



# Metformin as an energy restriction mimetic agent for breast cancer prevention

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## Abstract

**Background:** This study examined whether metformin administration inhibited chemically induced mammary carcinogenesis in rats. In cancer prevention, metformin may act (1) indirectly through reducing systemic risk factors; or (2) directly through AMPK-mediated signaling. To begin to delineate clinically relevant mechanisms for breast cancer prevention, metformin was also studied along with dietary energy restriction.

**Materials and Methods:** Mammary cancer was induced in female Sprague-Dawley rats (50 mg/kg MNU, i.p.). Metformin was fed alone (AIN93G + 0.05 to 1.0% w/w metformin) or combined with 40% dietary energy restriction. Plasma analytes (e.g., insulin, glucose, IGF-I) and protein expression (e.g., AMPK, mTOR, Akt) in mammary carcinomas and liver were evaluated. Additional studies included (1) aldehyde dehydrogenase flow cytometry, to gauge potential for cancer-initiated cells in mammary carcinomas to respond to metformin; (2) cell culture, to understand dose response (0.02--20 mM) of different cancer cell line molecular subtypes to metformin; and (3) analysis of a rat mammary epithelial cell microarray database, to examine expression of genes related to metformin pharmacokinetics (e.g., organic cation transporters) and pharmacodynamics (e.g., complex I of electron transport). **Results:** While a dosing regimen of 1.0%/0.25% metformin-reduced palpable mammary carcinoma incidence, multiplicity, and tumor burden and prolonged latency, lower doses of metformin failed to inhibit carcinogenesis despite effects on plasma insulin. Human breast cancer cell growth inhibition in response to metformin was only observed at high concentrations. Poor in vivo and in vitro response to metformin may be the result of pharmacokinetic (OCT-1 expression was low in rat mammary cells; OCT-3 was downregulated in mammary carcinoma) and pharmacodynamic (complex I transcripts were higher in mammary epithelial cells from carcinomas versus uninvolved gland) effects. In combination with dietary energy restriction, metformin offered protection against new tumor occurrence following release from combined treatment. Flow cytometry indicated the presence of cancer-initiated cells in mammary carcinomas. **Conclusions:** As a single agent, metformin possessed limited cancer inhibitory activity. However, metformin may be an effective component of multiagent interventions that target cancer-initiated cells. There is a clear need to identify the conditions under which metformin is likely to benefit prevention and control of breast cancer.

**Keywords:** AMP-activated protein kinase, insulin, mammalian target of rapamycin, mammary carcinogenesis, metformin

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## INTRODUCTION

To date, the most powerful physiological inhibitor of the carcinogenic process that has been identified is dietary energy restriction (DER), also referred to as caloric restriction.<sup>[1-3]</sup> Though not all cancer sites have been shown to be sensitive to energy balance, breast cancer, a highly prevalent disease, is responsive.<sup>[4]</sup> While there is certainly a hormonal component to this sensitivity, emerging evidence indicates that additional host-systemic and cell-autonomous mechanisms are also involved.<sup>[5,6]</sup>

As we have recently reported,<sup>[3]</sup> DER is widely recognized for its effectiveness; however, it is also one of the most misunderstood interventions because of the perception that severe restriction of caloric intake is required for benefit. To the contrary, both the clinical and preclinical literature show that modest differences in patterns of energy balance are associated with meaningful differences in cancer risk.<sup>[7]</sup> Yet given the almost universal lapses in the regulation of energy intake relative to energy expenditure that occur as part of daily activities leading to overweight and obesity, we have suggested the potential value of identifying energy restriction mimetic agents for use in individuals at increased risk for breast cancer.<sup>[8]</sup>

One such agent, 2-deoxyglucose, accumulates in transformed cells that have undergone the Warburg switch in metabolism.<sup>[8]</sup> We hypothesized that 2-deoxyglucose would therefore affect similar mechanisms targeted by DER.<sup>[9]</sup> Experimental evidence from our group has not refuted this hypothesis.<sup>[8,10]</sup> However, given that an estimated 30% of human cancers do not undergo the Warburg switch in metabolism,<sup>[11,12]</sup> a new approach was formulated based on the recognized need for multiagent strategies to successfully prevent disease.<sup>[13-15]</sup> To this end, we sought to determine the feasibility of creating an energy deficit within the cell by targeting oxidative phosphorylation. For this proof in the principle set of experiments, a weak complex I electron inhibitor was selected, metformin.<sup>[16]</sup>

Metformin, a biguanide, is a widely used pharmaceutical agent in the management of type-2 diabetes.<sup>[17]</sup> In the diabetes literature, the most commonly cited mechanisms discussed for the beneficial effects of metformin are the blocking of liver gluconeogenesis, increased skeletal muscle uptake of glucose, and the reduction in absorption of glucose by the intestinal mucosa.<sup>[18-20]</sup> Evidence of reduced insulin resistance has also been reported. However, mechanisms that underlie these effects are not yet clearly defined, particularly regarding their application to cancer prevention.

Metformin and the related compounds phenformin and buformin are mitochondrial poisons that target complex I

in the electron transport chain.<sup>[16]</sup> While one adverse event that can occur in response to treatment with biguanides is lactic acidosis, a problem that resulted in phenformin and buformin being withdrawn from clinical use, the mechanistic implications are frequently not discussed.<sup>[21-24]</sup> Of the four currently recognized classes of intracellular energy sensors, AMP-activated protein kinase (AMPK) is a primary sensor of the intracellular ratio of AMP to ATP. Suppression of complex I activity by metformin changes the intracellular energy charge resulting in activation of AMPK.<sup>[16]</sup> AMPK is a component of a complex regulatory network (LKB1/AMPK-mTOR-IRS/Akt) that integrates signals from the extracellular and intracellular environments to control processes critical to the maintenance of tissue size homeostasis.<sup>[25-29]</sup> Often, one or more components of this network are frequently deregulated during carcinogenesis.<sup>[30,31]</sup> Dietary energy restriction also impacts these pathways and processes.<sup>[9,10]</sup> So, when combined, do DER and metformin offer greater protection together than either alone?

From our experience in rat mammary carcinogenesis, stopping DER results in rapid rebound in cancer endpoints.<sup>[32]</sup> This rebound would require individuals to maintain the restricted state indefinitely to slow the progression of the disease, whereas, ideally, the disease is eliminated by the prevention strategy. Emerging research indicates cancer-initiated cells are a potential target to effectively prevent disease through its elimination.<sup>[33]</sup> To investigate the role of metformin alone or in combination with DER to potentially target populations of cancer-initiated cells, the work reported in this paper addressed four questions (1) can metformin administration inhibit chemically induced mammary carcinogenesis; (2) do administered doses of metformin affect host systemic factors; (3) does metformin alter the effects of DER in inhibiting mammary carcinogenesis; and (4) what are the pharmacokinetic and pharmacodynamic (PK/PD) considerations for inhibition of mammary carcinogenesis by metformin?

## MATERIALS AND METHODS

### Chemicals and cell lines

Primary antibodies used in this study were rabbit anti-phospho-AMPK (Thr172), anti-AMPK, anti-phospho-mTOR (Ser2448), anti-mTOR, anti-TORC1, anti-phospho-PRAS40 (Thr246), anti-PRAS40, anti-phospho-p70S6K (Thr389), anti-p70S6K, anti-phospho-4E-BP1 (Thr37/46), anti-4E-BP1, anti-phospho-Akt (Ser473), anti-Akt, anti-phospho-ACC (Ser79), anti-ACC, anti-PI3Kp110, anti-LKB1, anti-rabbit immunoglobulin-horseradish peroxidase-conjugated secondary antibody, and LumiGLO reagent with peroxide, all from Cell Signaling Technology (Beverly, MA, USA). Mouse anti- $\beta$ -Actin primary antibody and metformin were

obtained from Sigma Chemical Co. (St. Louis, MO, USA). Anti-IGF1R $\alpha$  and antimouse immunoglobulin-horseradish peroxidase-conjugated secondary antibody were purchased from Santa Cruz Corporation (Santa Cruz, CA, USA). Carcinogen: 1-methyl-1-nitrosourea (MNU) was obtained from Ash Stevens (Detroit, MI, USA) and stored at  $-80^{\circ}\text{C}$  prior to use. Glucose-hexokinase liquid stable reagent was purchased from Thermo Fisher Scientific Inc. (Waltham, MA, USA). Enzyme-linked immunosorbent assay (ELISA) kit of adiponectin, multiplex kit for insulin and leptin, and signalplex kit for insulin growth factor-1 were from Millipore (Billerica, MA, USA). Dulbecco's modified Eagle's medium Ham's F12 50/50 mix with L-glutamine (DMEM/F12), fetal bovine serum (FBS, 5.1 mM glucose) were purchased from Invitrogen Corp. (Carlsbad, CA, USA). Human breast cancer cell lines of MCF-7, MDA-MB-231, MDA-MB-453, MDA-MB-468, SK-BR-3, BT-20, and BT-549 were obtained from American Type Culture Collection (Manassas, VA, USA). ALDEFLUOR kit and Collagenase/hyaluronidase was from StemCell Technology (Vancouver, Canada), medium 199 and Hanks' Balanced Salt Solution (HBSS) were from Invitrogen Corp. (Carlsbad, CA, USA).

### Cell culture

Human breast cancer cells were plated at the rate of  $3 \times 10^4$  cells per well in flat-bottomed 96-well plates in 100  $\mu\text{l}$  of DMEM/F12 medium supplemented with 10% fetal bovine serum and cultured overnight in 5%  $\text{CO}_2$  atmosphere at  $37^{\circ}\text{C}$ . The next day, cells were provided fresh medium including metformin at doses of 0.02, 0.1, 0.2, 1.0, 2.0, 5.0, 10.0, and 20.0 mM. At days 1, 2, and 3 after metformin exposure, cells were fixed with 1% glutaraldehyde, replaced with phosphate-buffered saline (PBS) and stored at  $4^{\circ}\text{C}$ . At the end of an experiment, all of the plates were stained with 0.02% aqueous crystal violet for 30 min and rinsed with deionized water. After redissolving the bound crystal violet in 70% ethanol, the absorbance was determined at 590 nm using a Spectromax Plus Microplate Spectrophotometer System (Molecular Devices, Sunnyvale, CA, USA).

### Dissociation of rat mammary carcinoma, ALDEFLUOR assay and separation of the ALDH-positive cell population by FACS

Freshly harvested rat mammary carcinomas that had been induced by treatment of rats with MNU were minced with scalpels in a tissue culture dish and dissociated in a 50 ml tube containing digestion medium (collagenase/hyaluronidase:medium 199 = 1:10) in  $37^{\circ}\text{C}$  water both for 30 min. The dissociation was stopped by adding 2% HBSS-FBS solution and the cell suspension was filtered with a cell strainer. Then, the cells were washed with 2% HBSS-FBS solution. The ALDEFLUOR assay was completed by following the vender's manual and as described by Ginestier in 2007. Briefly,

cells obtained from freshly dissociated rat mammary carcinoma were suspended in an ALDEFLUOR assay buffer containing aldehyde dehydrogenase (ALDH) substrate and incubated at  $37^{\circ}\text{C}$  for 40 min. As a negative control, for each sample of cells an aliquot was treated with 50 mM diethylaminobenzaldehyde, a specific ALDH inhibitor. The sorting gates were established using negative controls and the cells stained with PI only and cells were sorted using MoFlo (Dako Colorado, Inc.) Flow Cytometer and High Speed Cell Sorter that is located in the Core Facility at Colorado State University.

### Carcinogenesis experiments

Three experiments were conducted to determine how dietary metformin and/or DER affected the carcinogenic response in the mammary gland during the postinitiation phase of chemically induced mammary carcinogenesis. For all experiment female Sprague--Dawley rats were obtained from Charles River, Wilmington MA at 20 days of age. At 21 days of age, rats were injected with 50 mg/kg MNU, i.p., as previously described.<sup>[34]</sup> Rats were housed three per cage in solid-bottomed polycarbonate cages equipped with a food cup unless otherwise specified.

#### Experiment 1

Six days following carcinogen injection, 90 rats were randomized into one of three groups, 30 rats per group, and were fed *ad libitum* either AIN-93G diet containing no metformin, or that diet supplemented with a loading dose of metformin at 0.5% or 1.0% (w/w) metformin for 5 days. Thereafter, rats were continued on 0.05 or 0.25% metformin w/w for the remainder of the study (28 days).

#### Experiment 2

Six days following carcinogen injection, 60 rats were randomized into two groups (30/group): (1) control AIN-93G diet; (2) 0.3% metformin fed in AIN-93G diet (w/w). Animals were *ad libitum* fed for 9 weeks.

#### Experiment 3

In this experiment, rats were individually housed. Six days following carcinogen injection, 120 rats were randomized into one of four groups (30/group): (1) control; (2) 40% DER; (3) 40% DER + 0.25% metformin (w/w); 4) 40% DER + 0.25% metformin (w/w). The approach used for feeding rats has been described in detail.<sup>[35]</sup> Briefly, rats were *ad libitum* meal fed with AIN-93G diet for group 1, restricted to 60% the amount of fed control animals consumed in groups 2--4 for 8 weeks. During the last 2 weeks of the experiment, the animals in groups 1--3 were maintained on the same diet and fed in the same manner. The animals in group 4 were switched to AIN-93G diet and fed in the same manner as group 1, i.e., they were released from 40% DER + 0.25% (w/w) metformin.

For all experiments, animal rooms were maintained at  $22 \pm 1$  °C with 50% relative humidity and a 12-h light/12-h dark cycle. Rats were weighed three times per week and were palpated for detection of mammary tumors twice per week starting from 19 days postcarcinogen. The work reported was reviewed and approved by the Institutional Animal Care and Use Committee at Colorado State University and conducted according to the committee guidelines.

### Necropsy

Following an overnight fast, rats were killed over a 3-hour time interval via inhalation of gaseous carbon dioxide. The sequence in which rats were euthanized was stratified across groups so as to minimize the likelihood that order effects would masquerade as treatment associated effects. After the rats lost consciousness, blood was directly obtained from the retroorbital sinus and gravity fed through heparinized capillary tubes (Fisher Scientific, Pittsburgh, PA, USA) into EDTA-coated tubes (Becton Dickinson, Franklin Lakes, NJ, USA) for plasma. The bleeding procedure took approximately 1 min/rat. Plasma was isolated by centrifugation at  $1000 \times g$  for 10 min at room temperature. Following blood collection and cervical dislocation, rats were skinned and the skin was examined under translucent light for detectable mammary pathologies. All grossly detectable mammary gland lesions and abnormalities were excised and processed for histological classification as described.<sup>[36,37]</sup> Only mammary adenocarcinomas are reported.

### Assessment of circulating molecules

Glucose was determined using a kit obtained from Thermo Fisher Scientific Inc. (Waltham, MA, USA). Insulin growth factor-1 was determined using a commercial signalplex kit, insulin and leptin were determined using a multiplex kit, and adiponectin was determined by a commercial enzyme-linked immunosorbent assay kit from Millipore (Billerica, MA, USA). All analyses were performed according to the manufacturer's instructions.

### Western blotting

Mammary carcinomas and liver were homogenized in the lysis buffer (40 mM Tris-HCl [pH 7.5], 1% Triton X-100, 0.25 M sucrose, 3 mM EGTA, 3 mM EDTA, 50  $\mu$ M  $\beta$ -mercaptoethanol, 1 mM phenyl-methylsulfonyl fluoride, and complete protease inhibitor cocktail [Calbiochem, San Diego, CA, USA]). The lysates were centrifuged at  $7500 \times g$  for 10 min at 4 °C and supernatant fractions collected and stored at -80 °C. Supernatant protein concentrations were determined by the Bio-Rad protein assay (Bio-Rad, Hercules, CA, USA). Western blotting was performed as described previously.<sup>[25]</sup> Briefly, 40  $\mu$ g of protein lysate per sample was subjected to 8--16% sodium dodecyl sulfate-polyacrylamide gradient gel electrophoresis (SDS-PAGE)

after being denatured by boiling with the SDS sample buffer (63 mM Tris-HCl [pH 6.8], 2% SDS, 10% glycerol, 50 mM DTT, and 0.01% bromophenol blue) for 5 min. After electrophoresis, proteins were transferred to a nitrocellulose membrane. The levels of phospho-AMPK (Thr172), AMPK, phospho-mTOR (Ser2448), mTOR, TORC1, phospho-PRAS40 (Thr246), PRAS40, phospho-p70S6K (Thr389), p70S6K, phospho-4E-BP1 (Thr37/46), 4E-BP1, phospho-Akt (Ser473), Akt, , phospho-ACC (Ser79), ACC, PI3Kp110, LKB1, IGF1R $\alpha$ , and  $\beta$ -actin were determined using specific primary antibodies, followed by treatment with the appropriate peroxidase-conjugated secondary antibodies and visualized by LumiGLO reagent Western blotting detection system. The chemiluminescence signal was captured using a ChemiDoc densitometer (Bio-Rad) that was equipped with a CCD camera having a resolution of  $1300 \times 1030$ . Quantity One software (Bio-Rad) was used in the analysis of the actin-normalized scanning density data.

### Statistical analyses

Differences among groups in the incidence of mammary adenocarcinomas were evaluated by chi-square analysis.<sup>[38]</sup> Differences among groups in the number of mammary adenocarcinomas per rat (multiplicity) were evaluated by ANOVA after square root transformation of tumor count data.<sup>[38]</sup> Differences in final body weight, gene expression, and circulating molecules were evaluated by ANOVA with *post hoc* comparisons by the method of Tukey.<sup>[39]</sup> Differences in the number of human cancer cells following exposure to metformin at different doses and time points were evaluated by factorial ANOVA of the absorbance data.<sup>[39]</sup> For Western blots, representative bands are shown in the figures. The data displayed in the bar graphs of the figures were either the actin-normalized scanning data or the ratio of the actual scanning units derived from the densitometric analysis of each Western blot for the phospho-proteins involved in energy sensing pathways. For statistical analyses, the actin-normalized scanning density data obtained from the ChemiDoc scanner using Quantity One (Bio-Rad) were first rank transformed. This approach is particularly suitable for semiquantitative measurements that are collected as continuously distributed data, as is the case with Western blots. The ranked data were then subjected to multivariate analysis of variance.<sup>[40]</sup> Ratio data were computed from the scanning units derived from the densitometric analysis, i.e., the arbitrary units of optical density for variables stated and then the ratios were rank transformed and evaluated via multivariate analysis of variance. All analyses were performed using Systat statistical analysis software, version 13. The principle component analysis for the data from cell culture, plasma analytes, and western blotting was completed using Partek (Partek, Inc., St. Louis, MO, USA).



## RESULTS AND DISCUSSION

Interest in metformin for breast cancer prevention evolved based on epidemiological evidence showing associations between diabetic patients receiving metformin and cancer rates; individuals who received higher doses of metformin were better protected against cancer.<sup>[41-43]</sup> The breast is one of the organ sites for which the protective effects were observed.<sup>[44]</sup> As such, over the past several years, use of metformin for breast cancer prevention and control has been discussed.<sup>[45,46]</sup> Since elevated insulin and IGF-1 levels are associated with increased cancer risk and metformin improves insulin sensitivity, the approach has mechanistic traction.<sup>[45]</sup> Nonetheless, consideration must be given to the patient population from which most clinical and epidemiological evidence has been reported, as metformin may be utilized in both diabetic and nondiabetic individuals for breast cancer risk reduction. Therefore, the investigation of mammary carcinogenesis in nondiabetic animals is important. Recently, metformin was reported to not affect MNU-induced mammary carcinogenesis (MNU, i.v., three doses) despite significant effects on circulating levels of insulin.<sup>[47]</sup> Herein, a focused set of experiments was performed to further understand this result using additional preclinical model systems. Bearing in mind the previous finding,<sup>[47]</sup> we carried out several metformin dosing studies in a rapid emergence MNU-induced mammary carcinogenesis rat model.<sup>[48]</sup> In addition to metformin alone, we investigated combined treatment with metformin + DER as a potential strategy to affect populations of cancer-initiated cells in an environment sensitized by physiologic energy restriction.

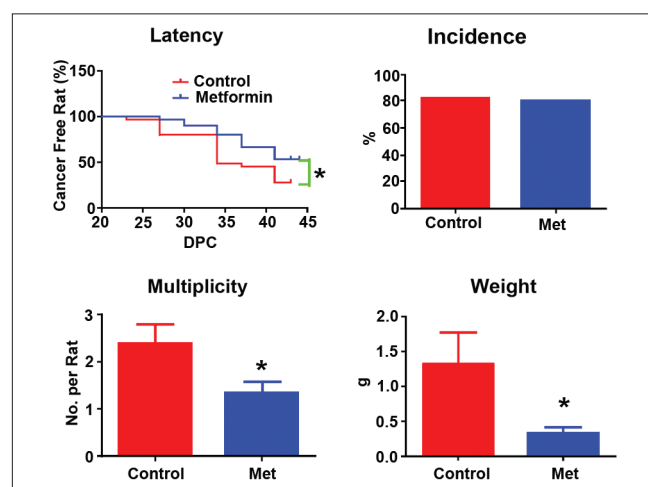
### Effect of metformin on chemically induced mammary carcinogenesis

Initially, rats were fed diet containing a loading dose of 0.5% or 1.0% (w/w) metformin beginning 7 days postcarcinogen. Recognizing that high metformin doses have the potential to induce lactic acidosis and retard growth thus confounding data interpretation, maintenance dosing of 0.05% or 0.25% w/w was begun after 5 days of metformin loading and was continued for the remainder of the study (28 days). As shown in Figure 1 (palpable) and Supplementary Table 1 (final), only the group of animals on the 1.0/0.25% dosing regime was protected against the development of mammary carcinogenesis. Metformin reduced the incidence, multiplicity, and tumor burden (mass per rat) of palpable mammary tumors, confirmed to be adenocarcinomas, with prolongation of latency to occurrence of palpable tumors ( $P < 0.05$  for each endpoint).

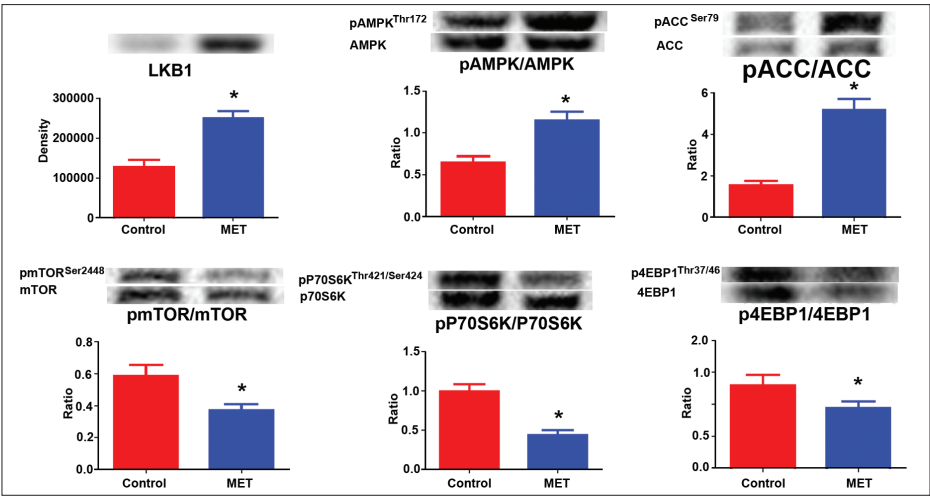
In an effort to gain mechanistic insight into whether the 1.0/0.25% metformin dosing regimen inhibited mammary carcinogenesis by a direct effect on cell signaling, mammary

carcinomas from the control group and 1.0/0.25% metformin were assessed for effects on the signaling network of which AMPK is a component. As shown in Figure 2, AMPK was activated in mammary carcinomas from metformin-treated rats with concomitant activation of acetyl co-A carboxylase, a specific downstream target of activated AMPK. These findings are consistent with a direct effect of metformin on the intracellular environment of the tumor, as recently described in an *in vitro* model<sup>[16]</sup> whereby metformin inhibition of complex I results in changes in cellular energy charge and AMPK activation. Along with our observation of AMPK activation, levels of phospho AKT were reduced along with key downstream targets of mTOR, S6 kinase, and 4EBP-1. Though metformin was inducing direct effects in mammary carcinomas, these findings do not rule out the possibility that systemically mediated mechanisms were also involved.

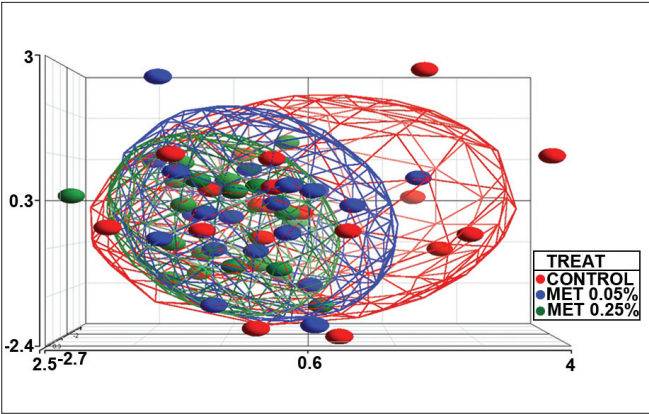
To explore this issue, plasma was assessed for systemic factors [Table 1 and Supplementary Figure 1] and liver protein expression was analyzed for the same signaling components evaluated in the mammary carcinomas [Figure 3 and Supplementary Table 2]. Only the cancer inhibitory dose (1.0/0.25% w/w) of metformin induced AMPK in liver while downregulating mTOR signaling. This metformin dose also influenced plasma insulin and leptin relative to control. Of note, when metformin failed to induce effects in the liver, it also failed to inhibit the carcinogenic process in the mammary gland. The interplay between systemic factors and cell autonomous signaling cannot be discriminated based on these results; however, the finding highlights that responsiveness of systemic factors to metformin treatment may serve as a



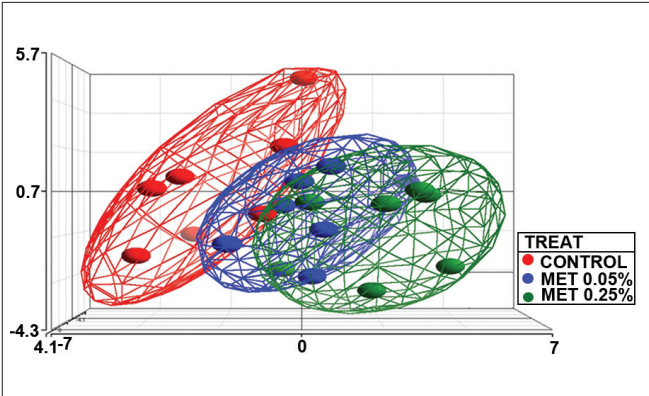
**Figure 1: Effect of metformin (MET) incorporated into a purified diet formulation at 1.0% for 5 days/0.25% w/w for 28 days versus control on palpable mammary cancer incidence, multiplicity (carcinoma number per rat), tumor burden (carcinoma mass/rat), and latency. Experimental design 1 in the Materials and Methods section. DPC, days postcarcinogen.**



**Figure 2:** A composite image of representative western blots for key components in the LKB1-AMPK--mTOR-AKT signaling network: LKB1, phosphorylated and total AMPK, ACC, mTOR, p70S6K, and 4EBP1. The images shown are those directly acquired from the ChemiDoc workstation that is equipped with a CCD camera having a resolution of 1300 × 1030 as described in the Materials and Methods. For nonratio data, values represent a mean relative absorbance values for each protein, normalized to β-actin. Control, no metformin; MET, 1.0%/0.25% metformin as described in the Materials and Methods, Experiment 1.



**Supplementary Figure 1:** Principle component analysis (PCA) of 60 rat plasma samples from three groups (20 each): control (no metformin), 0.5/0.05% w/w metformin (MET 0.05%), and 1.0/0.25% metformin w/w (MET 0.25%) in the animal diet as described in the Materials and Methods section, Experiment 1. Each dot represents a plasma sample.



**Figure 3:** Principle Component analysis mapping of 21 rat liver samples from three groups (seven each): control (no metformin), 0.5/0.05% metformin (MET 0.05%), and 1.0/0.25% metformin (MET 0.25%) in the animal diet as described in the Materials and Methods, Experiment 1. Blotted proteins included: IGF1R-PI3Kp110-AMPK-ACC-Akt-mTOR-TORC1-PRAS40-P70S6K-4EBP1 network associated protein expression. Each dot represents a liver sample.

**Table 1: Effect of dietary metformin on plasma analytes\***

Dietary treatment	Control	0.5/0.05% MET	1.0/0.25% MET
IGF-I (ng/ml)	323 ± 18	326 ± 13	312 ± 14
Insulin (ng/ml)	1.02 ± 0.06 <sup>a</sup>	0.81 ± 0.05 <sup>b</sup>	0.79 ± 0.04 <sup>b</sup>
Leptin (ng/ml)	1.03 ± 0.05 <sup>a</sup>	0.99 ± 0.04 <sup>a</sup>	0.84 ± 0.04 <sup>b</sup>
Adiponectin (µg/ml)	18.9 ± 1.1	18.1 ± 1.0	18.2 ± 0.9
Glucose (mg/dl)	72.4 ± 4.5	72.2 ± 5.1	67.6 ± 3.7

\*Values are means ± SEM. Data were analyzed by one-way ANOVA. Values within a row with different superscripts are statistically different (P < 0.05) MET, metformin.

criterion for identifying clinical subpopulations for breast cancer prevention with metformin. As an example of the potential for personalizing treatment, in diabetes, gene variants of cation transporters (OCTs) determine subpopulation responsiveness to drug treatment.<sup>[49,50]</sup>

**Pharmacokinetic and pharmacodynamic considerations that may affect biguanide efficacy for breast cancer prevention and control**

As noted by Pollak,<sup>[46]</sup> metformin may have PK/PD limitations relative to cancer prevention and control that

**Table S1: Effect of dietary metformin on the carcinogenic response (palpable + non-palpable mammary carcinomas detected at necropsy)\***

Dietary treatment	Control	0.5/0.05% MET	1.0/0.25% MET
Cancer Incidence (%)	83.3 <sup>a</sup>	73.3 <sup>a</sup>	80.0 <sup>a</sup>
Cancer Latency (days)	35.9 ± 1.2 <sup>a</sup>	35.9 ± 1.4 <sup>a</sup>	39.8 ± 0.9 <sup>b</sup>
Cancer Multiplicity (No. carcinomas/rat)	2.4 ± 0.4 <sup>a</sup>	1.9 ± 0.4 <sup>a</sup>	1.3 ± 0.3 <sup>b</sup>
Cancer Burden (Ave. cancer mass/rat (g))	1.33 ± 0.45 <sup>a</sup>	0.78 ± 0.24 <sup>a</sup>	0.34 ± 0.09 <sup>b</sup>
Final Body Weight (g)	186 ± 2 <sup>a</sup>	182 ± 3 <sup>a</sup>	168 ± 3 <sup>b</sup>

\*Values are means ± SEM except incidence. Values within a row with different superscripts are statistically different ( $P < 0.05$ ). MET, metformin.

**Table S2: Effect of dietary metformin on liver autonomous factors\***

Dietary treatment	Control	0.5/0.05% MET	1.0/0.25% MET
pAMPKThr172	311 ± 29	367 ± 27	404 ± 42
AMPK	854 ± 47	847 ± 35	828 ± 28
pAMPK/AMPK	0.37 <sup>a</sup> ± 0.04	0.44 <sup>a</sup> ± 0.03	0.49 <sup>b</sup> ± 0.05
pmTORSer2448	264 ± 21	195 ± 14	183 ± 25
mTOR	500 ± 27	467 ± 28	511 ± 28
pmTOR/mTOR	0.53 ± 0.04 <sup>a</sup>	0.42 ± 0.03 <sup>a,b</sup>	0.35 ± 0.04 <sup>b</sup>
TORC1	65.5 ± 6.1	49.3 ± 3.8	44.9 ± 3.8
pPRAS40(Thr246)	75.2 ± 7.7	70.6 ± 6.8	72.2 ± 6.5
PRAS40	147 ± 8	156 ± 8	179 ± 9
pPRAS40/PRAS40	0.54 ± 0.08	0.46 ± 0.06	0.41 ± 0.04
p4EBP1Thr37/46	539 ± 61	510 ± 72	502 ± 58
4EBP1	106 ± 7	130 ± 4	133 ± 5
p4EBP1/4EBP1	5.22 ± 0.74	3.87 ± 0.52	3.73 ± 0.32
pP70S6KThr389	72.2 ± 4.1	57.2 ± 5.1	40.3 ± 4.5
P70S6K	797 ± 67	952 ± 31	962 ± 37
pP70S6K/P70S6K	0.094 ± 0.007 <sup>a</sup>	0.060 ± 0.005 <sup>b</sup>	0.043 ± 0.005 <sup>b</sup>
IGF1R $\alpha$	16.7 ± 0.5 <sup>a</sup>	16.4 ± 1.5 <sup>a</sup>	12.0 ± 0.9 <sup>b</sup>
pAktSer473	706 ± 72	607 ± 54	484 ± 78
Akt	1569 ± 35	1616 ± 86	1765 ± 89
pAkt/Akt	0.45 ± 0.05 <sup>a</sup>	0.38 ± 0.04 <sup>a</sup>	0.27 ± 0.04 <sup>b</sup>
pACCSer79	1075 ± 59	1097 ± 34	1108 ± 52
ACC	531 ± 36	505 ± 39	505 ± 24
pACC/ACC	2.06 ± 0.12	2.24 ± 0.14	2.23 ± 0.17
PI3Kp110	97.6 ± 4.2 <sup>a</sup>	94.0 ± 4.1 <sup>a,b</sup>	84.2 ± 2.6 <sup>b</sup>

\*Values are means ± SEM. Values within a row with different superscripts are statistically different ( $P < 0.05$ , one-tailed)MET, metformin.

**Table 2: Gene expression of organic cation transporters in the rat mammary gland epithelial cells and mammary adenocarcinoma epithelial cells\***

Accession number	Gene name	Symbol	MGEC	ACEC	P
NM_012697	Solute carrier family 22 (organic cation transporter), member 1	Slc22a1	14.6 ± 0.6	14.7 ± 0.6	0.869
NM_031584	Solute carrier family 22 (organic cation transporter), member 2	Slc22a2	12.6 ± 0.5	12.4 ± 0.4	0.715
NM_019230	Solute carrier family 22, member 3	Slc22a3	381 ± 46	18.8 ± 2.5a	<0.001

\*Values are means ± SEM. Data were analyzed by ANOVA. Values in a row with superscripts are statistically different from each other. MGEC, mammary gland epithelial cells; ACEC, adenocarcinoma epithelial cells.

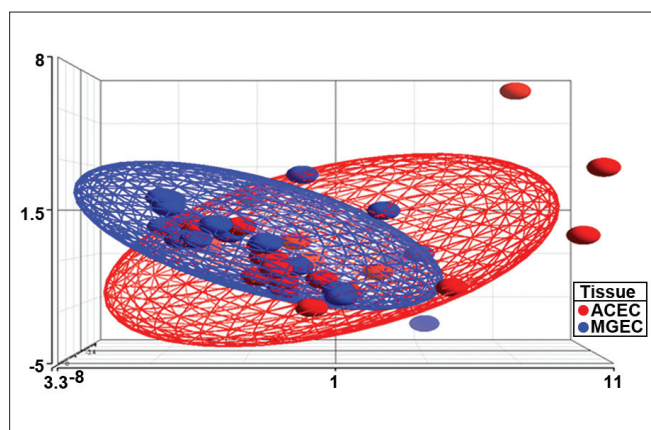
require more attention. Here we begin to investigate these issues in the rat preclinical model. Two questions were initially addressed: (1) Do epithelial cells from mammary gland and/or mammary carcinomas express transcripts for the genes that encode the organic OCTs

responsible for cellular uptake of metformin,<sup>[51-53]</sup> i.e., altered pharmacokinetics? and (2) Is complex I of the mitochondrial electron chain system suppressed in mammary carcinomas versus mammary epithelial cells, i.e., altered pharmacodynamics?

**Pharmacokinetics** Expression analysis was carried out using a previously published rat mammary epithelial cell microarray database.<sup>[54]</sup> The database was created using RNA extracted from mammary epithelial cells harvested by laser capture microdissection from mammary gland and mammary carcinomas and assayed using Affymetrix GeneChip Rat Genome 230 2.0 microarray chips. The expression database was analyzed for the gene transcripts encoding the OCTs to determine the potential for metformin to accumulate in mammary epithelial cells. Thirteen genes from this family of transport proteins were identified and found to be expressed at low levels in epithelial cells of the mammary gland [Supplementary Table 3]. The expression of three of these genes OCT1, 2, and 3 is shown in Table 2. Metformin has been reported to be a substrate of OCT 1 and 3.<sup>[55]</sup> As shown in Table 2, levels of OCT 1 and OCT 2 transcripts were very low in mammary epithelial cells isolated from either the mammary gland or mammary carcinomas. However, markedly higher levels of OCT3 in mammary gland epithelial cells were found but not in epithelial cells isolated from mammary carcinomas. While elevated OCT3 in untransformed epithelial cells would likely be necessary for accumulation of metformin, low-expression levels of OCT 1 and OCT 3 in the epithelial cells of the mammary

carcinomas implies metformin may not concentrate in mammary tumor epithelial cells.

**Pharmacodynamics** Assuming that metformin PK would allow exposure of mammary epithelial cells to metformin, we also investigated transcript levels of metformin target, complex I. As shown in Supplementary Table 3, genes encoding components of complex I of the electron transport chain are expressed in epithelial cells from both the mammary gland and mammary carcinomas. Given the number of transcripts involved, the expression data were subjected to multivariate analysis of variance. Overall expression of complex I transcripts was found to be higher in epithelial cells from carcinomas ( $P < 0.001$ ). The univariate  $P$ -value for each gene is provided in the Supplementary tables and a visual representation of that analysis is shown as a PCA plot [Figure 4]. Metabolic reprogramming in cancer cells makes interpretation of elevated complex I difficult, as complex I is only one component of a multi-component system. Elevated complex I in cancer cells may indicate that the system is potentially more sensitive to metformin treatment if metformin pharmacokinetics are not limiting. Alternatively, elevated complex I in cancer cells may indicate that the system will readily compensate for inhibition.



**Figure 4:** Principle Component analysis of the mammary gland epithelial cells (MGEC) and mammary adenocarcinoma epithelial cells (ACEC) representing complex I associated gene expression from 42 samples (21 each). Each dot represents one gene chip from each animal.

Evaluation of mammary epithelial cell transcripts indicates the importance of carrying out further PK/PD studies to investigate metformin as a breast cancer preventive agent. Additionally, evaluation of other biguanides (e.g., phenformin and buformin) reported to inhibit chemically-induced mammary carcinogenesis in the rat<sup>[56,57]</sup> should be investigated to determine how these compounds behave *in vivo*, as these biguanides are more potent inducers of AMPK than metformin.<sup>[16]</sup>

### Responsiveness of human breast carcinoma cell lines to metformin treatment

The data in Table 2 imply that metformin is unlikely to be universally effective against all types of breast cancer, given potential for downregulation of OCT-1 and OCT-3 in the epithelial cells from mammary carcinomas. A

**Table 3: Effect of dietary energy restriction and metformin on the carcinogenic response in the mammary gland\***

Dietary treatment	Control	40% DER	40% DER + 0.25% MET	40% DER + 0.25% MET - REL	P
Cancer incidence (%)	79.3 <sup>a</sup>	60.0 <sup>a,b</sup>	53.3 <sup>b</sup>	46.7 <sup>b</sup>	0.064
Cancer latency (days)	58.9 ± 3.0 <sup>a</sup>	74.0 ± 2.0 <sup>b</sup>	72.8 ± 2.2 <sup>b</sup>	71.8 ± 2.0 <sup>b</sup>	< 0.0001
Cancer multiplicity (No. carcinomas/rat)	3.03 ± 0.45 <sup>a</sup>	0.83 ± 0.17 <sup>b</sup>	0.73 ± 0.14 <sup>b</sup>	1.07 ± 0.28 <sup>b</sup>	< 0.0001
Cancer burden (Ave. cancer mass/rat (g))	2.18 ± 0.62 <sup>a</sup>	0.43 ± 0.16 <sup>b</sup>	0.25 ± 0.12 <sup>b</sup>	0.75 ± 0.31 <sup>b</sup>	0.001
Final body weight (g)	227 ± 4 <sup>a</sup>	166 ± 1 <sup>b</sup>	159 ± 2 <sup>b</sup>	194 ± 2 <sup>c</sup>	< 0.0001

\*Values are means ± SEM except incidence. Values within a row with different superscripts are statistically different ( $P < 0.05$ ). DER, dietary energy restriction; MET, metformin; REL, release



**Table S3: Gene expression of complex I and organic cation transporters in rat mammary gland epithelial cells and mammary adenocarcinoma epithelial cells\***

Accession no.	Gene name	Symbol	MGEC	ACEC	P
NM_00100550	NADH dehydrogenase (ubiquinone) Fe-S protein 1, 75kDa	Ndufs1	70.6 ± 7.4	70.5 ± 4.1	0.987
NM_001011907	NADH dehydrogenase (ubiquinone) Fe-S protein 2 (predicted)	Ndufs2_ predicted	570 ± 26	608 ± 22	0.277
	NADH dehydrogenase (ubiquinone) Fe-S protein 3 (predicted)	Ndufs3_ predicted	387 ± 19	461 ± 31	0.051
NM_001025146	NADH dehydrogenase (ubiquinone) Fe-S protein 4, 18kDa (NADH-coenzyme Q reductase)	Ndufs4	207 ± 8	235 ± 7 <sup>a</sup>	0.012
	NADH dehydrogenase (ubiquinone) Fe-S protein 5b, 15kDa (NADH-coenzyme Q reductase) (predicted)	Ndufs5b_ predicted	322 ± 30	412 ± 41	0.123
NM_019223	NADH dehydrogenase (ubiquinone) Fe-S protein 6	Ndufs6	284 ± 13	359 ± 28 <sup>a</sup>	0.022
NM_001008525	NADH dehydrogenase (ubiquinone) Fe-S protein 7 (predicted)	Ndufs7_ predicted	255 ± 14	286 ± 46	0.528
	NADH dehydrogenase (ubiquinone) Fe-S protein 8 (predicted)	Ndufs8_ predicted	172 ± 16	241 ± 21 <sup>a</sup>	0.013
NM_031064	NADH dehydrogenase (ubiquinone) flavoprotein 2	Ndufv2	919 ± 62	1013 ± 76	0.341
NM_012985	NADH dehydrogenase (ubiquinone) I alpha subcomplex 5	Ndufa5	123 ± 6	131 ± 8	0.448
	NADH dehydrogenase (ubiquinone) I alpha subcomplex, 2 (predicted)	Ndufa2_ predicted	281 ± 20	401 ± 32 <sup>a</sup>	0.003
	NADH dehydrogenase (ubiquinone) I alpha subcomplex, 6 (B14) (predicted)	Ndufa6_ predicted	141 ± 4	150 ± 6	0.194
XM_216859	NADH dehydrogenase (ubiquinone) I alpha subcomplex, 7 (B14.5a) (predicted)	Ndufa7_ predicted	513 ± 29	530 ± 40	0.723
XM_216044	NADH dehydrogenase (ubiquinone) I alpha subcomplex, 8 (predicted)	Ndufa8_ predicted	216 ± 9	253 ± 10 <sup>a</sup>	0.009
	NADH dehydrogenase (ubiquinone) I alpha subcomplex, 9 (predicted)	Ndufa9_ predicted	884 ± 54	992 ± 48	0.143
	NADH dehydrogenase (ubiquinone) I alpha subcomplex 10 /// NADH dehydrogenase I alpha subcomplex 10-like protein	Ndufa10	2276 ± 84	2037 ± 100	0.074
	NADH dehydrogenase (ubiquinone) I alpha subcomplex, assembly factor I (predicted)	Ndufa1_ predicted	92.1 ± 5.7	127 ± 8 <sup>a</sup>	0.001
	NADH dehydrogenase (ubiquinone) I beta subcomplex, 2 (predicted)	Ndufb2_ predicted	602 ± 27	640 ± 29	0.343
NM_001106912	NADH dehydrogenase (ubiquinone) I beta subcomplex 3 (predicted)	Ndufb3_ predicted	1099 ± 30	1200 ± 54	0.108
NM_001037338	NADH dehydrogenase (ubiquinone) I beta subcomplex, 4, 15kDa (predicted)	Ndufb4_ predicted	1185 ± 56	1251 ± 28	0.459
	NADH dehydrogenase (ubiquinone) I beta subcomplex, 5 (predicted)	Ndufb5_ predicted	663 ± 31	792 ± 37 <sup>a</sup>	0.011
XM_001058166	NADH dehydrogenase (ubiquinone) I beta subcomplex, 6, 17kDa (predicted)	Ndufb6_ predicted	205 ± 16	242 ± 13	0.081
	NADH dehydrogenase (ubiquinone) I beta subcomplex, 7 (predicted)	Ndufb7_ predicted	700 ± 37	901 ± 40 <sup>a</sup>	0.001
XM_216929	NADH dehydrogenase (ubiquinone) I beta subcomplex, 9 (predicted)	Ndufb9_ predicted	561 ± 29	625 ± 39	0.194
	NADH dehydrogenase (ubiquinone) I, alpha/beta subcomplex, I (predicted)	Ndufab1_ predicted	677 ± 41	777 ± 36	0.073

Table S3 (contd...)

Table S3: (Contd...)

Accession no.	Gene name	Symbol	MGEC	ACEC	P
NM_001009290	NADH dehydrogenase (ubiquinone) I, subcomplex unknown, 2 (predicted)	Ndudc2_ predicted	1748 ± 70	1665 ± 46	0.331
NM_022270	Solute carrier family 22 (organic cation transporter), member 4	Slc22a4	12.7 ± 0.6	30.9 ± 3.6 <sup>a</sup>	<0.001
NM_019269	Solute carrier family 22 (organic cation transporter), member 5	Slc22a5	33.1 ± 1.1	41.3 ± 1.0 <sup>a</sup>	<0.001
NM_017224	Solute carrier family 22 (organic cation transporter), member 6	Slc22a6	9.9 ± 0.3	10.2 ± 0.4	0.615
NM_053537	Solute carrier family 22 (organic cation transporter), member 7	Slc22a7	8.6 ± 0.4	8.1 ± 0.2	0.273
NM_031332	Solute carrier family 22 (organic cation transporter), member 8	Slc22a8	6.7 ± 0.1	6.9 ± 0.1	0.332
	Solute carrier family 22 (organic cation transporter), member 12	Slc22a12	6.6 ± 0.2	7.1 ± 0.2	0.136
	Solute carrier family 22 (organic cation transporter), member 13 (predicted)	Slc22a13_ predicted	11.9 ± 0.3	12.4 ± 0.4	0.290
	Solute carrier family 22 (organic cation transporter), member 15 (predicted)	Slc22a15_ predicted	155 ± 9	303 ± 32 <sup>a</sup>	<0.0001
NM_177421	Solute carrier family 22 (organic cation transporter), member 17	Slc22a17	107 ± 10	170 ± 17 <sup>a</sup>	0.003
	Solute carrier family 22, member 19	Slc22a19	9.8 ± 0.4	9.2 ± 0.2	0.209

\*Values are means ± SEM. Data were analyzed by ANOVA. Values within a row with superscripts are statistically different. MGEC, mammary gland epithelial cells; ACEC, adenocarcinoma epithelial cells.

Table S4: Types of breast cancer cell lines and their major molecular expressions\*

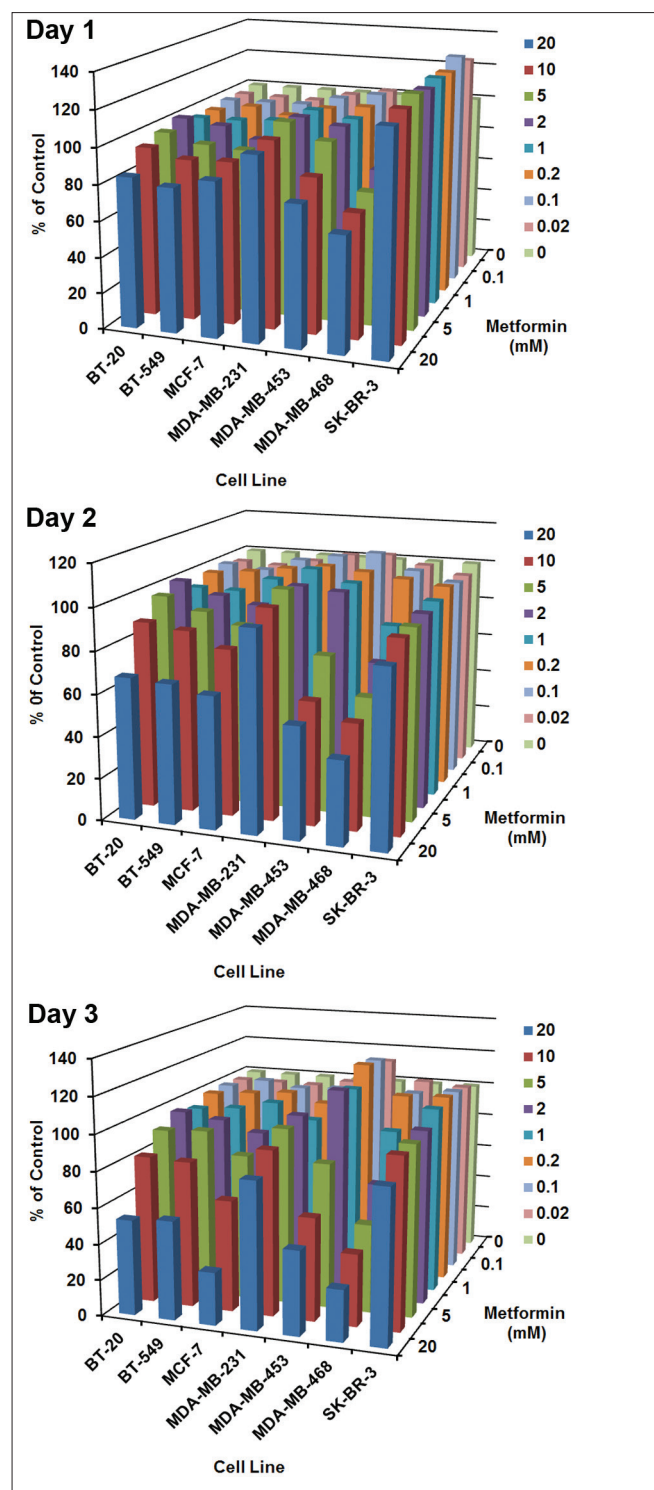
Cell name	Subtype	Disease	Receptors
BT-20	Basal A	Carcinoma	ER <sup>-</sup> , PR <sup>-</sup> , HER2/ neu <sup>-</sup> , WNT3 <sup>+</sup>
BT-549	Basal B	Ductal Carcinoma	ER <sup>-</sup> , PR <sup>-</sup> , HER2/ neu <sup>-</sup> , p53m <sup>++</sup>
MCF7	Luminal	Adenocarcinoma	ER <sup>+</sup> , PR <sup>+</sup> , HER2/ neu <sup>-</sup>
MDA-MB-231	Basal B	Adenocarcinoma	ER <sup>-</sup> , PR <sup>-</sup> , HER2/ neu <sup>-</sup> , EGF <sup>+</sup> TGFa <sup>+</sup>
MDA-MB-453	Luminal	Metastatic Carcinoma	ER <sup>-</sup> , PR <sup>-</sup> , HER2/ neu <sup>-</sup> , FGF <sup>+</sup>
MDA-MB-468	Basal A	Adenocarcinoma	ER <sup>-</sup> , PR <sup>-</sup> , HER2/ neu <sup>-</sup> , EGF <sup>+</sup>
SK-BR-3	Luminal	Adenocarcinoma	HER2/ neu <sup>+</sup> , ER <sup>-</sup> , PR <sup>-</sup>

\*Information regarding cells types was obtained from ACTT and referenced in the following papers:<sup>[1-4]</sup>

Farther reading: • Alimova, I.N., Liu, B., Fan, Z., Edgerton, S.M., Dillon, T., Lind, S.E., and Thor, A.D. (2009) Metformin inhibits breast cancer cell growth, colony formation and induces cell cycle arrest in vitro. *Cell Cycle*, 8, 909-915. • Liu, B., Fan, Z., Edgerton, S.M., Deng, X.S., Alimova, I.N., Lind, S.E., and Thor, A.D. (2009) Metformin induces unique biological and molecular responses in triple negative breast cancer cells. *Cell Cycle*, 8, 2031-2040. • Neve, R.M., Chin, K., Fridlyand, J., Yeh, J., Baehner, F.L., Fevr, T., Clark, L., Bayani, N., Coppe, J.P., Tong, F., Speed, T., Spellman, P.T., DeVries, S., Lapuk, A., Wang, N.J., Kuo, W.L., Stilwell, J.L., Pinkel, D., Albertson, D.G., Waldman, F.M., McCormick, F., Dickson, R.B., Johnson, M.D., Lippman, M., Ethier, S., Gazdar, A., and Gray, J.W. (2006) A collection of breast cancer cell lines for the study of functionally distinct cancer subtypes. *Cancer Cell*, 10, 515-527. • Haupt, B., Ro, J.Y., and Schwartz, M.R. (2010) Basal-like breast carcinoma: a phenotypically distinct entity. *Arch. Pathol. Lab. Med.*, 134, 130-133.

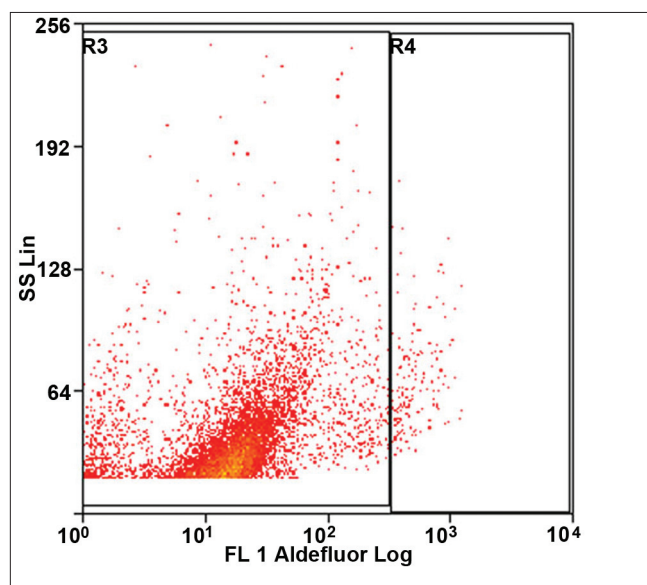
simple cell growth assay was used to evaluate whether the five recognized molecular subtypes of human breast cancer (Supplementary Table 4 for information on cell lines) show variation in the effectiveness of metformin treatment. As detailed in Figure 5, Supplementary Table 5, and Supplementary Figure 2, the dose response data from seven breast cancer cell lines showed marked differences in growth inhibition by metformin among the molecular subtypes. Growth inhibition ranged from as little as 22% in the SK-BR3 cell line to 71% in the MCF-7 cell line. Because eight doses of metformin were investigated in seven cell lines over 3 days, data were evaluated using multivariate analysis of variance and the overall effect was visualized

by the principal components analysis [Supplementary Figure 2]. The growth patterns differed sufficiently to permit identification of the cell lines by the unsupervised cluster technique, an approach similar to that was used to establish the molecular subtypes into which human breast cancers are currently categorized;<sup>[58]</sup> however, no clear relationship between molecular subtype of breast cancer and responsiveness to metformin was apparent. For the cell lines that were responsive to metformin, the doses required for growth inhibition were considerably higher than levels of metformin achieved in plasma during clinical management of diabetes,<sup>[59]</sup> indicating that significant barriers may exist to making metformin clinically useful.

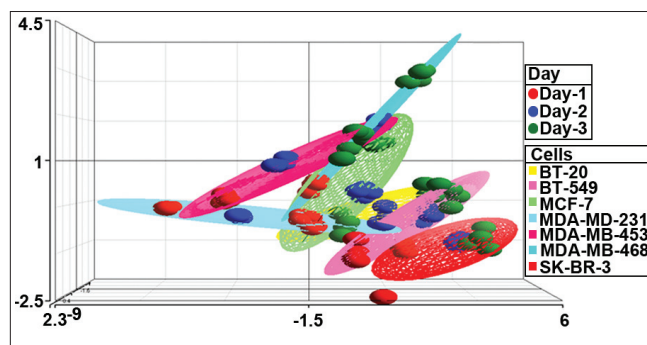


**Figure 5: Effect of metformin on the growth of seven different types of human breast cancer cells exposed to 0.0, 0.02, 0.1, 0.2, 1.0, 2.0, 5.0, 10.0, or 20.0 mM metformin in the cell culture medium for 1, 2, or 3 days.**

Nonetheless, these results underscore the importance of recognizing that metformin is unlikely to be effective against all molecular subtypes of breast cancer and that understanding resistant to treatment will likely involve PK/PD.



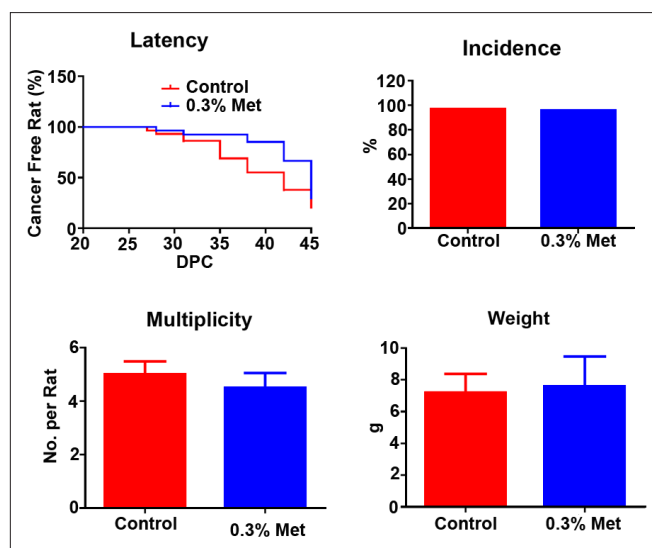
**Figure 6: The ALDEFLUOR-positive cell population from the rat mammary adenocarcinomas. Representative flow cytometry analysis of aldehyde dehydrogenase (ALDH) activity in cells derived from 10 rat mammary adenocarcinomas. The ALDEFLUOR assay was performed as described in the Materials and Methods and Ginestier's publication in 2007. The average percent of ALDH-positive cells as shown in the right box R4 was  $2.10 \pm 0.43$  (mean  $\pm$  SEM).**



**Supplementary Figure 2: Principle component analysis (PCA) of seven different types of human breast cancer cells exposed to 0.0, 0.02, 0.1, 0.2, 1.0, 2.0, 5.0, 10.0, and 20.0 mM metformin in the cell culture medium for 1, 2, or 3 days. Each dot represents a pooled effect of different concentrations of metformin on the cell growth for each day.**

### Effect of metformin in combination with DER on chemically induced mammary carcinogenesis

In order to determine whether the higher loading dose is important for metformin cancer inhibitory activity in our rapid emergence model for breast cancer, rats were fed 0.3% w/w metformin starting 7 days postcarcinogen (Experiment 2). As shown in Supplementary Figure 3, using this dosing scheme, metformin did not protect against mammary carcinogenesis. This finding suggests that early events in the carcinogenic process are more susceptible to high dose metformin. Early events in the rapid emergence model, such as progression of transformed cells into hyperplasia, are potential targets for intervention, especially if cancer-



**Supplementary Figure 3:** Carcinogenic response to dietary metformin (0.3%) showing final cancer latency, incidence, multiplicity (carcinoma number per rat), and tumor burden (carcinoma mass/rat). Rats were treated with no metformin (control) or with dietary metformin at 0.3% w/w (0.3% Met) as described in the Materials and Methods section, Experiment 2. DPC, days post carcinogen.

initiated cells can be eliminated. Given the preclinical model was more susceptible to metformin at an early stage, we further investigated enhancing sensitivity to metformin through combined treatment with DER. As shown in Table 3, treatment with 40% DER alone or in combination with metformin was highly effective in inhibiting mammary carcinogenesis. Final cancer incidence, multiplicity, and tumor burden were markedly reduced, and latency was prolonged. While the rats treated with metformin + DER had a lower carcinogenic response, the differences between DER versus metformin + DER were not statistically different. Given our published work that DER activates AMPK and the data in Figure 2 that indicate that metformin does as well, the results indicate the system may reach a plateau.

From our previous work,<sup>[32]</sup> it is known that the cancer inhibitory activity of DER is not retained when animals are released from DER and fed *ad libitum*. In fact, protective effects of DER on cancer incidence, multiplicity, and tumor burden were lost. After 8 days of release from DER, cancer incidence increased from 56.7% to 80% and cancer multiplicity from 0.73 to 2.60. However, the data in Table 3 show sustained protection against the occurrence of new tumors following release from metformin + DER treatment. Cancer incidence did not change and cancer multiplicity increased by only 25% in 14 days following release from combined treatment. However, tumor burden tripled in comparison to animals that remained on the combined treatment, indicating a loss of growth suppression

of established tumors when combined treatment was stopped. We interpret these results as being suggestive that metformin, when combined with a sensitizer such as DER, has a role in regulating and potentially deleting cancer-initiated cells.

As a follow-up to this study, we evaluated 10 mammary carcinomas for aldehyde dehydrogenase staining, a marker for cancer-initiated cells.<sup>[60]</sup> As shown in Figure 6, approximately 2% of the epithelial cells in MNU-induced mammary carcinomas stained positive. While this is not definitive evidence that chemically induced carcinomas contain cancer-initiated cells, the data are consistent with their existence in the MNU model system. We hypothesize that metformin, in the presence of a sensitizer such as DER, leads to deletion of cancer-initiated cells and extends protection once metformin + DER treatment is stopped. Future experiments will investigate how metformin combined with a sensitizing treatment may result in a curative-type prevention.

### Limitations

A widely used rodent model for mammary carcinogenesis was employed for the *in vivo* experiments reported herein;<sup>[48]</sup> however, the rats in this model are not obese and are not subject to the insulin resistance which is a common component of type-2 diabetes. As such, this work provides information about the potential use of metformin for breast cancer prevention in a population of women who do not experience either condition.<sup>[42,46]</sup> Nonetheless, the data reported may not adequately reflect the potential for metformin to prevent breast cancer in obese and/or diabetic women. Moreover, metformin-induced mechanisms related to the regulation of AMPK activity by adipokines such as adiponectin and leptin may not be detected under the circumstances investigated.<sup>[61]</sup>

### CONCLUSIONS

There is considerable interest in evaluating metformin for its efficacy in the prevention and control of breast cancer. While a strong rationale exists for clinical studies, the data reported herein suggest the need for more preclinical evaluation, particularly related to metformin PK/PD. In particular, the susceptibility of molecular subtypes of breast cancer to inhibition by metformin, as well as limitations in target site accumulation due to potential downregulation of OCT transporters, needs to be addressed. Metformin, when given at high doses early in the carcinogenic process, as well as when combined with DER, inhibits mammary carcinogenesis. Identification of mechanisms underlying



**Table S5: Effect of metformin on growth of human breast cancer cells\***

Cells	BT-20	BT-549	MCF-7	MDA-MB-231	MDA-MB-453	MDA-MB-468	SK-BR-3
Metformin (mM)	Day-1						
0.0	1.73 ± 0.01 <sup>a</sup>	2.10 ± 0.06 <sup>a</sup>	1.69 ± 0.02 <sup>a</sup>	0.79 ± 0.03 <sup>a,b,c</sup>	1.17 ± 0.03 <sup>a</sup>	1.97 ± 0.03 <sup>a</sup>	1.72 ± 0.05
0.02	1.70 ± 0.01 <sup>a,b</sup>	2.06 ± 0.01 <sup>a,b</sup>	1.66 ± 0.01 <sup>a</sup>	0.81 ± 0.01 <sup>a</sup>	1.24 ± 0.02 <sup>a</sup>	1.90 ± 0.03 <sup>a</sup>	2.20 ± 0.02
0.1	1.71 ± 0.01 <sup>a,b</sup>	2.08 ± 0.03 <sup>a,b</sup>	1.69 ± 0.01 <sup>a</sup>	0.83 ± 0.01 <sup>a</sup>	1.27 ± 0.02 <sup>a</sup>	1.97 ± 0.02 <sup>a</sup>	2.29 ± 0.02
0.2	1.68 ± 0.01 <sup>a,b</sup>	2.12 ± 0.02 <sup>a,b</sup>	1.65 ± 0.004 <sup>a</sup>	0.82 ± 0.01 <sup>a,b</sup>	1.23 ± 0.01 <sup>a</sup>	1.90 ± 0.02 <sup>a</sup>	2.21 ± 0.02
1.0	1.67 ± 0.01 <sup>a,b</sup>	2.04 ± 0.02 <sup>a,b</sup>	1.67 ± 0.02 <sup>a</sup>	0.84 ± 0.02 <sup>a</sup>	1.21 ± 0.02 <sup>a</sup>	1.74 ± 0.03 <sup>b</sup>	2.22 ± 0.01
2.0	1.75 ± 0.02 <sup>a</sup>	2.07 ± 0.03 <sup>a,b</sup>	1.57 ± 0.02 <sup>b</sup>	0.85 ± 0.01 <sup>a,b</sup>	1.21 ± 0.01 <sup>a</sup>	1.59 ± 0.02 <sup>c</sup>	2.18 ± 0.03
5.0	1.69 ± 0.01 <sup>a,b</sup>	1.95 ± 0.02 <sup>b,c</sup>	1.55 ± 0.01 <sup>b</sup>	0.86 ± 0.01 <sup>a,b</sup>	1.17 ± 0.04 <sup>a</sup>	1.46 ± 0.01 <sup>d</sup>	2.21 ± 0.01
10.0	1.63 ± 0.02 <sup>b</sup>	1.88 ± 0.03 <sup>c</sup>	1.53 ± 0.02 <sup>b</sup>	0.83 ± 0.01 <sup>b,c</sup>	1.01 ± 0.02 <sup>b</sup>	1.36 ± 0.03 <sup>d,e</sup>	2.16 ± 0.02
20.0	1.45 ± 0.02 <sup>c</sup>	1.69 ± 0.03 <sup>d</sup>	1.45 ± 0.01 <sup>c</sup>	0.81 ± 0.01 <sup>c</sup>	0.91 ± 0.02 <sup>b</sup>	1.27 ± 0.002 <sup>e</sup>	2.09 ± 0.02
P	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	0.406
Day-2							
0.0	2.21 ± 0.01 <sup>a</sup>	2.53 ± 0.02 <sup>a</sup>	2.12 ± 0.02 <sup>a</sup>	1.20 ± 0.02 <sup>a,b</sup>	1.65 ± 0.04 <sup>a</sup>	2.63 ± 0.04 <sup>a</sup>	2.68 ± 0.02 <sup>a</sup>
0.02	2.15 ± 0.02 <sup>a</sup>	2.44 ± 0.02 <sup>a,b</sup>	2.13 ± 0.02 <sup>a</sup>	1.26 ± 0.02 <sup>a,c</sup>	1.74 ± 0.02 <sup>a</sup>	2.67 ± 0.03 <sup>a</sup>	2.60 ± 0.01 <sup>a,b</sup>
0.1	2.19 ± 0.04 <sup>a</sup>	2.46 ± 0.06 <sup>a,b</sup>	2.20 ± 0.02 <sup>a</sup>	1.29 ± 0.02 <sup>c</sup>	1.80 ± 0.04 <sup>a</sup>	2.68 ± 0.04 <sup>a</sup>	2.61 ± 0.03 <sup>a</sup>
0.2	2.17 ± 0.03 <sup>a</sup>	2.53 ± 0.04 <sup>a</sup>	2.18 ± 0.03 <sup>a</sup>	1.27 ± 0.01 <sup>a,c</sup>	1.70 ± 0.03 <sup>a</sup>	2.67 ± 0.04 <sup>a</sup>	2.65 ± 0.03 <sup>a</sup>
1.0	2.08 ± 0.05 <sup>a,b</sup>	2.37 ± 0.03 <sup>a,b</sup>	2.14 ± 0.04 <sup>a</sup>	1.29 ± 0.01 <sup>c</sup>	1.67 ± 0.03 <sup>a</sup>	2.16 ± 0.01 <sup>b</sup>	2.56 ± 0.01 <sup>b</sup>
2.0	2.23 ± 0.03 <sup>a</sup>	2.40 ± 0.03 <sup>a,b</sup>	1.95 ± 0.01 <sup>b</sup>	1.23 ± 0.01 <sup>a,c</sup>	1.66 ± 0.03 <sup>a</sup>	1.81 ± 0.01 <sup>c</sup>	2.50 ± 0.01 <sup>b,c</sup>
5.0	2.15 ± 0.04 <sup>a</sup>	2.31 ± 0.05 <sup>b</sup>	1.83 ± 0.01 <sup>c</sup>	1.26 ± 0.01 <sup>a,c</sup>	1.23 ± 0.02 <sup>b</sup>	1.51 ± 0.03 <sup>d</sup>	2.45 ± 0.02 <sup>c</sup>
10.0	1.96 ± 0.02 <sup>b</sup>	2.18 ± 0.02 <sup>b</sup>	1.69 ± 0.02 <sup>d</sup>	1.20 ± 0.02 <sup>a,b</sup>	0.97 ± 0.02 <sup>c</sup>	1.34 ± 0.01 <sup>e</sup>	2.44 ± 0.02 <sup>c</sup>
20.0	1.49 ± 0.02 <sup>c</sup>	1.68 ± 0.02 <sup>c</sup>	1.34 ± 0.02 <sup>e</sup>	1.15 ± 0.02 <sup>b</sup>	0.88 ± 0.01 <sup>c</sup>	1.05 ± 0.004 <sup>f</sup>	2.23 ± 0.01 <sup>d</sup>
P	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
Day-3							
0.0	2.54 ± 0.04 <sup>a</sup>	2.66 ± 0.07 <sup>a,b</sup>	2.32 ± 0.06 <sup>a</sup>	1.91 ± 0.03 <sup>a</sup>	1.92 ± 0.03 <sup>a,b</sup>	2.97 ± 0.04 <sup>a</sup>	2.65 ± 0.08 <sup>a,b</sup>
0.02	2.52 ± 0.04 <sup>a</sup>	2.63 ± 0.05 <sup>a,b</sup>	2.30 ± 0.03 <sup>a</sup>	1.96 ± 0.03 <sup>a</sup>	2.23 ± 0.08 <sup>a</sup>	3.14 ± 0.07 <sup>a</sup>	2.75 ± 0.08 <sup>a,b</sup>
0.1	2.54 ± 0.04 <sup>a</sup>	2.77 ± 0.01 <sup>a</sup>	2.35 ± 0.05	1.96 ± 0.05 <sup>a</sup>	2.32 ± 0.08 <sup>a</sup>	3.06 ± 0.08 <sup>a</sup>	2.80 ± 0.05 <sup>a</sup>
0.2	2.52 ± 0.04 <sup>a</sup>	2.68 ± 0.05 <sup>a,b</sup>	2.39 ± 0.05 <sup>a</sup>	1.88 ± 0.04 <sup>a,b</sup>	2.34 ± 0.05 <sup>a</sup>	3.14 ± 0.05 <sup>a</sup>	2.83 ± 0.03 <sup>a</sup>
1.0	2.41 ± 0.02 <sup>a,b</sup>	2.57 ± 0.05 <sup>a,b</sup>	2.35 ± 0.02 <sup>a</sup>	1.78 ± 0.01 <sup>a,b</sup>	2.16 ± 0.04 <sup>a</sup>	2.68 ± 0.01 <sup>b</sup>	2.77 ± 0.06 <sup>a,b</sup>
2.0	2.48 ± 0.03 <sup>a,b</sup>	2.51 ± 0.03 <sup>b</sup>	2.06 ± 0.01 <sup>b</sup>	1.92 ± 0.05 <sup>a</sup>	2.22 ± 0.03 <sup>a</sup>	1.94 ± 0.02 <sup>c</sup>	2.59 ± 0.08 <sup>a,b</sup>
5.0	2.33 ± 0.02 <sup>b</sup>	2.47 ± 0.05 <sup>b</sup>	1.88 ± 0.03 <sup>b</sup>	1.88 ± 0.04 <sup>a,b</sup>	1.55 ± 0.03 <sup>b</sup>	1.46 ± 0.01 <sup>d</sup>	2.54 ± 0.03 <sup>b</sup>
10.0	2.03 ± 0.08 <sup>c</sup>	2.15 ± 0.04 <sup>c</sup>	1.44 ± 0.07 <sup>c</sup>	1.76 ± 0.03 <sup>b</sup>	1.10 ± 0.01 <sup>b</sup>	1.19 ± 0.02 <sup>e</sup>	2.52 ± 0.03 <sup>b</sup>
20.0	1.35 ± 0.04 <sup>d</sup>	1.47 ± 0.06 <sup>d</sup>	0.68 ± 0.05 <sup>d</sup>	1.56 ± 0.01 <sup>c</sup>	0.89 ± 0.02 <sup>c</sup>	0.85 ± 0.01 <sup>f</sup>	2.25 ± 0.01 <sup>c</sup>
P	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001

\*Values are means ± SEM. Data were analyzed by ANOVA. Values within a column for the same day with different superscripts are statistically different ( $P < 0.05$ ).

early stage susceptibility of the carcinogenic process to metformin may uncover novel preventive strategies. One such strategy is aimed at deleting cancer-initiated mammary epithelial cells to achieve sustained protection after ending treatment.

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## REFERENCES

1. Kritchevsky D. Caloric restriction and cancer. *J Nutr Sci Vitaminol (Tokyo)* 2001;47:13-9.
2. Thompson HJ, Zhu Z, Jiang W. Dietary energy restriction in breast cancer prevention. *J Mammary Gland Biol Neoplasia* 2003;8:133-42.
3. Thompson HJ, Jiang W, Zhu Z. From energetics and cancer: Exploring a road less traveled. In *Physical Activity, Dietary Calorie Restriction, and Cancer*. In: McTiernan A, editor. Heidelberg: Springer Science + Business Media, LLC; 2011. p. 55-67.
4. WCRF/AICR. Food, Nutrition, Physical Activity, and the Prevention of Cancer: A Global Perspective. 2007. Washington, DC: AICR; 2008.
5. Subbaramaiah K, Howe LR, Bhardwaj P, Du B, Gravaghi C, Yantiss RK, et al. Obesity is associated with inflammation and elevated aromatase expression in the mouse mammary gland. *Cancer Prev Res (Phila)* 2011;4:329-46.
6. Brown KA, Simpson ER. Obesity and breast cancer: Progress to understanding

- the relationship. *Cancer Res* 2010;70:4-7.
7. Thompson HJ, Playdon M, Matthews S, McTiernan A. Weight cycling and breast cancer risk: A critical review and analysis. *Cancer Prev Res (Phila)* (in press)
  8. Zhu Z, Jiang W, McGinley JN, Thompson HJ. 2-Deoxyglucose as an energy restriction mimetic agent: effects on mammary carcinogenesis and on mammary tumor cell growth in vitro. *Cancer Res* 2005;65:7023-30.
  9. Jiang W, Zhu Z, Thompson HJ. Dietary energy restriction modulates the activity of AMP-activated protein kinase, Akt, and mammalian target of rapamycin in mammary carcinomas, mammary gland, and liver. *Cancer Res* 2008;68:5492-9.
  10. Jiang W, Zhu Z, Thompson HJ. Modulation of the activities of AMP-activated protein kinase, protein kinase B, and mammalian target of rapamycin by limiting energy availability with 2-deoxyglucose. *Mol Carcinog* 2008;47:616-28.
  11. Vander Heiden MG, Cantley LC, Thompson CB. Understanding the Warburg effect: The metabolic requirements of cell proliferation. *Science* 2009;324:1029-33.
  12. DeBerardinis RJ, Mancuso A, Daikhin E, Nissim I, Yudkoff M, Wehrli S, et al. Beyond aerobic glycolysis: Transformed cells can engage in glutamine metabolism that exceeds the requirement for protein and nucleotide synthesis. *Proc Natl Acad Sci USA* 2007;104:19345-50.
  13. Lippman SM, Hawk ET. Cancer prevention: From 1727 to milestones of the past 100 years. *Cancer Res* 2009;69:5269-84.
  14. Lippman SM. The future of molecular-targeted cancer chemoprevention. *Gastroenterology* 2008;135:1834-41.
  15. Lippman SM. The dilemma and promise of cancer chemoprevention. *Nat Clin Pract Oncol* 2006;3:523.
  16. Hawley SA, Ross FA, Chevtzoff C, Green KA, Evans A, Fogarty S, et al. Use of cells expressing gamma subunit variants to identify diverse mechanisms of AMPK activation. *Cell Metab* 2010;11:554-65.
  17. Goodwin PJ, Stambolic V, Lemieux J, Chen BE, Parulekar WR, Gelmon KA, et al. Evaluation of metformin in early breast cancer: A modification of the traditional paradigm for clinical testing of anti-cancer agents. *Breast Cancer Res Treat* 2011;126:215-20.
  18. Vella S, Buetow L, Royle P, Livingstone S, Colhoun HM, Petrie JR. The use of metformin in type 1 diabetes: A systematic review of efficacy. *Diabetologia* 2010;53:809-20.
  19. Bosi E. Metformin--the gold standard in type 2 diabetes: What does the evidence tell us? *Diabetes Obes Metab* 2009;11 Suppl 2:3-8.
  20. Correia S, Carvalho C, Santos MS, Seica R, Oliveira CR, Moreira PI. Mechanisms of action of metformin in type 2 diabetes and associated complications: An overview. *Mini Rev Med Chem* 2008;8:1343-54.
  21. Meisheri YV. Phenformin induced lactic acidosis. *J Assoc Physicians India* 1995;43:147.
  22. Salpeter SR, Greyber E, Pasternak GA, Salpeter EE. Risk of fatal and nonfatal lactic acidosis with metformin use in type 2 diabetes mellitus: Systematic review and meta-analysis. *Arch Intern Med* 2003;163:2594-602.
  23. Scheen AJ. Clinical pharmacokinetics of metformin. *Clin Pharmacokinet* 1996;30:359-71.
  24. Sirtori CR, Pasik C. Re-evaluation of a biguanide, metformin: Mechanism of action and tolerability. *Pharmacol Res* 1994;30:187-228.
  25. Hardie DG. AMPK: A key regulator of energy balance in the single cell and the whole organism. *Int J Obes (Lond)* 2008;32 Suppl 4:S7-12.
  26. Hardie DG. New roles for the LKB1-->AMPK pathway. *Curr Opin Cell Biol* 2005;17:167-73.
  27. Hardie DG. The AMP-activated protein kinase pathway--new players upstream and downstream. *J Cell Sci* 2004;117:5479-87.
  28. Hardie DG. AMP-activated protein kinase: A master switch in glucose and lipid metabolism. *Rev Endocr Metab Disord* 2004;5:119-25.
  29. Towler MC, Hardie DG. AMP-activated protein kinase in metabolic control and insulin signaling. *Circ Res* 2007;100:328-41.
  30. Efeyan A, Sabatini DM. mTOR and cancer: Many loops in one pathway. *Curr Opin Cell Biol* 2010;22:169-76.
  31. Zoncu R, Efeyan A, Sabatini DM. mTOR: From growth signal integration to cancer, diabetes and ageing. *Nat Rev Mol Cell Biol* 2011;12:21-35.
  32. Zhu Z, Jiang W, McGinley J, Wolfe P, Thompson HJ. Effects of dietary energy repletion and IGF-I infusion on the inhibition of mammary carcinogenesis by dietary energy restriction. *Mol Carcinog* 2005;42:170-6.
  33. Subramaniam D, Ramalingam S, Houchen CV, Anant S. Cancer stem cells: A novel paradigm for cancer prevention and treatment. *Mini Rev Med Chem* 2010;10:359-71.
  34. Thompson HJ. From methods for the induction of mammary carcinogenesis in the rat using either 7,12-dimethylbenz(a)anthracene or 1-methyl-1-nitrosourea. In *Methods in Mammary Gland biology and Breast Cancer Research*. Ip MM, Asch BB, editors. New York: Kluwer Academic/Plenum Publishers; 2000. p. 19-29.
  35. Zhu Z, Haegele AD, Thompson HJ. Effect of caloric restriction on pre-malignant and malignant stages of mammary carcinogenesis. *Carcinogenesis* 1997;18:1007-12.
  36. Thompson HJ, Singh M, McGinley J. Classification of premalignant and malignant lesions developing in the rat mammary gland after injection of sexually immature rats with 1-methyl-1-nitrosourea. *J Mammary Gland Biol Neoplasia* 2000;5:201-10.
  37. Singh M, McGinley JN, Thompson HJ. A comparison of the histopathology of premalignant and malignant mammary gland lesions induced in sexually immature rats with those occurring in the human. *Lab Invest* 2000;80:221-31.
  38. Sokal RR, Rohlf FJ. *Biometry the principles and practice of statistics in biological research*. New York: W.H. Freeman; 1995.
  39. Snedecor GW, Cochran WG. *Statistical Methods*. Ames, IA: Iowa State University Press; 1989.
  40. Morrison DF. *Multivariate Statistical Methods*. New York: McGraw-Hill Publishing Co.; 1990.
  41. Decensi A, Puntoni M, Goodwin P, Cazzaniga M, Gennari A, Bonanni B, et al. Metformin and cancer risk in diabetic patients: A systematic review and meta-analysis. *Cancer Prev Res (Phila)* 2010;3:1451-61.
  42. Dowling RJ, Goodwin PJ, Stambolic V. Understanding the benefit of metformin use in cancer treatment. *BMC Med* 2011;9:33.
  43. Goodwin PJ, Ligibel JA, Stambolic V. Metformin in breast cancer: Time for action. *J Clin Oncol* 2009;27:3271-3.
  44. Giovannucci E, Harlan DM, Archer MC, Bergsten RM, Gapstur SM, Habel LA, et al. Diabetes and cancer: A consensus report. *CA Cancer J Clin* 2010;60:207-21.
  45. Dowling R, Goodwin P, Stambolic V. Understanding the benefit of metformin use in cancer treatment. *BMC Med* 2011;9:33.
  46. Pollak M. Metformin and other biguanides in oncology: Advancing the research agenda. *Cancer Prev Res (Phila)* 2010;3:1060-5.
  47. Bojkova B, Orendas P, Garajova M, Kassayova M, Kutna V, Ahlersova E, et al. Metformin in chemically-induced mammary carcinogenesis in rats. *Neoplasma* 2009;56:269-74.
  48. Thompson HJ, McGinley JN, Rothhammer K, Singh M. Rapid induction of mammary intraductal proliferations, ductal carcinoma in situ and carcinomas by the injection of sexually immature female rats with 1-methyl-1-nitrosourea. *Carcinogenesis* 1995;16:2407-11.
  49. Becker ML, Visser LE, van Schaik RH, Hofman A, Uitterlinden AG, Stricker BH. Genetic variation in the organic cation transporter 1 is associated with metformin response in patients with diabetes mellitus. *Pharmacogenomics J* 2009;9:242-7.
  50. Takane H, Shikata E, Otsubo K, Higuchi S, Ieiri I. Polymorphism in human organic cation transporters and metformin action. *Pharmacogenomics* 2008;9:415-22.
  51. Nies AT, Koepsell H, Damme K, Schwab M. Organic cation transporters (OCTs, MATEs), in vitro and in vivo evidence for the importance in drug therapy. *Handb Exp Pharmacol* 2011;201:105-67.
  52. Shu Y, Sheardown SA, Brown C, Owen RP, Zhang S, Castro RA, et al. Effect of genetic variation in the organic cation transporter 1 (OCT1) on metformin action. *J Clin Invest* 2007;117:1422-31.
  53. Shu Y, Brown C, Castro RA, Shi RJ, Lin ET, Owen RP, et al. Effect of genetic variation in the organic cation transporter 1, OCT1, on metformin pharmacokinetics. *Clin Pharmacol Ther* 2008;83:273-80.
  54. Zhu Z, Jiang W, McGinley JN, Price JM, Gao B, Thompson HJ. Effects of dietary energy restriction on gene regulation in mammary epithelial cells. *Cancer Res* 2007;67:12018-25.
  55. Nies AT, Koepsell H, Winter S, Burk O, Klein K, Kerb R, et al. Expression of organic cation transporters OCT1 (SLC22A1) and OCT3 (SLC22A3) is affected by genetic factors and cholestasis in human liver. *Hepatology* 2009;50:1227-40.
  56. Anisimov VN. Effect of buformin on the life span, estrous cycle, and spontaneous tumors. *Vop Onkol* 1980;26:42-8.
  57. Anisimov VN, Dilman VM. Inhibitory effect of phenformin in the development of mammary tumors induced by N-nitrosomethylurea. *Vop Onkol* 1980;26:58.
  58. Neve RM, Chin K, Fridlyand J, Yeh J, Baehner FL, Fevr T, et al. A collection of breast cancer cell lines for the study of functionally distinct cancer subtypes. *Cancer Cell* 2006;10:515-27.

59. Stambolic V, Woodgett JR, Fantus IG, Pritchard KI, Goodwin PJ. Utility of metformin in breast cancer treatment, is neoangiogenesis a risk factor? *Breast Cancer Res Treat* 2009;114:387-9.
60. Ginestier C, Hur MH, Charafe-Jauffret E, Monville F, Dutcher J, Brown M, et al.

ALDH1 is a marker of normal and malignant human mammary stem cells and a predictor of poor clinical outcome. *Cell Stem Cell* 2007;1:555-67.

61. Lim CT, Kola B, Korbonsits M. AMPK as a mediator of hormonal signalling. *J Mol Endocrinol* 2010;44:87-97.

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