The MTR_{asym} is negative mainly due to the asymmetry of conventional magnetization transfer effect (as can be judged from the intercepts at ± 4.5 ppm). We applied a $1.6\mu T$ saturation power in our study so this background effect was relatively strong. There may also be a small contribution from the relayed NOEs of mobile proteins.

In order to verify that the DGE signal differences in tumor are due to **D**-glucose, localized 1H in vivo MRS were acquired pre- and post-glucose infusion for three of the inhibitor-treated mice (Figure 6). The difference NMR spectra showed strong peaks between 3.5 to 4.0 ppm, consistent with the H2-H6 proton resonances of **D**-glucose. Even though a single voxel was chosen to best encapsulate the tumor, the MRS voxel still contains tissue surrounding the tumor and it is difficult to quantify the exact glucose concentration in the tumor. The difference NMR spectra in the tumor region did not show noticeable change in the lactate signal.

Discussion

We report DGE MRI responses to mTOR inhibition therapy in the U87 brain tumor model. Different from DCE contrast studies, in which the gadolinium contrast agent is not metabolized in tissue, **D**-glucose is taken up and utilized by cells. Therefore, the DGE dynamic process is a more complicated process and the signal changes not yet completely understood(27,29). The current study provides additional insight into the contributions to the DGE signal from different tissue compartments.

Previous reports have shown the rapamycin and its derivatives causes hyperglycemia in human(51) and mice(23) with glioblastoma. Our study on healthy control mice group confirms that rapamycin induces hyperglycemia in healthy animals as well, which is intuitive since inhibition of mTOR down-regulates the cellular uptake and thus usage of glucose. In normal mice, the first 6–8 min after the infusion showed no difference before and after the inhibitor treatment, which we interpret to reflect mainly blood perfusion. The later part of the dynamic, however, consists of contribution from both perfusion and metabolism. When cellular glucose uptake and metabolism are reduced, more glucose remains in the vascular space. The DGE signal in the brain of healthy mice therefore likely originates from the glucose in the brain parenchymal microvessels and extravascular extracellular space (EES) due to incomplete inhibition of GLUT1 activity. The higher signal in the later part of the dynamic (8 min onwards) in the brain post treatment likely reflects the difference in uptake and metabolism due to administration of rapamycin.

The glucoCEST contrast in tumors can in principle originate from three compartments: the vascular space, the *EES*, and the intracellular space. Under normal physiological conditions, the glucose concentration in brain tissue (cells+ *EES*) is roughly a quarter of the blood glucose concentration(52,53) Thus, at the baseline blood glucose concentration of approximately 5 mM, the glucose concentration in the brain will be around 1–1.5 mM. At higher blood glucose concentrations this proportionality is expected to remain, unless the transporters become saturated. In line with that (figure 3), we observed that the glucoCEST contrast in the no-treatment group increased by about 4.8% in the blood vessels and 1.1% in the brain, which is in good agreement with known plasma to brain glucose ratio. In the presence of rapamycin treatment, the increase in the overall brain level remained, which we interpret as a reduction in metabolism. The elevated glucose level (2.9%) in the tumor region is a result of BBB disruption. When comparing with the DCE AUC image, it is evident that the DGE AUC image shows a greater area of enhancement beyond the tumor boundary defined by DCE and anatomical images. This extended area of enhancement might be a result of minor BBB leakage that makes the brain permeable for **D**-glucose but not for Gd contrast agent. The faster diffusion rate of glucose compared to Gd chelates may also contribute to the extended enhancement.

After six minutes post injection, a steady state seems to be established in the inhibitor treatment group, where the glucose equilibrates between the vascular space and the *EES*. It is clear that the glucoCEST contrast in the tumor and the contralateral brain of the treatment group did not go down to the pre-injection level within the observation period (Fig. 3a). We interpret this as the treatment group being less active in glucose utilization. The glucose

retention is further validated by the MRS results where high remaining glucose signal was observed in the difference spectrum (Fig. 6). Interestingly, the glucoCEST contrast in the tumor is comparable to (no significant difference found from two-sample t-test) that in the vessel for both the treatment and no-treatment group at 17 min post glucose injection (Fig. 3). From five minutes onwards after the glucose injection, the difference between the treatment and the no-treatment group becomes more apparent. The signal in the blood vessels in the inhibitor treatment group decreases at a slower rate indicating that more glucose is retained in the blood. This later part of the dynamic curve reflects the glucose utilization and the higher retention is consistent with lower overall glucose consumption in the mouse (note that the blood reflects the result of whole-body metabolism) over the measurement time period. Wei et al. suggested this is attributed to the inhibitor treatment reducing the glucose transporter (GLUT1) activity(23), resulting in a reduced glucose uptake that is not limited to the brain but globally in the mouse. The pronounced increase in glucoCEST contrast after the inhibitor treatment, especially in the tumor is attributed to an accumulation of **D**-glucose in the EES. Tumor has high vascular volume fraction and poor drainage, after treatment its glucose utilization is hampered, thus glucose concentration remains high in the vasculature and EES. This observation is further supported from the glucoCEST contrast from 6 min onwards; the glucoCEST contrast in the contralateral brain with treatment was maintained at around 1% while that in the no-treatment group went back to the baseline. This is similar to the observation in the brain of healthy mice. We also found that after the first pass of glucose, there was a gradual increase in glucoCEST contrast in the treatment group in both the tumor and the contralateral brain. This may indicate the accumulation of glucose after treatment.

In addition, an observation is that the signal increase in blood is higher in the tumor even though the glucose concentration is equal in blood and tumor EES. The reason is that the volume of tumor vasculature plus EES is generally about 15–35% of the tumor. So, while the concentration of **D**-glucose in EES is expected to be similar to that in blood, the MRI effect on the voxel level is lower due to the fact that negligible **D**-glucose signal is present in the tumor cells.

Finally, we ascribe the observed DGE response to the rapamycin treatment group mainly to higher glucose concentrations in vasculature and EES as a result of reduced glucose uptake in the tumor cells. This could assist in evaluating tumor responses to the mTOR inhibitor treatments.

Conclusions

GlucoCEST imaging of a glioma xenograft model enables the detection of glucose uptake in brain tumors, which can be readily separated from that of normal brain. Together with previous studies in breast tumors and colorectal tumors, these results show the potential of glucoCEST imaging as a practical approach for cancer glucose uptake detection.

The inhibitor rapamycin treated glioblastoma model provided some insight into the source of the glucoCEST contrast and has demonstrated its potential to monitor tumor responses to the inhibitor treatment. Comparing two groups of mice implanted with human brain tumors

with and without inhibitor treatment, a significant difference in glucoCEST contrast was seen. The stronger signal (close to doubling) observed in the inhibitor treatment group and the fact that the tumor signal is similar to the blood signal provides evidence that glucoCEST signal originates mainly from the EES and blood vessels.

The glucoCEST study using dynamic glucose-enhanced and the z-spectra reveals the permeability, uptake, retention and utilization of glucose in tumors. These parameters provide an overview about the tumor physiology and metabolism, especially to monitor the treatment response in brain tumor via imaging.

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Fig 1.

(a) The dynamic glucose images of a healthy mouse pre and post inhibitor treatment, each image is an average of 6 images acquired within one minute. This mouse represents the largest signal change post treatment among all mice. The start and completion time of infusion is labeled on the image; (b) DGE signal as a function of infusion time in the brain tissue and; (c) in the blood vessels.

Fig 2.

The anatomical (a), post-GdT1w (b), DGE- $AUC_{initial}^{5min}$ calculated at 1.2ppm (c) and DCE- $AUC_{initial}^{5min}$ (d) images of one mouse after inhibitor treatment. Both the DGE and DCE AUC images were calculated by integrating the images acquired in the first 5 min after the start of glucose infusion. (e) The DGE dynamic signal in a blood vessel, the tumor and the contralateral brain region. The yellow arrows in (c) indicate the blood vessels from which the dynamic signal was extracted.

Fig 3.

The DGE dynamic difference signal (mean $\pm$ SD, N=4) for the inhibitor and no-treatment groups in the tumor (a), contralateral brain region (b) and blood vessel (c). The signals of the tumor and contralateral brain regions for the no-treatment group were taken from our previous work(29).

Fig 4.

The time resolved $AUC_{dynamic}^{3min}$ values taken every 3 min (except the last period) for the inhibitor and no-treatment groups in the tumor (a), the contralateral brain region (b), and the blood vessels (c) showing a significantly higher signal in the inhibitor group (*, $P<0.05$; **, $P<0.001$).

Fig 5.

The z-spectra (a), difference spectra (b), and the MTR_{asym} (c) pre- and post- glucose injection of the mice ($n = 3$) with inhibitor treatment. (Left) The tumor; (middle) contralateral brain; and (right) blood vessel.

Fig 6.
The localized in vivo magnetic resonance spectra of the brain tumor in three mice with inhibitor treatment pre- and post- glucose infusion as well as their difference spectrum.