Lack of Effect of Metformin on Mammary Carcinogenesis in Nondiabetic Rat and Mouse Models

Matthew D. Thompson¹, Clinton J. Grubbs², Ann M. Bode³, Joel M. Reid⁴, Renee McGovern⁴, Philip S. Bernard⁵, Inge J. Stijlman⁵, Jeffrey E. Green⁶, Christina Bennett⁶, M. Margaret Juliana³, Fariba Moeinpour⁵, Vernon E. Steele⁷, and Ronald A. Lubet⁷

Abstract

Epidemiologic studies have shown that diabetics receiving the biguanide metformin, as compared with sulfonylureas or insulin, have a lower incidence of breast cancer. Metformin increases levels of activated AMPK (AMP-activated protein kinase) and decreases circulating IGF-1; encouraging its potential use in both cancer prevention and therapeutic settings. In anticipation of clinical trials in nondiabetic women, the efficacy of metformin in nondiabetic rat and mouse mammary cancer models was evaluated. Metformin was administered by gavage or in the diet, at a human equivalent dose, in standard mammary cancer models: (i) methyltrinitrosourea (MNU)-induced estrogen receptor–positive (ER⁺) mammary cancers in rats, and (ii) MMTV-Neu/p53KO ER⁻ (estrogen receptor–negative) mammary cancers in mice. In the MNU rat model, metformin dosing (150 or 50 mg/kg BW/d, by gavage) was ineffective in decreasing mammary cancer multiplicity, latency, or weight. Pharmacokinetic studies of metformin (150 mg/kg BW/d, by gavage) yielded plasma levels (Cmax and AUC) higher than humans taking 1.5 g/d. In rats bearing small palpable mammary cancers, short-term metformin (150 mg/kg BW/d) treatment increased levels of phospho-AMPK and phospho-p53 (Ser20), but failed to reduce Ki67 labeling or expression of proliferation-related genes. In the mouse model, dietary metformin (1,500 mg/kg diet) did not alter final cancer incidence, multiplicity, or weight. Metformin did not prevent mammary carcinogenesis in two mammary cancer models, raising questions about metformin efficacy in breast cancer in nondiabetic populations. Cancer Prev Res; 8(3); 231–9. ©2015 AACR.

Introduction

Population studies of metformin

Metformin (1,1-dimethylbiguanide) is an antihyperglycemic drug prescribed for the management of type 2 diabetes (1), and is used worldwide by approximately 120 million people (2). Recently, there has been great interest in the use of metformin as a potential chemopreventive/therapeutic agent based on epidemiologic studies showing a lower incidence of cancer and lower toxicity in diabetics taking this drug compared with those taking sulfonylurea or insulin (3, 4). In 1995, Evans and colleagues (3) reported an association of metformin use with reduced cancer risk in diabetic populations. Among the cancers shown to be lower was breast cancer (5, 6). These findings helped support a large ongoing clinical trial of metformin in a therapeutic adjuvant setting for breast cancer (7, 8).

Molecular, cellular, and physiologic studies with metformin

One area of this research is focused on how metformin alters energy metabolism in cancers. Metformin clearly leads to decreased liver gluconeogenesis and reduced circulating glucose and insulin in diabetics, although the mechanism is unclear. Metformin is thought to inhibit complex I in the electron transport chain in the inner mitochondrial membrane (9), increasing the AMP/ATP ratio in the cell and stimulating AMP-activated protein kinase (AMPK). Dependent on liver kinase B1 (LKB1) activity (10), AMPK is a primary intracellular energy sensor (11). Activated AMPK inhibits the mTOR signaling network to decrease cell proliferation and increase apoptosis. However, understanding mTOR is complicated by the fact that it modulates multiple intracellular and extracellular pathways; therefore, defining an exact mechanism may be complex (12). Furthermore, metformin may act indirectly at cancer sites by altering liver metabolism and reducing systemic risk factors (e.g., glucose, insulin, and IGF-1 signaling), or metformin may act directly to influence cancer cell metabolism at the target site. Though the role of LKB1/AMPK in the activity of metformin is convincing, published studies have led to multiple proposed alternative mechanisms of action such as altered glucagon signaling or altered growth factor signaling via...
receptor tyrosine kinases (13, 14). For breast cancer, the two major pathways that have been routinely discussed are: (i) activation of AMP kinase secondary to the primary effect on LKB1 (11–13) altering the expression of various genes involved in gluconeogenesis; and (ii) metformin reducing levels of IGF1, a mechanism that may be relevant for a variety of cancers (15).

Animal studies with metformin

Although numerous publications on metformin have focused on cell culture studies (1, 16), in vivo animal data using in situ arising mammary cancer models are more limited (17, 18). We examined the effects of metformin in three animal models of breast cancer routinely used to screen for chemopreventive agents. In all three studies, we used standard rodent chow. The specific models were: (i) the MNU-induced model of estrogen receptor–positive (ER+) mammary cancer in rats. These tumors respond to the same hormonal agents observed in ER+ human breast cancer (19, 20), and are similar by array analysis to well-differentiated human ER+ tumors (21); (ii) the MMTV-Neu/p53KO model of estrogen receptor–negative (ER−) mammary cancer in mice. This is a model of ER− Neu–overexpressing cancers in humans, which overwhelmingly have p53 alterations (22, 23); and (iii) a therapeutic xenograft assay with cells derived from the C31 T antigen model of breast cancer, which by array analysis appears similar to the human basal/triple-negative subtype of breast cancer (24). The data for this therapeutic model, which used a different mode of agent delivery, are presented as Supplementary Materials in this article. In all three assays, metformin failed to inhibit cancer formation or growth. Pharmacokinetic (PK) data in the MNU rat model showed that metformin achieved plasma levels slightly higher than those achieved at a standard dose in humans, and did modulate biochemical endpoints such as AMP kinase levels and phosphorylation of p53. Finally, short-term treatment with metformin failed to decrease Ki67 levels by IHC, and failed to decrease the expression of multiple proliferation-related genes (Mki67, Ccne1, and Top2a) determined by RT-PCR in MNU mammary cancers of rats, whereas similar treatment with tamoxifen strikingly decreased Ki67 levels. These latter studies showed that metformin was ineffective as a chemopreventive agent despite achieving substantial plasma levels and altering known pharmacodynamic endpoints, for example, phosphorylated p53.

Materials and Methods

MNU-induced rat mammary cancer model

The MNU-induced rat mammary cancer model was performed as previously described (20, 23). In brief, female Sprague–Dawley rats (Harlan Sprague-Dawley, Inc.), were placed on standard Teklad diet (4% fat by weight; 8% fat by caloric intake) and administered the carcinogen MNU (75 mg/kg BW) by i.v. injection at 50 days of age. Five days after MNU, the animals were dosed by gavage with metformin; 150 or 50 mg/kg BW/d in saline. The animals received the treatment until termination of the study at 126 days after MNU injection. The rats were weighed 1× per week, palpated for mammary tumors 2× per week, and observed daily for signs of toxicity. Mammary tumors were excised, weighed, and processed for histologic classification at the termination of the study. Differences in latency and cancer weights were determined by log-rank analysis and Mann–Whitney, respectively.

Short-term biomarker determination

A presurgical animal model was used to examine the effect of metformin on pharmacodynamic biomarkers. This model most closely approximates the presurgical intervention that has often been used in clinical breast cancer trials (25). For determination of biomarkers (IHC or RT-PCR), animals bearing small MNU-induced ER+ mammary cancers were treated for 7 days with metformin (150 mg/kg BW/d), or tamoxifen (100 mg/kg of diet) as a positive control. Animals were sacrificed and tumors were either snap frozen (RT-PCR), placed in Zamboni’s fixative (IHC), or fixed in formalin for 24 hours.

Immunohistochemistry and DAPI staining

Mammary cancers were harvested and fixed in Zamboni’s fixative and embedded in Optimal Cutting Temperature Compound (Tissue Tek, Sakura Finetex USA, Inc.). Samples were washed with TPBS (1×PBS-0.3% Triton X-100) and blocked overnight with 5% normal donkey serum. Primary antibodies were prepared in 1% normal donkey serum TPBS and incubated for 7 hours with samples at room temperature and then washed with TPBS overnight. Samples were washed with additional fresh TPBS for 1 hour. Secondary antibodies were prepared using 1% normal donkey serum TPBS and added to each well. Secondary antibodies were left on the samples 5 hours at room temperature, washed in TPBS overnight, and washed again in PBS a second overnight. After secondary antibody staining/washing, DAPI was diluted in PBS at 1:50,000. The samples were incubated for 30 minutes followed by several washes in PBS with gentle gyrotary shaking for 1 hour. After washing was complete, samples were mounted on coverslips using 1.35% to 1.5% Noble agar, then dehydrating using progressively greater percentages of alcohol, cleared with xylene and mounted on coverslips using cytoseal. All samples were attached to coverslips and allowed to set at room temperature until dry (1–5 minutes), and mounted onto slides using Cytoseal XYL (Richard-Allan Scientific, 8312-4). Slides were examined with a Nikon Confocal Microscope.


Proliferation by RT-qPCR

Sample preparation and first-strand cDNA synthesis. MNU-induced mammary tumors from nontreated, tamoxifen-treated, and metformin-treated (n = 10) rats were processed as formalin-fixed, paraffin-embedded tissue blocks. Two 5-μm scrolls were taken from each tumor block and total RNA was extracted using the Roche High Pure miRNA Isolation Kit (Roche Applied Science). The quantity of RNA was assessed using the NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies, Inc.). First-strand cDNA was synthesized from 600 ng RNA using a 2 pmol mixture of reverse primers, 100 ng of random hexamers, and Superscript III reverse transcriptase (1st Strand Kit; Invitrogen). The reaction

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was held at 55°C for 60 minutes followed by a 15-minute step at 70°C. The cDNA was washed on a QiAquick PCR purification column (Qiagen) and was stored at −80°C in TE (10 mmol/L Tris-HCl, pH 8.0, 0.1 mmol/L EDTA) until qPCR analysis.

**PCRs and relative quantification.** Primer sets were selected to have similar GC contents and Tms using the LightCycler Probe Design software (version: 2.0.B.22; Roche Applied Science). Each primer set was validated using a pool of cDNA from different mammary cancers processed as formalin-fixed, paraffin-embedded tissue blocks. PCR reactions (5-μL) were prepared in 384-well plates using a robotic Evolution P3 Precision Pipetting Platform (PerkinElmer Ltd.). Each reaction contained 2.5 μL Roche LC 480 SYBR Green I Master Mix 2X, 0.4 μmol/L of each primer, and 1.25 ng cDNA. A pool of cDNA from rat mammary cancers was provided in each run as a calibrator reference and assigned a value of 10 ng for each sample. Samples and calibrator were run in duplicate. PCR amplification was performed using the LC480 (Roche Applied Science) with an initial denaturation step (95°C, 8 minutes) followed by 45 cycles of denaturation (95°C, 4 seconds), annealing (56°C, 6 seconds with 2°C/s transition), and extension (72°C, 6 seconds with 2°C/s transition). Fluorescence (530 nm) from the SYBR Green I dsDNA dye was acquired for each cycle after the extension step. The specificity of the PCR was determined by post-amplification melting curve analysis. Reactions were automatically cooled to 65°C and slowly heated at 2°C/s to 99°C, while continuously monitoring fluorescence (10 acquisitions/°C). Relative copy numbers were calculated from an external standard curve (efficiency 1.8) and correcting to the Cp of the 10 ng calibrator. To control for variation in RNA quality, the copy number for each gene was further normalized to actb, which served as a housekeeper gene. Values from replicate samples were averaged and data were log 2 transformed. The primers used for the RT-PCR studies were ccnb1 R3F gaggtaggatgtagg ccnb1 R3R ggccttagaggatcatca; Mki67 R3F gcctgcactcag Mki67 R3R ttgcttagactctgagac; Top 2A R1F ccaggcagagactagact Top 2A R1R tigctttgcctcgtga.

**Pharmacokinetic studies.** Female Sprague-Dawley rats were administered metformin (150 mg/kg BW/d) i.p. for 14 days. Plasma samples were collected on days 1 and 14. Metformin concentrations in plasma were determined using an LC/MS-MS method as modified from Tucker and colleagues (ref. 26; See Supplementary Materials). Plasma concentration-time data were analyzed by noncompartmental methods using the program WinNonlin Version 4.1 (Pharsight Corporation). Additional materials can be found in the Supplementary Information (e.g. Supplementary Fig. S1).

**MMTV-Neu/p53KO ER− mouse mammary cancer model**

MMTV-Neu+/−/p53 KO−/− mice were generated and maintained in the Institutional Animal Care and Use Committee approved laboratories at the University of Alabama at Birmingham as previously described (23). MMTV-Neu transgenic mice (strain FVB/N-Tg (MMTV-Neu) 202 Mul/J) were purchased from The Jackson Laboratory. A p53-deficient line (p53 NS-T) was purchased on a C57BL background (Taconomic) and back-crossed at least five times onto a FBV/N background. For generation of MMTV-Neu+/−/p53 KO−/− females, p53−/− males were crossed to MMTV-Neu+/− females. MMTV-Neu+/− females were generated by crossing MMTV-Neu+/− males with FVB/NJ females. The animal rooms were maintained at 22°C ± 2°C and lighted 12 hours per day in a facility specially designed for administering chemical carcinogens to animals. Animals were allowed free access to the diet (Teklad 4% mash) and water throughout the duration of the experiments. Mice were placed on control diet or diet containing 1,500 mg metformin/kg of diet beginning at 60 days of age. Metformin was obtained from the National Cancer Institute (NCI) Prevention Repository and incorporated into the mash diet by mixing with a liquid–solid blender (Patterson-Kelly Co.). Mice were examined weekly for the development of palpable mammary tumors beginning at 4 months of age. Mice were kept on the control or metformin-containing diets until approximately 11 months of age.

**Results**

**Effects of metformin on ER+ mammary cancers**

Two different doses of metformin (150 or 50 mg/kg BW/d) were administered by gavage to rats beginning 5 days after MNU administration; with the higher dose being equivalent to a human dose of 1.5 g/d based on the FDA human equivalent dosing calculation. Neither dose significantly altered tumor latency (Fig. 1A) or multiplicity (Fig. 1B). However, metformin was associated, with a slight increase (0.1 > P > 0.05) in the final tumor weights at the end of the study (Fig. 1C).

**Effects of metformin in the ER− mouse transgenic model**

Metformin administered in the diet (1,300 mg/kg) to MMTV-Neu/heterozygous p53/KO mice failed to alter survival of the mice (Fig. 2A), tumor multiplicity (Fig. 2B), or decrease tumor weights of treated mice (Fig. 2C). Metformin in an ER− mouse mammary tumor xenograft model (Supplementary Fig. S2) was administered at 100 or 150 mg/kg BW i.p., twice daily. Neither dose significantly inhibited the growth of the C3(1)/Tag tumor xenografts.

**Biomarker studies on metformin in the MNU rat model**

As can be seen in Fig. 3A and B, a limited (albeit statistically significant) increase in phospho-AMPK was observed in the tumors of rats treated for 7 days with metformin. Interestingly, a substantial increase in the levels of phosphorylated p53 at serine 20 and a smaller increase at position 392 were observed, along with greatly increased levels of phosphorylated p70S6 kinase at threonine 389. Upon further examination of levels of p53 phosphorylation in MCF cells treated in cultures with metformin, p53 was similarly phosphorylated at these two sites confirming the in vivo results (Supplementary Fig. S3). We also examined the effects of metformin on the proliferation of metformin-treated tumors using Ki67. Treatment with metformin did not decrease the proliferation index (~30% increase, 0.1 < P < 0.05; Fig. 4A). In contrast, tamoxifen, a highly effective preventive agent, decreased...
the Ki67 index by roughly 75% \((P < 0.05)\). Finally, the effect of metformin on proliferation-associated genes (Fig. 4B) was examined. Gene expression of the proliferation-related genes mki67, cccb1, and top2a was decreased by tamoxifen but not metformin, in agreement with the effects on Ki67 observed with IHC.

**Pharmacokinetic studies with metformin in female Sprague-Dawley rats**

Plasma concentrations were measured in female Sprague-Dawley rats on days 1 and 14 after treatment with 150 mg/kg BW/d by gavage. Metformin plasma profiles and PK data are presented in Supplementary Fig. S1 and Supplementary Tables S1 and S2. Mean plasma concentrations of 7.47 ± 0.79 μg/mL and 6.72 ± 0.39 μg/mL were achieved 1 hour after the oral dose on days 1 and 14, respectively (Table 1). The steady-state \(\text{AUC}_{0-24\text{h}}\) value of 44.6 μg/mL × h on day 14 was similar to the \(\text{AUC}_{0-24\text{h}}\) value of 47.7 μg/mL × h on day 1, indicating that the drug did not accumulate in plasma during the daily administration schedule.

**Discussion**

Metformin, an antidiabetic, has generated a great deal of interest in recent years as a potential preventive and therapeutic agent (1, 2). Part of the initial interest was based on epidemiologic studies showing that individuals taking metformin (as contrasted with other antidiabetic agents) had a lower incidence of cancer, although certain recent epidemiologic studies have not confirmed this observation. Among the cancers shown to be lower was breast cancer (5, 6). Although certain of the more recent epidemiologic data have shown more limited efficacy. There has been great enthusiasm for the use of metformin because of its relatively low toxicity, and the fact that it alters energy metabolism, which is felt to be altered in most cancers. Furthermore, the main mechanistic alterations induced by metformin are (i) effects on LKB1, which secondarily affects activity of cAMP kinase and the mTOR pathway and/or (ii) effects on the IGF1 pathway (15). Both of these pathways would appear relevant for a wide variety of cancers. *In vitro* studies have confirmed the expected mechanistic results, and show that metformin is preferentially effective in tumor cells (1, 16). There have also been a variety of xenograft studies (27). However, there have been relatively few studies using *in situ* arising mammary cancers that are typically used in prevention studies. Given this limited number of studies in prevention models, we examined the use of metformin in two standard mammary cancer prevention models, and in a graft model more closely approximating the basal subtype of breast cancer. The MNU model of mammary cancer that develops ER \(^-\) cancers appears by gene array analysis to be similar to highly differentiated ER \(^+\) mammary cancers in humans (21). The model has been shown to be sensitive to hormonal manipulations that decrease ER \(^+\) tumors in women, including SERMs, aromatase inhibitors, and ovariectomy (19, 20). We failed to observe any preventive activity of metformin at a dose comparable with standard human doses. The higher dose was chosen to be the equivalent of a human dose of approximately 1.5 g/d based on standard FDA scaling factors. Somewhat surprisingly, we found that the \(C_{\text{max}}\) and AUC values (Table 1) of this metformin dose were roughly 2- to 3-fold higher than the \(C_{\text{max}}\) (3.1 ± 0.93 μg/mL) and AUC (18.4 ± 6.5 μg/mL × h) values for a dose in humans of 1.5 g/d (26). Despite the fact that the PK data were higher than that achieved in humans, no cancer-preventive activity in the rat model was observed. Of note, the peak concentration of metformin achieved in plasma of approximately 7.5 μg/mL, equivalent to a concentration of 60 μmol/L, is markedly less than a media concentration of ≥1 mmol/L (and more typically 2.5–10 mmol/L) used in cell culture to achieve efficacy (Supplementary Fig. S2; ref. 16). We observed qualitative increases in final tumor weights at both doses of metformin, which was marginally significant at the 150 mg/kg BW/d dose \((0.05 < P < 0.075)\). A prior study by Zhu and colleagues (17) in MNU-treated rats with a modified method similarly failed to significantly inhibit tumor formation until doses >3× higher were used.

We used two mouse models of breast cancer to test the efficacy of metformin. The first model was the MMTV-Neu p53/KO...
model, which develops ER− tumors with an altered p53 and overexpression of Neu (22, 23). This is similar to ER− Neu-overexpressing tumors in humans, which similarly show overexpression of Neu and mutations in P53. The Neu model has been shown to be sensitive to the preventive effects of both EGFR inhibitors and RXR agonists (28, 29), which can profoundly increase latency and decrease tumor incidence. In the MMTV-Neu model, metformin was administered in the diet at a dose similar to the human equivalent dose. The reason we used diet for the mouse study was due to the practical limitations of administering metformin by gavage for an extended time period. The dose used (1,500 mg/kg diet) is in line with the human equivalent dose of 1.5 g/d using the standard scaling factors. The results show that metformin failed to demonstrate any preventive activity (Fig. 2A and B). There was a prior report showing a significant, albeit not very striking, effect of metformin at a similar dose when given in water (30). The PK of the two is likely to be somewhat different. However, even the metformin data in the prior study, although administered early in life, were only marginally effective, increasing tumor latency by only 7%. This must be viewed in the context of highly effective agents that can increase latency more than 60% and strongly decrease tumor incidence (28, 29). We also performed a more limited therapeutic study of metformin using a tumor graft derived from a transgenic C31 T antigen mouse (31) and administered to immunosuppressed SCID mice. These tumors appear by array analysis to be quite similar to basal-type breast cancer in humans (24). This study (Supplementary Fig. S2) examined the therapeutic effects of metformin following i.p. administration, and no efficacy was observed. The i.p. dose was used because a lung cancer prevention model had found it to be highly effective (32), although administration by this route is unlikely for a prevention study in humans. We have examined the efficacy of metformin in additional in situ models in other organs, including colon, head and neck, and urinary bladder, and failed to achieve positive results in animals on a standard chow diet (data not shown; Lubet and Grubbs). There is, however, a preclinical study in a pancreatic model, which achieved strong efficacy after dietary administration of metformin at doses similar to those we used in the MMTV-Neu/p53KO model, which was intended to parallel the human equivalent dose (33).

We determined whether the treatments used altered potential biomarkers in the MNU model (25). The model parallels a presurgical model in humans. Specifically, a small palpable ER+ tumor is allowed to develop and the rat was treated short-term with metformin (or tamoxifen as a positive control; Fig. 4A). We first looked at Ki67 labeling in cancers treated short-term with metformin, and found that metformin increased proliferation in the treated tumors. In contrast, tamoxifen (which greatly

Figure 2.
Effects of metformin on the development of mammary carcinomas in MMTV-Neu/p53KO mice. Weanling mice were characterized for the presence both the MMTV-Neu transgene and the KO of one copy of the tumor-suppressor gene p53. Mice were placed on control diet or diet containing 1,500 mg/kg metformin at 60 days of age. Metformin did not significantly increase survival (P < 0.05; (A), and also did not impact incidence, multiplicity (B), or cancer weight (C).
decreases tumor incidence) decreased Ki67 roughly 75%. These studies must be seen in the context of our prior studies that showed that a wide variety of agents, which inhibit mammary cancer formation (SERMs, aromatase inhibitors, EGFR inhibitors, RXR agonists etc.), inhibit proliferation, whereas ineffective agents do not (25). The effects of metformin and tamoxifen were then examined for their effects on expression of \(Mki67\), \(Ccnb1\), and \(Top2A\) by RT-PCR (Fig. 4B). The specific genes used were initially defined as part of a set of genes associated with proliferation in human breast cancer (34, 35). Genes involved in cell-cycle regulation (i.e., proliferation) are prognostic for determining risk of recurrence in women with ER\(^+\) breast cancer receiving endocrine therapy alone (34–36). We selected three proliferation genes (\(Mki67\), \(Ccnb1\), and \(Top2A\)) that are important for prognosis in ER\(^+\) human breast tumors, and determined their change in expression in ER\(^+\) cancers in the rat after treatment with tamoxifen or metformin. The proliferation markers MKI67 and CCNB1 are both included in the risk algorithms for Oncotype Dx and Prosigna (i.e., PAM50; refs. 34–36). Topoisomerase 2\(a\) protein expression is highly correlated with Ki67 protein expression in breast cancer and may provide additional predictive information in response to anthracycline regimens (37–39). The effects of

**Figure 3.**
Effects of metformin on expression of multiple biomarkers using IHC. Rats bearing MNU-induced cancers were treated with vehicle or metformin (150 mg/kg BW/d) for a period of 7 days. At that time, rats were sacrificed and tumors removed. Tissue slices were prepared and stained as described in Materials and Methods. Representative staining is shown. A, alterations of expression in the various phosphorylated proteins in control mammary tumors following short-term metformin exposure; B, IHC slides staining for the various phosphoproteins, DAPI, and a merged image.
metformin kinase and a number of phosphorylated proteins involved in cell cycling (Fig. 3) were also evaluated. A limited, but significant, increase in AMP kinase was observed. We also saw an increased phosphorylation of p53 at serines 20 and 392 sites. Unexpectedly, an increased level of P30S6 kinase, a ribosomally associated kinase, was also seen. Increased levels of the latter might be expected with an agent that causes increased proliferation of tumors (as shown in Fig. 4A). It was observed almost 10 years ago that p53 is a substrate for AMPK.

In fact, p53 (via downstream proteins) significantly contributes to the overall response to gluconeogenesis and may induce autophagy (38–40). We subsequently determined whether phosphorylation was increased at these sites in MCF-7 cells exposed in cell culture to metformin (Supplementary Fig. S3). Similar increases in these p53 phosphorylation sites in MCF-7–treated cells occurred. However, the doses used in vitro (>1 mmol/L) are much higher than the C_{max} achieved in plasma either in our studies or in human studies. These changes are obviously not efficacy biomarkers, because metformin was ineffective as a preventive agent in this model. Nevertheless, they may be pharmacodynamic biomarkers, indicating that metformin has reached the target organ and has a physiologic effect. One of the more interesting aspects of metformin efficacy was an early article by Buzzai and colleagues (40) showing metformin was preferentially active in cells with a knockout (KO) of p53. Although our data does not include animal cancers with a clear p53 mutation or KO p53, the MMTV-Neu ER^- mammary cancer model is heterozygous for p53 deletion and is likely to lose the second copy of p53 (16).

Our lack of efficacy in commonly used mammary cancer models is disconcerting. These results argue for testing agents using standard prevention models at doses close to the human equivalent dose using the same route of administration. Our PK data show that reasonable doses were administered. These results encourage clinical trials at the phase IIA levels using biomarkers more directly related to preventive efficacy (41, 42), and not purely biochemical parameters (activated AMP kinase) as the primary endpoint. In fact, there were two recent biomarker studies in breast cancer using metformin, with proliferation as a potential biomarker. Although the smaller trial that lacked a placebo control yielded positive results (41), the larger trial was negative and, in fact, observed an increase in proliferation index in women with a body mass index <25 (42), similarly to our present data (Fig. 4A). It is hoped that this will temper enthusiasm for large phase II or III trials in the absence of clear phase IIA data, particularly in the more general population. Perhaps the greatest clinical question deals with the ongoing phase III adjuvant trial using metformin in an adjuvant setting at a dose of 1.7 g/d, which is close to the dose we used (7, 8). The majority of these participants are likely either to have ER^- and/or Neu–overexpressing tumors, in which our present data look clearly ineffective. This trial specifically precludes the inclusion of diabetics where the epidemiologic data look somewhat promising. It is emphasized that the present data are in animals that were on a standard diet, that is, animals were neither diabetic nor prediabetic. It certainly is possible that in a model with the altered physiology associated with diabetes or prediabetes, one may observe preventive activity.

### Table 1. Mean plasma concentration-time data and PK estimates

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<th>Group 1 (day 1)</th>
<th>Group 2 (day 14)</th>
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<tr>
<td>1</td>
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<th>PK estimates</th>
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<td>C_{max} (ng/mL)</td>
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<td>AUC_{0-24 h} (ng/mL × h)</td>
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**Figure 4.**

Effects of metformin on expression of proliferation-related biomarkers. Rats were treated as described in Materials and Methods. Both expression of proliferation-related genes and Ki67 values were determined in formalin-fixed samples. A, effects of metformin and a positive control (tamoxifen) on proliferation index in mammary cancers. Results are based on counting at least 2,000 cells for each of the tumors. The Ki67-labeling index was significantly increased in cancers treated with metformin and decreased in cancers treated with tamoxifen (P < 0.05) when compared with controls. B, effects of metformin and tamoxifen on expression of proliferation-related genes.
Disclosure of Potential Conflicts of Interest

Philip S. Bernard has ownership interest (including patents) in BioClassifier LLC. No potential conflicts of interest were disclosed.

Authors’ Contributions

Conception and design: C.J. Grubbs, J.E. Green, V.E. Steele, R.A. Lubet
Development of methodology: C.J. Grubbs, J.M. Reid, R. McGovern, V.E. Steele
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): C.J. Grubbs, A.M. Bode, J.M. Reid, R. McGovern, J.J. Stijlenman, J.E. Green, C. Bennett, F. Moepionpur
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): M.D. Thompson, A.M. Bode, J.M. Reid, R. McGovern, P.S. Bernard, J.E. Green, C. Bennett, M.M. Juliana, R.A. Lubet
Writing, review, and/or revision of the manuscript: M.D. Thompson, C.J. Grubbs, A.M. Bode, J.M. Reid, P.S. Bernard, C. Bennett, F. Moepionpur, V.E. Steele, R.A. Lubet

Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): C.J. Grubbs

Grant Support

NCI Contract Number HHSN261201200021I awarded to Dr. Clinton J. Grubbs, University of Alabama at Birmingham (Birmingham, AL).

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Received June 9, 2014; revised December 12, 2014; accepted December 28, 2014; published OnlineFirst February 13, 2015.

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