Reprogramming Metabolism with Metformin Improves Tumor Oxygenation and Radiotherapy Response

Vanessa E. Zannella¹,²,³, Alan Dal Pra¹,³,⁴,⁵, Hala Muaddi¹,⁵,⁶, Trevor D. McKee¹, Shawn Stapleton¹,⁶, Jenna Sykes¹, Rachel Glicksman³,⁴, Selim Chaib¹,⁷, Paul Zamiara¹,², Michael Milosevic¹,²,³,⁴, Bradly G. Wouters¹,⁴,⁶,⁷, Robert G. Bristow¹,²,³,⁵, Mariianne Koritzinsky¹,²,⁴,⁵*

¹Princess Margaret Cancer Centre, University Health Network, Toronto, ON, Canada.
2Institute of Medical Science, University of Toronto, Canada.
3Radiation Medicine Program, Princess Margaret Cancer Centre, University Health Network, Toronto, ON, Canada
4Department of Radiation Oncology, University of Toronto, Canada.
5Faculty of Medicine, University of Toronto, Canada.
6Department of Medical Biophysics, University of Toronto, Canada.
7Department of Radiation Oncology (Maastro Lab), GROW School for Oncology & Developmental Biology, Maastricht University, Maastricht, The Netherlands.
8Selective Therapies Program, Ontario Institute for Cancer Research, Toronto, ON, Canada.

These first authors contributed equally
These senior authors contributed equally

*Corresponding author: Dr. Mariianne Koritzinsky
610 University Avenue, Rm 10-628
Toronto M5G 2M9, ON, Canada
Phone: +1-416-581-7841, Fax: +1-416-946-2984
Email: mkoritzi@uhnresearch.ca / rob.bristow@rmp.uhn.on.ca
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Statement of Translational Relevance

In this study, we demonstrate that metformin can decrease tumor hypoxia and consequently increase radiation response. This provides a scientific rationale for combining metformin with radiotherapy in the clinic. Metformin is commonly prescribed as first line of treatment for patients with type 2 diabetes. It is generally well tolerated and already taken by numerous diabetic patients undergoing radiotherapy for cancer. Introduction of metformin to non-diabetic patients receiving radiation treatment is therefore of high feasibility, and could counteract tumor hypoxia that currently limits treatment efficacy. Identification of a novel mechanism by which metformin can improve radiation response will guide future biomarker evaluation and patient selection.
Abstract

Purpose: Tumor hypoxia is a negative prognostic factor in multiple cancers, due in part to its role in causing resistance to radiotherapy. Hypoxia arises in tumor regions distal to blood vessels as oxygen is consumed by more proximal tumor cells. Reducing the rate of oxygen consumption is therefore a potential strategy to reduce tumor hypoxia. We hypothesized that the anti-diabetic drug metformin, which reduces oxygen consumption through inhibition of mitochondrial complex I, would improve radiation response by increasing tumor oxygenation.

Experimental Design: Tumor hypoxia was measured in xenografts before and after metformin treatment using 2-nitroimidazole hypoxia markers quantified by immunohistochemistry (IHC), flow cytometry and positron emission tomography (PET)-imaging. Radiation response was determined by tumor growth delay and clonogenic survival in xenografts with and without administration of metformin. The impact of metformin use on outcome was assessed in 504 localized prostate cancer patients treated with curative-intent, image-guided radiotherapy (IGRT) from 1996 to 2012. Three-year biochemical relapse-free rates were assessed using the Kaplan-Meier method.

Results: Metformin treatment significantly improved tumor oxygenation in two xenograft models as measured by IHC, flow cytometry and PET imaging. Metformin also led to improved radiotherapy responses when mice were administered metformin immediately prior to irradiation. Clinically, metformin use was associated with an independent and significant decrease in early biochemical relapse rates (p=0.0106).

Conclusion: Our data demonstrate that metformin can improve tumor oxygenation and response to radiotherapy. Our study suggests that metformin may represent an effective and inexpensive means to improve radiotherapy outcome with an optimal therapeutic ratio.
**Introduction**

The presence of tumor hypoxia is a negative prognostic factor in tumors treated with curative intent by radiotherapy (RT) across multiple sites, including squamous cell carcinoma of the head and neck (1) and prostate carcinoma (2). Tumor cells located further than ~150µm from vessels experience ‘diffusion-limited’ hypoxia as oxygen is consumed by the tumor cells more proximal to the vessels (3). Severely hypoxic yet viable cells are well documented to surround necrotic volumes (4), and these hypoxic cells are resistant to ionizing radiation (IR) (3). Several randomized clinical trials have shown the potential for improving RT efficacy by modifying the tumor microenvironment (5, 6). In these trials, attempts to increase tumor oxygenation prior to therapy were made using hyperbaric oxygen or an oxygen-rich gas like carbogen (95% O₂, 5% CO₂) in combination with systemic administration of vasodilating agents. In spite of positive results (5, 6), these strategies have not gained clinical traction due to practical limitations, toxicity and relatively modest clinical benefit (7).

An alternative strategy to achieve improved tumor oxygenation is to decrease cellular oxygen consumption; a rational choice given that oxygen gradients arise from tumor cell respiration. Mathematical modeling suggests that decreasing oxygen consumption could be significantly more efficient at improving tumor oxygenation than increasing oxygen supply (8). There is therefore ample rationale for combining pharmaceutical agents that decrease oxygen consumption with curative RT. This strategy has been explored *in vitro*, where a variety of respiration inhibitors have been shown to radiosensitize 3-D spheroid models that contain viable hypoxic radioresistant cells in their interior (9-12). The respiration inhibitors metaiodobenzylguanadine and arsenic trioxide have also been shown to increase radiation response in
experimental tumor models (13, 14), but these agents are infrequently prescribed and may be associated with toxicities when combined with RT.

Metformin is a commonly-prescribed biguanide used as a first-line treatment for type II diabetes. It efficiently and safely lowers blood glucose levels, and subsequently insulin, by inhibiting liver gluconeogenesis. Metformin’s primary mechanism of action is considered to be direct inhibition of complex I activity in the mitochondrial electron transport chain (ETC)(15-17). The ETC provides cellular ATP by transferring electrons from substrates provided by the citric acid cycle to molecular oxygen. Given that metformin is a well-tolerated drug already consumed by millions of diabetic patients undergoing RT, we hypothesized that metformin’s activity as a respiration inhibitor could lead to increases in tumor RT response through inhibition of tumor cell oxygen consumption and improved tumor oxygenation. This would constitute a novel mechanism in which tumor radioresponse is improved secondary to metabolic remodeling, rather than a change in the intrinsic radiosensitivity of the tumor cell.

**Methods**

*Cell lines and culture*

LNCaP (CRL-1740™) prostate carcinoma and HCT116 (CCL-247™) colorectal carcinoma cells were from ATCC. The SCC-74B cell line was a generous gift from Dr. R. Grenman at Turku University Hospital (Finland), established from a squamous cell carcinoma of the tongue metastasized to a neck lymph node. The POP-092S cell line was created at Princess Margaret Cancer Center from a primary xenograft established from an adenocarcinoma of the sigmoid colon. LNCaP, HCT116 and SCC-74B cells were grown as adherent monolayers and
kept in exponential growth phase. LNCaP and HCT116 cells were kept in RPMI and SCC-74B in MEM:F15, all supplemented with 10% fetal bovine serum (FBS) (all Gibco). POP-092S cells were grown in suspension in growth media to enrich for stem cells (18). Oxygen consumption was measured using a Seahorse XF96 Extracellular Flux Bioanalyzer (Seahorse Bioscience).

**Irradiation and clonogenic survival**

Cells were irradiated using a 137Cs Nordion Gammacell unit at a dose rate of 1.03Gy/min. Metformin was present for 30 minutes before, and 1 hour after, irradiation. Cells were then trypsinized and single-cells plated for clonogenic survival at low density in triplicate. After 14 days, colonies were fixed, stained (0.2% methylene blue in 80% ethanol) and counted if containing more than 50 cells. Surviving fraction was calculated as the number of colonies divided by the number of seeded cells in the treated population, corrected by the same ratio for the untreated.

**Xenograft establishment and growth**

All animal experiments were performed under protocols approved by the Ontario Cancer Institute’s Animal Care Committee, according to the regulations of the Canadian Council on Animal Care. HCT116 and POP-092S donor tumors were grown in the right biceps femoris muscle and under the left renal capsule, respectively. Tumor fragments measuring 2.0 mm were implanted subcutaneously into the right flank of syngeneic mice. SCID mice were used for all experiments except tumor growth delay after irradiation, where we used non-radiosensitive athymic CD-1 nude mice. Tumor growth was monitored by caliper measurements 3 times a week. All surgical procedures and tumor imaging were carried out under anesthesia (3%
isofluorane induction, 1% maintenance). For measurements of oxygen consumption rates, tumor-bearing mice were sacrificed and 23 tumor fragments weighing 7-10mg were immediately placed in individual wells in the Seahorse XF96 Extracellular Flux Bioanalyzer (Seahorse Bioscience).

**Quantification of tumor hypoxia**

All animal experiments were performed under protocols approved by the Ontario Cancer Institute’s Animal Care Committee, according to the regulations of the Canadian Council on Animal Care. Mice carrying 200mm³ HCT116 tumors or 1000mm³ POP-092S tumors were injected i.p. with 120mg/kg pimonidazole (Hydroxyprobe-1, Chemicon International). After 2 hours, mice breathed carbogen (95%O₂, 5%CO₂) or received 100mg/kg metformin i.v.. After 10 minutes, 10mg/kg EF5 (2-(2-Nitro-1H-imidazole-1-yl)-N-(2,2,3,3,3-pentafluoropropyl)acetamide), a kind gift from Dr. C. Koch (University of Pennsylvania) was injected i.p.. 3 hours later, 50mg/kg Hoechst 33342 (Invitrogen) was injected i.v. and mice sacrificed by cervical dislocation. One tumor half was immediately frozen in liquid nitrogen, the other half was processed for flow cytometry.

**Immunohistochemistry**

Tumor sections measuring 5µm were fixed with 2% paraformaldehyde and scanned for Hoechst autofluorescence using an Olympus Fluorescence upright Microscope with Metamorph software. They were stained with FITC/Cy3/Cy5-conjugated antibodies recognizing pimonidazole (Hydroxyprobe, Chemicon International), EF5 (Dr. C. Koch, University of Pennsylvania), and CD31 (BD Pharmingen) respectively and DAPI (Sigma) for DNA. Scans were aligned with ImagePro and analyzed with Definiens TissueStudio™ suite, including only
viable tumor tissue (Figure S1A). A threshold for background was applied to each individual stain signal and subsequently either the average intensity or a binarized fractional positive area (Figure S1B) was calculated.

Flow cytometry

Tumors were disaggregated using DNase I, *Streptomyces griseus* protease and collagenase IX, filtered, fixed and permeabilized using formaldehyde and Triton X-100 (all Sigma). Cells were blocked with 10% skim milk, 1.5% lipid-free albumin, 5% mouse serum and stained with FITC-anti-pimonidazole, Cy5-conjugated antibody against EF5 and DAPI. Flow cytometry was performed on a BD Biosciences LSR II and analyzed with FlowJo (Tree Star Inc.). Only the larger POP-092S tumors yielded sufficient cell numbers for analysis.

$^{18}$F-FAZA-PET imaging and analysis

Hypoxia was assessed using μPET imaging of the hypoxia tracer $^{18}$F-FAZA (fluoroazomycinarabinofuranoside). Mice were administered 100mg/kg metformin i.v., and 30 minutes later injected with ~11MBq of $^{18}$F-FAZA i.v. (Centre for Probe Development and Commercialization). After 140 minutes, were anesthetized using isoflurane, scanned for 20 minutes with the Focus 220 μPET (Siemens Preclinical Solutions), and computed tomography (80 kV, 70 mA, eXplore Ultra, GE Healthcare). Image registration and contouring was performed using Inveon Research Workplace (Siemens). Tumor and muscle tissues were contoured and the % injected dose / gram was exported for each voxel of each tissue and further analyzed in MatLab (Mathworks). A normal distribution was fit to the %ID/g muscle histogram,
defining well-oxygenated tissue. The distribution of tumor voxels was then fit to a function defined as the sum of two normal distributions; the first distribution representing well-oxygenated tissue derived from muscle and the second distribution representing the hypoxic voxels. The hypoxic tumor fraction was defined as the fractional area under the curve of the hypoxic distribution.

Xenograft irradiation and growth delay

Mice bearing 200mm³ HCT116 tumors were injected i.v. with 100mg/kg metformin or saline 30 minutes prior to irradiation. Anesthetized mice underwent a cone-beam CT scan followed by image-guided tumor radiation at 2.92 Gy/min using two beams from XRAD 225. Mice affected by gastro-intestinal toxicity (less than 5%) were excluded from the study. Tumor growth was monitored 3 times a week by caliper measurements.

Ex-vivo clonogenic assays

Mice bearing 200mm³ HCT116 tumors, established by subcutaneous injection of 2x10⁶ cells, were irradiated without anesthesia using a Gammacell. To create completely hypoxic tumors, one group of mice was sacrificed 5 minutes prior to irradiation, while the other group was sacrificed immediately following irradiation. Tumors were excised, dissociated, and colony-forming assays were performed, correcting for viability using trypan-blue exclusion.

Patient cohort and treatment characteristics

This study was approved by the UHN Research Ethics Board (REB# 12-5365-CE). A detailed retrospective chart review was performed of 504 patients treated with curative external
beam radiotherapy for clinical stage T1-T4, N0-X, M0 adenocarcinoma of the prostate cancer at Princess Margaret Hospital between 1996 and 2012. The clinical data are summarized in Table S1, including the use of metformin or other oral hypoglycemic drugs (e.g. sulfonylureas, thiazolidinediones, meglitinide) and/or insulin. The total radiotherapy dose was escalated over the period of accrual in a series of separate phase I/II studies as previously published (19). The median radiation dose for the entire cohort was equivalent to 78Gy in 2Gy fractions. Patients were followed at 6 monthly intervals after completing treatment with clinical examination and prostate specific antigen (PSA).

**Statistical analysis**

For studies *in vitro* or in experimental tumor models, two-sided t-tests or log-rank tests were used to assess statistical significance. For clinical data, the primary outcome was biochemical relapse free rate (bRFR) following the start of radiotherapy. Biochemical relapse was defined by the Phoenix criteria as a post-treatment PSA nadir plus 2 ng/ml or treatment of salvage hormones due to a rising PSA as previously described (19). Patients who did not experience a biochemical relapse by their last known PSA date were considered censored. A Cox proportional-hazards model was used to determine whether metformin use influenced biochemical failure after RT independent of standard clinical prognostic factors. The proportional hazards assumption was checked for each variable by looking at the Schoenfeld residuals. As the proportional hazards assumption was found to be violated for metformin use, a Cox regression model was used to model metformin as a time-varying coefficient, adjusting for clinical T-category (T1 vs T2+), Gleason score (6 vs 7 vs 8+), pre-treatment PSA (continuous) and the use of hormonal treatment (Y vs N) as previously described (2). All analyses were done...
using SAS version 9.2 and R version 2.12.1. A two-sided p-value of 0.05 was used to assess statistical significance.

As a complimentary analysis, patients with and without record of metformin use were propensity score matched according to a list of important clinicopathologic covariates. The logit of the propensity score was calculated by forming a logistic regression model for metformin use with all the main effects and all two-way interactions of the following variables: Gleason score (6 vs. 7 vs. 8-9), pre-treatment PSA (continuous), age at the start of treatment (continuous), follow-up time (years, continuous), Biological Equivalent Dose (BED, continuous), hormone therapy (yes vs. no), and T-category (T1 vs. >T2). The best model was chosen using backwards selection. A one-to-one greedy matching algorithm was performed using a width of 0.2 of the standard deviation of the logit of the propensity score (SAS macro gmatch available at Mayo Clinic website) (20). Missing values of covariates were excluded. Covariate balance of the resulting cohort was assessed by ensuring all standardized differences were less than 10 (21).

Results

We measured oxygen consumption rates in vitro in the presence and absence of metformin in prostate carcinoma (LNCaP), head and neck squamous cell carcinoma (SCC-74B) and colorectal adenocarcinoma (HCT116 and POP-092S) cells. Metformin caused a significant dose- and time-dependent decrease in oxygen consumption in all cell lines (Fig.1A). Substantial differences were observed across the lines in terms of both metformin sensitivity and response kinetics (Fig.1B). The most metformin sensitive line was the prostate cell line LNCaP, which demonstrated a significant (p<0.05) reduction in respiration at an in vitro concentration of
metformin typically achieved in the plasma of diabetic patients (0.02mM) (Fig.1A, B). To determine if metformin could also inhibit oxygen consumption in vivo, we injected mice bearing HCT116 xenografts with 100mg/kg metformin and measured oxygen consumption rates in multiple tumor pieces ex vivo. This acutely administered dose corresponds to 25% of the daily oral dose taken by diabetic patients (22). Metformin-treated tumors demonstrated reduced rates of oxygen consumption compared to controls (Fig.1C - 7% reduction, p=0.005). These data show that metformin can reduce oxygen consumption in vitro and in vivo at clinically relevant doses.

Since tumor hypoxia arises in large part from oxygen consumption, we determined if metformin could improve oxygen distribution in tumors. To this end, we first injected tumor-bearing mice with the 2-nitroimidazole pimonidazole (pimo) which binds and labels hypoxic cells in the absence of oxygen. After pimo had cleared the bloodstream (2 hours), we injected 100mg/kg metformin or administered carbogen, followed by injection of a second 2-nitroimidazole, EF5, along with a perfusion marker, Hoechst. This strategy allows for detection of hypoxic tumor cells before and after the intervention, using antibodies specific for pimo and EF5. Using quantitative immunohistochemistry (IHC) (Fig.S1A), we found that metformin substantially reduced hypoxia in HCT116 tumors. Figure 2A shows examples of tumor sections where hypoxic cells prior to treatment (pimo+) are pseudo-colored green, and hypoxic cells following treatment (EF5+) are pseudo-colored red. In untreated tumors, most cells appear yellow since there are minimal changes in oxygenation in the time period between administrations of the two hypoxic cell markers (Fig.2A). A few cells stain for only one marker, consistent with transient hypoxia due to changes in red blood cell flux. In tumors from mice treated with metformin or carbogen, there was a significant decrease in EF5 (red) relative to pimo (green), reflecting tumor reoxygenation. Both the relative fraction of EF5 to pimo positive
cells, as well as the EF5 to pimo intensity ratio (Fig.2B) dropped upon treatment with metformin. Similar results were observed in the POP-092S colon xenograft model (Fig.S1C, D). There were no consistent changes in tumor perfusion upon metformin treatment as measured by Hoechst staining (data not shown).

IHC allows spatial analysis of the tumor microenvironment with single-cell resolution, but represents only a sub-region of the tumor. To probe the tumor microenvironment on a scale representative of the whole tumor, we dissociated POP-092S tumors and measured hypoxia before and after metformin treatment using flow cytometry. Similar to IHC results, the fraction of hypoxic (EF5+) cells dropped significantly following metformin treatment, reflecting widespread tumor reoxygenation (Fig.2C,D). The same approach was undertaken for LNCaP xenografts. These tumors supported very low levels of hypoxia, allowing quantitative analysis of only 4. Among these, metformin resulted in a substantial drop in hypoxia following metformin treatment (Fig.S1E). This robust response is consistent with the superior sensitivity of LNCaP cells to metformin (Fig.1B), but firm conclusions cannot be drawn in light of the low numbers. Finally, in SCC-74B xenografts we experienced a much increased inter-tumor variability compared to the other models, and no evidence of metformin-induced altered oxygenation (data not shown). Hence, 3 out of 4 tumor models provided data supporting a substantially reduced hypoxic fraction following metformin treatment.

Since non-invasive hypoxia imaging is entering clinical practice, we assessed whether this modality could also resolve metformin-induced changes in tumor oxygenation. Groups of mice bearing HCT116 tumors were injected with the positron-emitting 2-nitroimidazole $^{18}$F-FAZA and the percentage of tumor voxels containing hypoxia was estimated from $\mu$PET imaging. Consistent with results obtained using antibody-based IHC and flow cytometry, $^{18}$F-
FAZA-PET analysis demonstrated a significant (p=0.01) reduction in the hypoxic tumor volume after metformin treatment (Fig.2E). Taken together, results presented in Figure 2 demonstrate that metformin improves oxygenation as assessed at both micro- and macroscopic levels.

To test if the reduction in tumor hypoxia in metformin treated animals was sufficient to improve RT response, we irradiated HCT116 xenografts with a sub-curative, single dose of 15Gy and measured the time required for tumors to reach 4 times the irradiated volume. As expected, a single dose of metformin alone did not alter tumor growth (Fig.3A,B). However, metformin administrated 30 minutes prior to irradiation significantly increased tumor growth delay (log rank p=0.0003 in A and t-test p=0.004 in B). This increase in tumor response was not due to a change in cellular intrinsic radiosensitivity of oxygenated cells, since metformin treatment in vitro did not influence response (Fig.S2A). To corroborate this conclusion, we assessed the colony-forming ability of tumor cells in vitro following irradiation in vivo. We irradiated half of the tumor-bearing mice immediately following cervical dislocation, which renders all tumor cells hypoxic regardless of oxygen consumption rates. Consistent with the proposed mechanism, we observed that metformin only potentiated radiation-induced cell death in tumors from live, air-breathing mice (Fig.S2B). These results strongly suggest that metformin increases tumor radiation response in vivo by reducing the hypoxic tumor fraction.

We reasoned that if metformin causes increased tumor oxygenation and radiation response, diabetic patients taking metformin might have better outcomes following curative RT. We had previously shown that the presence of intra-glandular hypoxia leads to early biochemical failure (based on biochemical relapse-free rate; bRFR) following RT for localized prostate cancer (2). We therefore determined whether metformin altered early biochemical failure in 504 men undergoing similar curative prostate cancer RT that had differential metformin use (see
cohort characteristics in Table S1), with a median follow up from start of RT of 81.6 months (range 2.4 - 153.6). Within this cohort, we identified 114 patients taking metformin at the time of radiotherapy. On univariate analysis, the effect of metformin on bRFR was maximal early in follow-up (HR = 0.32, (0.11-0.92), p=0.034) and diminished with increasing time (p=0.019). Within a multivariate model, metformin use and a metformin interaction-with-time term, were both used to determine an independent effect of metformin on bRFR (see Table 1 and Fig. 4). Consistent with the pre-clinical data, the use of metformin was a significant and independent factor reducing early biochemical relapse after RT (after correcting for clinical covariates). As a complementary statistical approach, we also assessed the independent prognostic role of metformin using a propensity-score, matched analysis of 172 patients (Fig. S3) in which 86 patients taking metformin were matched based on previously reported covariates to patients in a control group (yielding proper balance among all covariates). In this sub-analysis, the bRFR at 3 years for patients on metformin was 92% as compared to 81% in the control group (adjusted hazard ratio= 0.63; 95% CI: 0.32 – 1.24, p = 0.06, stratified log-rank; see Figure S4). A cumulative incidence analysis considering deaths without prior biochemical recurrence to be competing risks (data not shown) confirmed these findings.

Taken together, our data suggest that metformin can provide a benefit to cancer patients treated with RT through its effects on reprogramming tumor metabolism and increasing tumor oxygenation.
Discussion

Epidemiological and retrospective studies have suggested that metformin may act as a cancer prevention and/or therapeutic agent (23). Two different mechanisms of anticancer activity have been proposed for metformin and other biguanides. The first is due to their ability to reduce circulating insulin levels, removing a potential growth-stimulating factor in cancer cells expressing the insulin receptor (23, 24). The second is through direct effects on metabolic activity of cancer cells, resulting in activation AMPK and subsequent inhibition of mTOR activity (23-26). Here we propose a new mechanism for metformin with specific application to cancers treated with radiotherapy – the inhibition of oxygen consumption (16, 17), with consequential re-oxygenation of radio-resistant hypoxic cells; this effect is independent of an effect on tumor cell radiosensitivity.

In addition to tumor hypoxia, intrinsic cellular radiation resistance and high proliferation rates limit the efficacy of RT. In the preclinical tumor model used here, we found no evidence that metformin affects intrinsic radiosensitivity. Some studies have reported that metformin can function as a cellular radiosensitizer (27-29), but this is clearly cell-line dependent (30, 31). Furthermore, it is uncertain whether metformin associated changes in radiosensitivity would offer any therapeutic ratio benefit, since the response of normal tissues to combined RT-metformin is currently unknown. Reports have suggested that in cell lines in which metformin increases radiosensitivity, continuous administration of metformin before and after radiation can increase tumor growth delay (26, 28, 30). In these studies, it is difficult to attribute the effects of metformin to specific mechanism(s), since they may include alterations of radiosensitivity, and tumor cell proliferation. Metformin alone also affects tumor growth in this setting, rendering the possible additive versus synergistic effect with radiation unclear. Our xenograft study was
designed to provide proof-of-principle that metformin improves radiation response specifically by the mechanism of increased tumor oxygenation in vivo. This was achieved by selecting a cell line that was not radiosensitized by metformin in vitro or in vivo (Fig.S2), and by delivering a single dose of metformin prior to radiation that alone had no effects on xenograft growth. Metformin treatment did also not alter blood glucose levels in the experimental animals (Fig.S5).

According to standard and FDA-recommended guidelines on dose-conversion between species (22), the single i.v. dose of 100mg/kg metformin in mice, that was used in all in vivo experiments here, corresponds to about 25% of the daily dose taken orally by diabetic patients (2000mg). Metformin is known to accumulate in some tissues, rendering the concentrations substantially higher than the steady-state plasma level (32). Tissue accumulation is likely dependent on expression of plasma membrane organic cation transporters (OCT) and multidrug and toxin extrusion (MATE) proteins (15). Ultimately, the efficacy of metformin to inhibit oxygen consumption will rely on the local concentration at the mitochondria, which may depend on other additional factors. These considerations combined with our data demonstrating 7% reduction in oxygen consumption rates in tumors upon metformin administration (Figure 1C) strongly suggest that clinically relevant metformin doses can modify tumor respiration. Mathematical modeling by Secomb and colleagues indicates that this magnitude of reduced oxygen consumption could have substantial impact on the hypoxic tumor fraction (8), in line with our measurements (Figure 2).

Taken together, our results indicate that metformin can substantially reduce the fraction of viable hypoxic cells that currently limit the efficacy of radiation treatment. This observation provides a novel mechanism and scientific rationale for combining metformin with RT in the clinic. The knowledge of metformin’s specific mechanism of action provided here has profound
implications for translating these findings into future clinical application. Metformin may be particularly useful in the context of altered fractionation RT schedules such as hypofractionation (doses greater than 2 Gy per fraction). According to results from mathematical modeling of tumor responses to RT, hypofractionation can result in a significant decrease in tumor cell killing compared to standard fractionation as a result of tumor hypoxia (33). Hypoxia modifying agents have therefore been suggested to be particularly important during hypofractionated RT schedules (7, 34). Metformin may thus have a specific role in prostate cancer, where hypofractionated RT schemes are increasingly used.

We observed that prostate cancer patients taking metformin had decreased early biochemical failure after RT. Although this effect cannot be directly attributed to reduced tumor hypoxia, it is consistent with the expected impact that an increase in tumor oxygenation would have. It is enticing that metformin improves rates of early biochemical relapse with very similar effect size and interaction with time as oxygen status itself in a similar patient cohort (2). Importantly, early biochemical failure after RT (e.g. within 18 months) has been observed to be a surrogate of prostate-cancer specific lethality (35) and if metformin use specifically reduced this risk, it could lower prostate cancer specific mortality. Indeed, a recent retrospective study observed lower rates of biochemical relapse, distant metastasis, prostate cancer specific mortality and overall mortality after combined RT-metformin use (27). In a large population-based retrospective study, Margel et al. have shown that for every additional 6 months of metformin treatment, there is a 24% decrease of prostate cancer-specific mortality and a decrease in all-cause mortality that declines over time, independent of treatment modality. Despite a lack of important details of the treatment, a subgroup analysis of 937 patients (24.4% of the entire cohort) who received RT as primary treatment showed a 48% decrease in prostate cancer-
specific mortality (adjusted HR, 0.56; 95% CI, 0.3 to 0.85, p = 0.09) (36). Other results from a smaller cohort of head and neck cancer patients treated with postoperative RT or esophageal cancer patients treated with neoadjuvant chemo-radiation showed that metformin use leads to lower loco-regional relapse rates and improved complete pathologic response (30, 37). These data are all consistent with metformin enhancing RT response in vivo.

The findings presented here from mechanistic studies in experimental tumor models and subsequent supportive clinical data, indicate that changes in tumor oxygenation contribute to the scientific rationale for pursuing metformin (and similar-acting agents) as a novel RT-enhancing drug. Novel strategies to overcome the barrier of tumor hypoxia in radiation treatments with curative intent are needed, and metformin may represent a safe and efficacious approach. Application of this strategy should incorporate use of biomarkers to help identify those patients most likely to benefit of the combination. Pre-treatment tumor hypoxia by gene signatures or extrinsic markers such as pimonidazole (IHC) or FAZA (PET) represent potentially important biomarkers for patient stratification. Furthermore, assessment of perfusion-limited versus diffusion-limited hypoxia may be important since metformin is only expected to affect diffusion-limited hypoxia. In fact, the large inter-tumor heterogeneity in (EF5+/Pimo+) observed in SCC-74B (data not shown) suggests that tumor hypoxia arises predominantly from the lack of perfusion in this model and could explain the lack of response in vivo. Other genetic tumor characteristics including the expression of OCT/MATE transporters represent additional potential biomarkers of response. Evaluation of biomarkers and patient stratification will be key to applying metformin as a means to modify the tumor microenvironment in combination with RT for future personalized cancer medicine.
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Table 1. Multivariate predictive models for prostate cancer radiotherapy bRFR including time-dependent effect

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ADT: Androgen Deprivation Therapy; bRFR: biochemical relapse free rate

a Effect of metformin on bRFR

b Time-dependent effect of metformin

* Used as reference variable
Figure legends

Figure 1. Metformin inhibits oxygen consumption. A: The oxygen consumption rates (OCR) of LNCaP, SCC-74B, HCT116 or POP-092S cells were measured over time after injection of various concentrations of metformin using the Seahorse Bioanalyzer. Data are normalized to basal OCR prior to metformin injection and represent 3 independent experiments +/- SEM. B: Relative OCR compared across the 4 cell lines at 3 hours post metformin injection. LNCaP was more sensitive than other cell lines, which was statistically significant (p<0.05) for doses >0.2mM metformin. C: HCT116 xenografts were excised 30 minutes after i.v. administration of 100mg/kg metformin to the tumor-bearing mice. Oxygen consumption rates (OCR) were measured on 23 pieces from each tumor, weighing 7-10mg. Data show average OCR +/-SEM, n=7.

Figure 2. Metformin increases tumor oxygenation. A: Mice bearing HCT116 xenografts were administered the hypoxic cell marker pimonidazole (pimo) 2 hours prior to i.v. injection of 100mg/kg metformin or carbogen breathing (95% O₂, 5% CO₂), followed by administration of a second hypoxic cell marker, EF5. After 3 hours, tumors were excised, sections stained and signals binarized. Green: Pimo+, Red: EF5+, Yellow: Pimo+EF5+. Examples from each group are shown. Scale bar represents 500µm. B: Quantification of tumor hypoxia across all HCT116 tumors from IHC. Left: the ratio of EF5+/Pimo+ from binarized images. Right: the intensity ratio of EF5/Pimo from non-binarized images (n= 4-5). C: Mice bearing POP-092S tumors were treated as in A. After tumor excision, cells were dissociated and fraction of pimo+ and EF5+ cells quantified by flow cytometry. Examples from each group are shown. D: Quantification of tumor hypoxia across all POP-092S tumors from flow cytometry. The ratio of EF5+/Pimo+ is
shown for each separate tumor. E: Groups of mice carrying HCT116 tumors were injected with $^{18}$F-AZA after 100mg/kg metformin and imaged with µPET. An example image is shown on the left. Right: The average fold change in hypoxic tumor fraction +/- SEM (n=3).

**Figure 3:** Mice bearing HCT116 xenografts were injected with saline or 100 mg/kg metformin 30 minutes prior to irradiation with 15Gy. Tumor growth was monitored over time and endpoint was defined as 4x the irradiated tumor volume. A: Kaplan-Meier representation of the data, B: Individual tumors’ time to reach 4x irradiated volume.

**Figure 4:** This plot shows biochemical recurrence free rates over time in a cohort of 504 prostate cancer patients, adjusting for known clinical factors including PSA, T category, Gleason score and androgen deprivation therapy use.
Figure 2

A. Control, Metformin, Carbogen

B. Box plots showing IHC (EF5+/Pimo+) for Control, Metformin, and Carbogen. Statistical significance: p=0.058

C. Flow cytometry data for Pimo and EF5 in DNA. Control: 41%, Metformin: 41%, Carbogen: 42%

D. Box plots showing IHC intensity (EF5/Pimo) for Control, Metformin, and Carbogen. Statistical significance: p=0.003

E. Bar chart showing % Voxel with Hypoxia for Control and Metformin. Statistical significance: p=0.01
Figure 4

Adjusted HR=0.23, 95% CI=0.07-0.71, Wald p=0.011

- No Metformin, 3y bRFR=85.1%
- Metformin, 3y bRFR=94.3%
Reprogramming Metabolism with Metformin Improves Tumor Oxygenation and Radiotherapy Response

Vanessa Zannella, Alan Dal Pra, Hala Muaddi, et al.

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