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Everolimus decreases [U-13C]glucose utilization by pyruvate carboxylase in breast cancer cells in vitro and in vivo

Gerke Ariaans a,1, Jiske F. Tiersma a,1, Bernardus Evers b, Albert Gerding b, Stijn J.H. Waaijer a, Remco A. Koster c, Daan J. Touw c, Barbara M. Bakker b, Dirk-Jan Reijngoud b, Steven de Jong a,1,2, Mathilde Jalving b,1,2

a Department of Medical Oncology, University of Groningen, University Medical Center Groningen, Groningen, the Netherlands
b Department of Pediatrics, Center for Liver, Digestive and Metabolic Diseases, University of Groningen, University Medical Center Groningen, Groningen, the Netherlands
c Department of Clinical Pharmacy and Pharmacology, Laboratory for Clinical and Forensic Toxicology and Drugs Analysis, University of Groningen, University Medical Center Groningen, Groningen, the Netherlands

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ABSTRACT

Reprogrammed metabolism is a hallmark of cancer, but notoriously difficult to target due to metabolic plasticity, especially in response to single metabolic interventions. Combining mTOR inhibitor everolimus and mitochondrial complex 1 inhibitor metformin results in metabolic synergy in in vitro models of triple-negative breast cancer. Here, we investigated whether the effect of this drug combination on tumor size is reflected in changes in tumor metabolism using [U-13C]glucose labeling in an MDA-MB-231 triple negative breast cancer xenograft model. The in vitro effects of everolimus and metformin treatment on oxidative phosphorylation and glycolysis reflected changes in 13C-labeling of metabolites in MDA-MB-231 cells. Treatment of MDA-MB-231 xenografts in SCID/Beige mice with everolimus resulted in slower tumor growth and reduced tumor size and tumor viability by 35%. Metformin treatment moderately inhibited tumor growth but did not enhance everolimus-induced effects. High serum levels of everolimus were reached, whereas levels of metformin were relatively low. Everolimus decreased TCA cycle metabolite labeling and inhibited pyruvate carboxylase activity. Metformin only caused a mild reduction in glycolytic metabolite labeling and did not affect pyruvate carboxylase activity or TCA cycle metabolite labeling. In conclusion, treatment with everolimus, but not metformin, decreased TCA cycle and inhibited pyruvate carboxylase activity. By using in-depth analysis of drug-induced changes in glucose metabolism in combination with measurement of drug levels in tumor and plasma, effects of metabolically targeted drugs can be explained, and novel targets can be identified.

1. Introduction

Breast cancer is the second most common cause of cancer-related death in women in western countries and triple-negative breast cancer (TNBC) has the poorest prognosis of all subtypes [1]. TNBC accounts for 10–20% of diagnosed breast cancers and is defined by the lack of expression of estrogen receptor (ER), progesterone receptor (PR) and human epidermal growth factor receptor 2 (HER2) [2]. Although conventional chemotherapy regimens can induce temporary responses, in advanced disease resistance rapidly develops. Although drugs targeting poly(ADP-ribose) polymerase (PARP) in BRCA mutated tumors and anti-body drug conjugates targeting HER2 low cancers are effective for defined TNBC subgroups [3–5], TNBC is generally characterized by a lack of targetable mutations and, so far, a disappointing lack of sensitivity to immune checkpoint inhibitors [6,7]. Evidence is emerging that reprogrammed metabolism is important in TNBC, potentially driven by the PI3K/AKT/mTOR pathway which is hyperactive in TNBC and involved in both survival and chemoresistance [8].

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Targeting the mTOR pathway is therefore of interest in TNBC. Inhibitors of mTOR reduce growth factor signaling and mitochondrial respiration by interfering with mTOR complex 1 (mTORC1) and/or mTOR complex 2 (mTORC2). The mTORC1 inhibitor everolimus is approved for treatment of ER-positive breast cancer in combination with the aromatase inhibitor letrozol [9]. However, clinical trials adding everolimus to various standard treatment regimes in TNBC showed only limited success at the cost of increased toxicity [10–12].

Metformin is the most widely prescribed diabetes drug. In epidemiological and retrospective studies, metformin use was associated with reduced breast cancer risk and improved response to neoadjuvant chemotherapy [13]. Metformin lowers blood glucose levels through reduction of gluconeogenesis in the liver and by increasing glucose uptake in skeletal muscle, thus reducing insulin resistance. The anti-cancer effects may be due to these systemic alterations in combination with anti-mitotic activity in tumor cells due to metformin-induced inhibition of mitochondrial complex 1 and activation of AMPK resulting in inhibition of the mTOR-signaling pathway [14]. Previously, we demonstrated direct anti-proliferative effects of metformin and everolimus on both luminal A (ER+ or PR+ and HER2-) breast cancer cells and TNBC cells in vitro [15]. Metformin also potentiated the effect of everolimus through metabolic synergy. It is unknown whether this phenomenon is conserved in vivo.

Here, we first investigated whether metabolic synergy caused by treatment with metformin and everolimus on TNBC cells in vitro can be detected using [U-13C]glucose tracing via GC/MS-based metabolomics. Then, we investigated whether adding metformin to everolimus treatment in a TNBC xenograft model in mice was more effective than everolimus alone. Furthermore, we applied in vivo [U-13C]glucose tracing to detect whether effects of metformin and/or everolimus treatment on xenograft metabolism reflected treatment efficacy.

2. Materials and methods

2.1. Reagents and cell culture

Everolimus was obtained from LC Laboratories (Woburn, MA, USA, E-4040). Everolimus was dissolved in DMSO and stored at −20°C. Prior to in vivo use it was formulated in a stable suspension with 1 x PBS containing 0.5% Tween-80 (Sigma Aldrich, Zwijndrecht, the Netherlands). Metformin was obtained from Sigma Aldrich (D150959). D-glucose (U-13C6, 99%) was purchased from Cambridge Isotope Laboratories (Tewksbury, MA, USA). For in vitro experiments, stock concentrations of everolimus were first dissolved in PBS and subsequently diluted to a final concentration of 10 nM in the culture medium. Metformin was dissolved in PBS and added to the cell culture medium at 0.005 or 5 mM. The TNBC cell line MDA-MB-231 was acquired from American Type Culture Collection (ATCC, Manassas, VA, USA). Cells were cultured in DMEM (Gibco by Thermo Scientific, Bleiswijk, the Netherlands; 11965092 mixed with 11966025 to yield an 11 mM glucose concentration) supplemented with 1 mM additional glutamine and 10% fetal calf serum (FCS).

2.2. Western blot analysis

MDA-MB-231 cells were lysed in MPER (Thermo Scientific) and diluted 1:1 with SDS sample buffer (4% SDS, 20% glycerol, 0.5 mol/L Tris-HCl (pH 6.8), 0.002% bromophenol blue). Lysates were resolved by SDS-PAGE and transferred to PVDF membranes. Membranes were incubated overnight at 4°C and probed with the following antibodies: rabbit-anti-S6, rabbit-anti-pS6, (Cell Signaling Technologies, Leiden, the Netherlands) in a 1:1000 dilution or mouse-anti-actin (MP Biomedicals, Santa Ana, USA) in a 1:10,000 dilution. Primary antibodies were tagged using HRP-coupled goat anti-rabbit or rabbit anti-mouse IgG and visualized with Lumi-Light (Roche, Almere, the Netherlands). Images were captured with the ChemiDoc MP imaging system (Bio-Rad, Veenendaal, the Netherlands) and Image Lab Software.

2.3. Seahorse analysis

Preparation of MDA-MB-231 cells for Seahorse XF measurements has been described elsewhere [15]. In short, MDA-MB-231 cells were seeded at an appropriate density in specialized V7 Seahorse tissue culture plates. After 2 days cells were treated with indicated concentrations of metformin, everolimus or a combination and incubated for another 2 days. On day 4, a mitochondrial stress test using ATP-synthase inhibitor oligomycin, the mitochondrial uncoupler carbonyl cyanide 4-(tri-fluoromethoxy)phenylhydrazide (FCCP) the complex I inhibitor rotenone and the cytochrome C reductase inhibitor antimycin A (all Sigma-Aldrich) was carried out according to the manufacturer’s protocol. Oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) were normalized for the amount of cellular protein in each well determined by Bradford assay.

2.4. Breast cancer xenograft model

All animal experiments were approved by the Institutional Animal Care and Use Committee of the University of Groningen (Groningen, the Netherlands, 6929A). Animal studies were conducted in 8-week-old female SCID/Beige mice (Harlan) of around 18 g at the start of the experiment. A priori power analysis was performed with an estimated effect size of 0.5, yielding a sample size of 6 animals. All animals (n = 26 in total) were housed under a controlled light–dark regime (12 h dark and 12 h light) and temperature-controlled (20°C) conditions. During tumor growth, animals were fed ad libitum with standard chow.

As a pilot, untreated, naive mice (n = 2) received an intraperitoneal (IP) injection with [U-13C]glucose (2 mg/g bodyweight) and 13C label enrichment was measured between 5 and 120 minutes after injection. An approximation of the steady-state was reached at 50% molar percent excess (MPE) after 30 minutes (Figure S1). For all mice, the tip of the tail was clipped, and a blood sample was spotted on filter paper to determine 13C label enrichment. Blood spots were air-dried and stored at room temperature until analysis. Isolation of glucose from blood spots was done as described earlier [16].

Under isoflurane anesthesia, mice (n = 24) were xenografted subcutaneously in each flank with 5 x 106 MDA-MB-231 cells suspended in 0.3 mL of a 1:1 mixture of PBS and Matrigel (BD Biosciences, Franklin Lakes, NJ, USA). The day before treatment commenced, mice were placed in individual cages to minimize confounding effects and ensure metabolic uniformity and mice were randomized into four groups of six animals based on individual tumor volume. The four groups consisted of a) mice treated with saline as a control group, b) mice treated with metformin or c) everolimus alone and d) mice treated with both drugs.

Treatment for 14 days with metformin, everolimus or the combination was started 19 days after inoculation, when individual tumors had a size of ~350 mm3. Mice received 10 mg/kg everolimus daily for 14 days via IP injection, as described previously [17]. Metformin was dissolved in PBS and stored at −20°C until use. Mice received 250 mg/kg metformin daily for 14 days via IP injection, as described earlier [18]. Mice treated with both drugs received injections with both drugs daily. A priori, humane end point leading to premature sacrifice and exclusion of animals were set in case of tumor ulceration or total tumor volume of ≥ 1500 mm3.

One day before sacrifice, mice were fasted from 11 pm to 8 am the next day with unlimited access to water. Five mice from each group received an IP injection with [U-13C]glucose (2 mg/g bodyweight). The remaining single mice from the saline, everolimus and combination group served as a correction group and were injected with unlabeled glucose at the same concentration as used for the [U-13C]glucose injections. The tissue of these mice was used to correct for natural abundance of glucose according to Evers et al. [19]. The metformin-treated
mouse was excluded from the analysis, since it already reached its humane endpoint on day 6 of the treatment. Thirty minutes after injection of uniformly labeled glucose all mice were sacrificed by heart puncture under inhalational isoflurane anesthesia. Blood was collected via heart puncture in K3-EDTA tubes (41.1504.005, Sarstedt, Nümbrecht, Germany) and centrifuged at 8000 rpm for 10 min at 4°C to separate blood cells from plasma. Plasma was stored at −80°C. One of the two tumors, the liver and a femoral muscle of each mouse were freeze-clamped and temporarily stored on dry ice. Samples were then stored at −80°C until analysis. The other tumor was fixed in formaldehyde and paraffin-embedded according to standard protocols.

The following parameters were assessed: tumor volume, which was the primary outcome measure, tumor weight, tumor viability, expression of pS6, Ki67 and cleaved caspase 3 in tumor tissue, everolimus and metformin levels in plasma and tumor tissue, and 13C enrichment in metabolites in tumor tissue. Analysis of immunohistochemistry results, everolimus and metformin levels in plasma and tumor tissue, and 13C enrichment in tumor tissue was blinded. The ARRIVE checklist was used when writing this manuscript [20].

2.5. Immunohistochemistry

Four μm slices were cut from paraffin-embedded tumors using a microtome and placed on coated glass slides. Hematoxylin-eosin staining was performed to determine viability and morphology of tumor samples. Heat-induced antigen retrieval was conducted in 10 mM citrate buffer 400 W rotary microwave for 15 min. Incubation with 0.3% H2O2 in PBS for 30 minutes blocked endogenous peroxidase activity. Endogenous avidin/biotin activity was blocked using a commercially available blocking kit (Vector Laboratories, Burlingame, CA, USA). Slices were incubated overnight with primary antibody directed against pS6 (Cell Signaling Technologies, cat. no. 2211), cleaved caspase 3 (Cell Signaling Technologies, cat. no. 9661) or Ki67 (Abcam, Cambridge, UK, cat. no. ab136912). PBS and IgG incubated slides served as a control for specificity of antibody binding. After staining with a secondary biotinylated antibody and a tertiary avidin and peroxidase coupled antibody staining was visualized using D-3,3’-diaminobenzidine (DAB). Hematoxylin staining was visualized as counterstaining. Scoring of hematoxylin-eosin-stained slides for cell death was done by quantifying the percentage of tissue with condensed nuclei blindly by two independent researchers. Quantification was done by manual annotation using ndp.view2 software (Hamamatsu Photonics, Shizuka, Japan). Ki67-stainings were quantified by taking the average of positive and negative nuclei counts in 10 equal sections throughout the whole slide.

2.6. Quantification of metformin and everolimus concentrations

Everolimus and metformin were quantified in plasma and in tissue homogenate using a liquid chromatography-tandem mass spectrometry (LC-MS/MS) method. Plasma and tissue were stored at −80°C for further analysis. Tissue samples were homogenized to a fine powder at liquid nitrogen temperature using mortar and pestle. The liquid everolimus plasma and tissue homogenate samples were processed and analyzed according to the method published by Koster et al. [21]. For all metformin specimens, samples were processed and analyzed according to the method published by Posma et al. [22].

2.7. Determination of isotopologue distribution in cell lysates and tumor samples using GC/MS

For in vitro labeling, MDA-MB-231 cells were plated and treated with indicated concentrations of metformin, everolimus or a combination. Glucose metabolite labeling was done for 30 min in DMEM-medium containing a 1:1 ratio of [U-13C]glucose and unlabeled glucose (in total 11 mM). Cells were washed twice with ice-cold PBS, resuspended in 1 mL PBS and further processed using 1 mL methanol and 2 mL chloroform and stored at −20°C until sample analysis. The rest of the procedure was the same as for tumor samples indicated below.

Frozen tumor samples were weighed and ground in a mortar. Liquid nitrogen was added to the mortar to keep the samples frozen. Using 200 mg of pulverized tissue, a 15% homogenate in PBS (w/v) was made. Homogenization in a Precells Evolution homogenizer (Bertin Technologies, Montigny-le-Bretonneux, France) was done by vortexing with 0.1 mm zirconia/silica beads (BioSpec Products, Bartlesville, OK, USA) in 2.0 mL screw cap tubes from BIORACKS (Landgraaf, the Netherlands). Tumor sample preparation was done according to the Bligh and Dyer technique [23]. A mixture of 1 mL methanol and 2 mL chloroform was subsequently added to 1 mL of tumor homogenate and samples were vortexed for 30 min at 4°C. After centrifugation at 1258 × g for 10 min, upper and lower phase were separated. The upper polar phase was dried in a constant flow of nitrogen at 37°C. Dried polar metabolites (organic acids and amino acids) were dissolved in 40 μL 2% methoxyamine hydrochloride (MOX) in pyridine and incubated for 90 min at 37°C and subsequently cooled down. N-tert-Butyldimethylsilyl-N-methyltrifluoroacetamide with 1% tert-Butyldimethylchlorosilane (MTBSTFA + 1% TDBMCS), in total 60 μL, was added to samples and derivatized at 55°C for 1 h. Mass isotopologue distributions were measured using an Agilent 7890 A GC coupled to an Agilent 5975 series GC/MS (Agilent Technologies, Santa Clara, CA, USA) using an Agilent DB-5 MS column (cat. no. 122-1932) in 1.5 mL GC/MS vials with 0.2 mL micro-inserts and Teflon septum screw caps (APG Pharma, Uithoorn, the Netherlands). Agilent MassHunter software was used for peak integration. The normalized mass isotopologue spectra measured by GC/MS were corrected for natural abundance of stable isotopes by multiple linear regression as described by Evers et al. [19].

2.8. Statistics

Data are presented as mean ± standard error of the mean (SEM). Different experimental conditions were compared using unpaired Student’s t-tests, two-way ANOVA with multiple comparisons or mixed effects model analysis as indicated in the figure legends, after testing for normal distribution using the Shapiro-Wilk test. Statistical analyses were performed using Prism 8 (GraphPad). A P-value of < 0.05 (two-tailed) was considered significant.

3. Results

3.1. In vitro characterization of the metabolic response to everolimus and metformin

We previously demonstrated growth inhibitory and metabolic effects of everolimus (10 nM) combined with high concentrations of metformin (5 mM and higher) in MDA-MB-231 cells [15]. In patients, however, metformin trough serum levels are 100–1000-fold lower [24,25]. To determine whether metformin can induce metabolic changes at clinically relevant concentrations, metformin was therefore also used at a concentration of 0.005 mM. Everolimus alone reduced phosphorylation of S6, and this effect was maintained in combination with low dose metformin (Fig. 1A, Fig. S2). Metformin resulted in reduced S6 phosphorylation (pS6) at the 5 mM but not the 0.005 mM dose. No effect on the oxygen consumption rate (OCR) was observed with everolimus or 0.005 mM metformin alone. However, everolimus combined with low dose metformin showed a trend towards decreased OCR and a significant reduction in the OCR/ECAR ratio (Fig. 1B). The decrease in OCR with 5 mM metformin was accompanied by a strong decrease in the OCR/ECAR ratio (Fig. 1B).

In short, though metformin at a clinically relevant dose did not affect the metabolic phenotype, it potentiated the effect of everolimus on the OCR/ECAR ratio.

To trace glucose utilization in vitro, MDA-MB-231 cells were labeled with a 1:1 ratio of [U-13C]glucose and unlabeled glucose for 30 min and...
the percentage of labeled glucose-derived metabolites was calculated (Fig. 1C). Around 40% of cytoplasmic metabolites pyruvate and lactate were M+3-labeled in the saline-treated group, indicating that these metabolites are in isotopic equilibrium. M+3 labeling of alanine (25–35% of alanine labeled) was lower than that of pyruvate and lactate. Less than 5% of aspartate, malate, α-ketoglutarate (α-KG), the mitochondrial activity marker succinate and the alternative TCA-cycle fuel glutamate were M+2-labeled. Fumarate labeling (M+2) was very low, even compared to the other TCA intermediates. Glutamine, the precursor of glutamate, was not labeled at all, likely due to the presence of ample glutamine in the medium, thereby eliminating the need to convert glutamate into glutamine. Also, glutamine production of MDA-MB-231 cells is negligible due to low expression of glutaminase [26]. M+3 labeling of malate and aspartate was much higher than M+2 labeling.
suggesting pyruvate carboxylase (PC) activity in these cells (Fig. 1D, Fig. 2). A comprehensive overview of all isotopologues can be found in Figure S3.

Labeling analysis of everolimus-treated cells revealed a trend towards increased labeling of α-KG (p=0.08), and reduced labeling of lactate and alanine, consistent with a partial switch from glycolysis towards a more oxidative phenotype. Addition of low-dose metformin to everolimus slightly but significantly potentiated the reduction of pyruvate labeling by everolimus. Interestingly, everolimus, alone or in combination with low-dose metformin, reduced M+3 labeling of malate and aspartate, suggesting that everolimus treatment reduces PC activity (Fig. 1D).

A low dose of metformin had little effect on metabolite labeling, consistent with the lack of effect on OCR and ECAR. Metformin at 5 mM reduced labeling of the mitochondria-related metabolites citrate, glutamate, and succinate, but also of malate and aspartate, corresponding to the decreased OCR/ECAR ratio. Detailed analysis of the isotopologues indicated that the high dose of metformin decreased the M+2 isotopologue abundance in malate and aspartate, whereas M+3 isotopologue abundance was similar to the control (Fig. 1D, Figure S3). This suggests that metformin decreases TCA cycle activity but does not affect the conversion of pyruvate to oxaloacetate by PC (Fig. 2).

In short, everolimus led to a clear reduction in M+3 label of malate and aspartate, suggesting that everolimus reduced PC activity. In contrast, metformin did not affect M+3 isotopologues of malate and aspartate, indicating that metformin did not affect PC activity. Furthermore, the clinically achievable concentration of 0.005 mM metformin potentiated the effect of everolimus in vitro, supporting further in vivo evaluation of the combination.

3.2. Effects of everolimus and metformin on tumor growth in vivo

To determine the effect of everolimus and metformin in vivo, MDA-MB-231 xenograft bearing SCID/beige mice were treated with everolimus, metformin or the combination for 14 days. Both everolimus and everolimus-metformin treated groups showed a clear reduction in tumor growth compared to control mice, whereas metformin treatment moderately reduced tumor growth (Fig. 3A). Effect sizes ranged from 0.53 for metformin and 0.74 and 0.86 for everolimus and the combination, respectively. Tumor weights at the end of the 14-day treatment regime were lower in the everolimus-treated group as compared to the control (Fig. 3B-C). Tumors from the metformin-treated group had a more heterogeneous tumor volume as compared to all other groups (Fig. 3D). Saline-treated tumors contained ~30% non-vital regions, whereas tumors from everolimus-treated mice had a higher percentage of non-vital regions (up to 80%). To determine the effect of everolimus and metformin on the mTOR signaling pathway, immunohistochemical staining for pS6 was performed (Fig. 3E). Positive pS6 staining was especially found in the viable border of the tumors and in the viable regions within the tumors. Non-viable areas were negative for pS6. Ki67 staining was mutually exclusive with cleaved caspase 3 staining, with Ki67 positive staining in the viable tumor areas and cleaved caspase 3 positive staining on the border between viable and necrotic areas. There was no difference in pS6, Ki67 or cleaved caspase 3 staining between treatment groups.

In summary, everolimus decreased tumor growth, size and viability, whereas metformin only mildly inhibited tumor growth.

3.3. Plasma and intra-tumoral concentrations of everolimus and metformin

To further understand the observed drug effects on the MDA-MB-231
xenografts, we determined everolimus and metformin concentrations in mouse plasma and in the tumors (Fig. 4).

Everolimus mean trough plasma concentrations in mice were 1.3 μM (Fig. 4A), which is more than 40-fold higher than trough levels of everolimus found in the blood of cancer patients during everolimus treatment [27]. Everolimus concentrations of up to 8 mg/kg were detected in xenografts 24 hours after the last dose. Assuming a similar density of tumors and plasma, this suggests a tumor retention of approximately 6.7-fold. Everolimus levels in plasma and xenografts were not affected by the co-treatment with metformin (Fig. 4A/B).

Metformin trough plasma concentrations in mice treated with metformin alone or in combination with everolimus were 0.6 μM (± 0.2) and 0.5 μM (± 0.2), respectively, which is at the lower end of the range in trough levels observed in cancer patients [28]. Metformin plasma levels were not influenced by everolimus co-treatment of the mice (Fig. 4A). Tumor metformin concentrations in mice treated with metformin alone or in combination with everolimus were 2.7 mg/kg (± 0.3) and 5.6 mg/kg (± 0.8), respectively, and are in line with tumor concentrations found in cancer patients [28] (Fig. 4B). Metformin tumor concentrations in mice, assuming a similar density of tumors and plasma, were 40- to 80-fold higher (21–43 μM) than plasma levels 24 hours after the last dose (Fig. 4C).

Fig. 3. Effect of metformin and everolimus on MDA-MB-231 tumors in vivo. Normalized MDA-MB-231 tumor growth in female SCID/Beige mice over 14 days of daily IP injection with 250 mg/kg metformin or 10 mg/kg everolimus or combination (A). MDA-MB-231 tumor weight at the end of the study (B). Visualization of tumors after excision (C). Non-viable area in the tumor determined by condensed nuclei on hematoxylin-eosin staining scored blindly by two independent researchers (D). Representative images of Ki67, cleaved caspase 3 and pS6 expression in tumor tissue (E). Data represent mean ± SEM of 4–6 mice. (A) Mixed effects model analysis, * p < 0.05, *** p < 0.001, **** p < 0.0001, treated vs. control. (B,D) Student’s t-test, * p < 0.05, *** p < 0.001, treated vs. control.
In short, everolimus concentrations in vivo were much higher than the effective concentration in our in vitro experiments. Despite retention in the tumor tissues, metformin concentration levels in the xenografts were very low compared to effective metformin concentrations in vitro, but similar to the concentrations achieved in patients. Interestingly, co-treatment with everolimus increased tumor levels, but not plasma levels of metformin. These results likely explain the observed efficacy of everolimus and the lack of effect of metformin in the xenograft experiments.

3.4. [U-\textsuperscript{13}C]glucose metabolite labeling of xenografts treated with everolimus and metformin

To study tumor glucose metabolism in vivo, MDA-MB-231 xenograft-carrying mice were given [U-\textsuperscript{13}C]-glucose 30 minutes before being euthanized. Labeling time was based on a pilot study determining the optimal ratio of fully labeled to unlabeled glucose over time in untreated mice (Figure S1). In the main study, the enrichment of M+6 (unmetabolized) [U-\textsuperscript{13}C]glucose was higher in the metformin treated mice compared to the other treatment groups (Figure S4). Metabolite enrichments were quantified using GC/MS (Fig. 5). Lactate labeling in tumor tissues was around 35%. However, only around 20% of its precursor pyruvate was labeled. Alanine, of which pyruvate is also a precursor, showed slightly lower \textsuperscript{13}C-enrichments than pyruvate. The tumor samples contained labeled glutamine, suggesting that the \textsuperscript{13}C-labeled glutamine was produced by murine cells, either by cells in the tumor microenvironment or by cells in the blood. Due to the low enrichment, the labeling of \(\alpha\)-KG could not be measured. All other TCA metabolites were clearly detectable. Pyruvate labeling was increased by everolimus treatment, but not by the combination of everolimus and metformin. Everolimus with or without metformin reduced the labeling of alanine, citrate, glutamate, glutamine, fumarate, malate, and aspartate, indicating a decrease in oxidative metabolism. Although metformin had no effect on tumor size, metformin reduced the labeling of lactate and alanine, indicating a decrease in glycolysis. Interestingly, M+3 isotopologues from the TCA cycle were less abundant compared to M+2 in MDA-MB-231 xenografts than in MDA-MB-231 cells cultured in vitro. Strikingly, PC-mediated M+1 labeling of citrate, malate, aspartate, and fumarate was the second most dominant isotopologue in xenografts (Figure S6D), whereas M+1 labeling of citrate and the other metabolites
in vitro was negligible (Figure S3D). Remarkably, these M+1, M+2 and M+3 isotopologue labels were all decreased after everolimus or combination treatment, indicating that everolimus influenced the TCA cycle as well as PC activity.

In summary, quantification of [U-13C]glucose labeling for detecting metabolic response was feasible in our in vivo model. Metformin reduced labeling of glycolytic metabolites, whereas everolimus decreased labeling of glycolytic as well as TCA cycle metabolites.

4. Discussion

In the present study, we demonstrate that, in contrast to in the in vitro setting, metformin added to everolimus did not have additional effect on MDA-MB-231 growth in vivo at the applied dose. Through [U-13C]glucose labeling and drug concentration analyses in plasma and tumor, we observed differences between the in vitro and in vivo setting that may account for this discrepancy. Metformin at a high concentration inhibited oxidative phosphorylation in MDA-MB-231 cells in vitro, which was reflected in reduced M+2 labeling of TCA metabolites. In vivo, however, almost no effect of metformin was observed on 13C-metabolite labeling, possibly due to the low intratumoral metformin levels reached in vivo. Everolimus, in contrast, reduced PC activity both in vitro and in vivo, assessed as reduced M+1 and M+3 labeling of TCA cycle metabolites. These results demonstrate that in vivo [U-13C]glucose labeling studies can be used to gain insight in drug-induced metabolic changes and putative mechanisms of action.

In vivo experiments with MDA-MB-231 xenografts demonstrated that treatment with everolimus reduced tumor weight and viability. However, no effect of everolimus was observed on S6 phosphorylation, the downstream readout for mTORC1 activity. Since tumors were harvested 24 h after the last dose of everolimus, it is possible that the inhibitory effect of everolimus on mTORC1 signaling was no longer visible either because the affected tumor area was no longer viable or because the effect of everolimus on pS6 is fleeting and therefore no longer visible after 24 h. Compensatory mechanisms after 2 weeks of treatment could also play a role. The in vivo [U-13C]-glucose labeling experiments clearly showed the effect of everolimus on glucose metabolism, suggesting that long-term everolimus treatment had a long-lasting effect on glucose metabolism. The 13C-metabolite analysis revealed that everolimus treatment caused a reduction in TCA-cycle related metabolites but did not influence lactate labeling. In-depth analysis of glucose metabolites showed that everolimus reduced M+2 isotopologue enrichment of TCA cycle intermediates only in vivo, while a decrease in M+3 isotopologues was observed both in the in vitro and in vivo setting.

The main source of M+3 metabolites in the TCA cycle is PC activity. PC can convert pyruvate directly into oxaloacetate, giving rise to M+3 oxaloacetate, which can then be converted to M+3 malate or aspartate [29]. This pathway is considered the most important pathway for...
anaplerosis and has been shown to be essential for MDA-MB-231 cell growth, migration, and \textit{in vitro} invasion ability [30]. Since everolimus reduced M+3, but not M+2 isotopologue levels in the \textit{in vitro} experiment, these results suggest that the drug causes an inhibitory effect on PC activity, either directly or indirectly. The \textit{in vitro} results also suggest an inhibitory effect of everolimus on the TCA cycle as indicated by the reduction in M+2 isotopologues. This might be related to the longer exposure of the xenografts to high concentrations of everolimus relative to the \textit{in vitro} cell cultures. Expression of mTORC1 is required for the production of mitochondrial proteins, which may be how long-term everolimus treatment affects mitochondrial metabolism [31]. In addition, M+1 citrate and other TCA metabolites were elevated in xenografts compared to \textit{in vitro} cell cultures, which was recently also shown by Duan et al. [32]. In their study, they showed that the elevation was due to extensive recycling of endogenous CO$_2$ by PC, leading to labeled M+1 CO$_2$ being incorporated into yet unlabeled TCA metabolites. Here, we showed that M+1 TCA metabolites in vivo were also reduced by everolimus, further supporting our hypothesis that everolimus treatment results in decreased PC activity. Further research is warranted to confirm the importance of the PC activity in vivo, especially since a small molecule inhibitor of PC was recently synthesized [33].

We previously demonstrated that everolimus in combination with metformin was more effective than single drug treatment in MDA-MB-231 cells, albeit using relatively high metformin concentrations [15]. It has been suggested that metformin can be retained in tumor cells due to expression of organic cation transporters (OCTs) [34]. We therefore reasoned that \textit{in vitro} effective concentrations might be achieved in the xenografts. However, the concentrations measured in the tumors in the present study are 2–3 orders of magnitude lower than the effective concentrations in \textit{in vitro}. In addition, metformin did not potentiate the everolimus mediated effects in the xenografts. Since serum and tumor concentrations in mice were measured 24 h after the last injection of metformin, accumulation in the tumors could not be accurately determined. The half-life of metformin is short and to support real accumulation versus longer retention in tumors, peak metformin plasma levels are needed [35,36], but were not measured in this study. Interestingly, our tumor metformin levels correspond with results from a previous study that reported a similar level of uptake of metformin in colon cancer xenografts, even though metformin dosage was 125 mg/kg, half of what was used here. In that study, both tumor and plasma levels were determined 30 minutes after IP injection of metformin, and no specific accumulation in the tumors was detected [37]. The increased level of metformin in tumors of mice that were co-treated with everolimus suggests that everolimus either increases metformin uptake or inhibits its release from tumors. Many transporters have been implicated in metformin uptake including multidrug and toxin extrusion protein 1 (MATE-1) and OCTs [22] and these may well be influenced by everolimus.

It is remarkable that the degree of $^{13}$C-labeling of lactate in the \textit{in vivo} tumors is higher than $^{13}$C-labeling of pyruvate. In theory, both metabolites should be labeled to the same extent because pyruvate and lactate can be rapidly and reversibly converted into one another. The higher amount of lactate labeling suggests a source of $^{13}$C-labeled lactate from outside the tumor with higher enrichment of labeled lactate than within the tumor. This is in line with a study by Hui et al. stating that lactate can be a primary source of carbon for the TCA cycle in mice [38]. The xenograft tumors also contain mouse stromal cells, which contribute to the $^{13}$C-labeling pattern of the analyzed tumor samples. For example, metabolic symbiosis between tumor cells and associated fibroblasts has been described, where the highly glycolytic breast tumor cells secrete lactate, which is then consumed by the surrounding fibroblasts [39]. This line of reasoning might hold true for $^{13}$C-labeled glutamine in the tumors as well, since the MDA-MB-231 cells are known to have low levels of glutamine synthetase, an enzyme involved in the conversion of glutamate into glutamine, and to depend more on glutaminase synthase converting glutamine into glutamate [26]. With current techniques, we are not able to determine to what extent different cell types contribute to the metabolic phenotype of the whole tumor. Limitations of this study include suboptimal everolimus dosing in mice, resulting in plasma and tumor tissue levels that are not comparable to the clinical situation. Also, PC activity should be measured directly to validate our interpretation of the $^{13}$C-labeling results. Furthermore, only relative abundance of $^{13}$C-labeling of metabolites was determined and not the absolute metabolite concentrations. In addition, $^{13}$C-labeling was measured at a single timepoint and only included the well-studied MBA-MB-231 xenograft model to show proof-of-concept. Therefore, these results should be validated in additional tumor models.

In conclusion, our study shows that in-depth analysis of drug-induced changes in glucose metabolism in combination with measuring drug levels in tumor and plasma offer new insights in the effect of metabolic targeting in breast cancer. This method also offered insights into the differential effects of everolimus and metformin on PC, which is an essential enzyme in TNBC cells. $^{13}$C-labeling showed clear differences between the \textit{in vitro} and \textit{in vivo} setting. Further investigation into the rational combination of metabolic drugs for the treatment of TNBC based on \textit{in vivo} labeling studies is warranted.

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CRediT authorship contribution statement

Gerke Ariaans: Conceptualization, Formal analysis, Investigation, Writing – original draft. Jiske F. Tiersma: Validation, Formal analysis, Investigation, Writing – original draft. Bernardus Evers: Investigation, Writing – review & editing. Albert Gerding: Investigation, Writing – review & editing. Stijn J.H. Waaijer: Investigation, Writing – review & editing. Remco A. Koster: Investigation, Writing – review & editing. Daan J. Touw: Resources, Writing – review & editing. Barbara M. Bakker: Conceptualization, Writing – review & editing. Dirk-Jan Reijingoud: Conceptualization, Writing – review & editing. Steven de Jong: Conceptualization, Writing – review & editing, Supervision. Mathilde Jalving: Conceptualization, Writing – review & editing, Supervision.

Declaration of Competing Interest

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Appendix A. Supporting information

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