Effects of Metformin, Buformin, and Phenformin on the Post-Initiation Stage of Chemically Induced Mammary Carcinogenesis in the Rat

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Abstract

Metformin is a widely prescribed drug for the treatment of type II diabetes. Although epidemiologic data have provided a strong rationale for investigating the potential of this biguanide for use in cancer prevention and control, uncertainty exists about whether metformin could be expected to have an impact in nondiabetic patients. Furthermore, little attention has been given to the possibility that other biguanides may have anticancer activity. In this study, the effects of clinically relevant doses of metformin (9.3 mmol/kg diet), buformin (7.6 mmol/kg diet), and phenformin (5.0 mmol/kg diet) were compared with rats fed control (9.3 mmol/kg diet), buformin (7.6 mmol/kg diet), and phenformin (5.0 mmol/kg diet) were compared with rats fed control diet (AIN93-G) during the post-initiation stage of 1-methyl-1-nitrosourea-induced (50 mg/kg body weight) mammary carcinogenesis (n = 30/group). Plasma, liver, skeletal muscle, visceral fat, mammary gland, and mammary carcinoma concentrations of the biguanides were determined. In comparison with the control group, buformin decreased cancer incidence, multiplicity, and burden, whereas metformin and phenformin had no statistically significant effect on the carcinogenic process relative to the control group. Buformin did not alter fasting plasma glucose or insulin. Within mammary carcinomas, evidence was obtained that buformin treatment perturbed signaling pathways related to energy sensing. However, further investigation is needed to determine the relative contributions of host systemic and cell autonomous mechanisms to the anticancer activity of biguanides such as buformin. Cancer Prev Res; 8(6); 518–27. ©2015 AACR.

Introduction

Considerable attention is being given to using metformin, one of the most widely prescribed drugs for the treatment of type II diabetes, for the prevention and control of a number of types of cancer, including breast cancer (1–3). Although epidemiologic studies, coupled with in vitro mechanistic evidence, have propelled metformin into clinical trials as a potential chemopreventive and adjuvant, preclinical observations have raised concerns about metformin efficacy (4–8). Magnifying the problem are conflicting reports about mechanism of action, particularly in the nondiabetic state (3, 9, 10).

Metformin is prescribed for the treatment of type II diabetes, but understanding of its mechanism of action in this context is limited and has recently been challenged (11,12). The prevailing view has been that metformin induces activation of AMP-activated protein kinase (AMPK) in the liver, presumably by reducing intracellular energy charge via partial inhibition of complex I activity. However, evidence from mice has been inconsistent, with the absence of either LKB1 or AMPK activity failing to block the glucose lowering effects of metformin (12). Rather, new evidence has been published indicating that metformin, as well as a related biguanide, phenformin, induce AMP accumulation resulting in inhibition of adenylyl cyclase and downregulation of protein kinase A activity (11). It is hypothesized that reduced activity of protein kinase A decreases the expression of genes that regulate hepatic gluconeogenesis, thereby decreasing hepatic glucose output. Nonetheless, this work failed to clarify the mechanism that accounts for AMP accumulation. One possible alternative to the proposed inhibition of complex I is that metformin and other biguanides inhibit the activity of AMP deaminase, the enzyme that catalyzes the conversion of AMP to IMP (13–15). If AMP deaminase is inhibited, AMP would accumulate intracellularly, not only activating AMPK, but also inhibiting adenylyl cyclase and leading to decreases in cyclic AMP and the activity of protein kinase A.

Recognizing that other biguanides differ from metformin in their biologic activities and that phenformin and buformin have been reported to inhibit mammary carcinogenesis in various experimental models (7, 16, 17), the experiments reported herein were conducted to compare effects of these biguanides. On the basis of the results of the carcinogenesis study, additional experiments were conducted to generate hypotheses about potential targets of the most effective anticancer biguanide, buformin, and about the likely involvement of host systemic and cell autonomous mechanisms in accounting for cancer inhibitory activity.

Materials and Methods

Chemicals

Primary antibodies used in this study were purchased from Cell Signaling Technology: anti-pPKAThr198/PKA, anti-pCREBSer133/Ser143/...
CREB, anti-pSTAT5 Tyr705/STAT3, anti-pSrc Ser127/137/Src, anti-pACC Ser79/ACC, anti-pAKT Ser473/Akt, anti-pAMPK Thr172/AMPK, anti-p70S6K Thr389/p70S6K, anti-Beclin2/AR, anti-FAK, anti-FASN, anti-GLUT4, anti-OCT3, anti-NOTCH1, anti-PGC1, anti-PPARα Ser246/PPARα, anti-pRaptor Ser792/anti-Raptor, and anti-PIK110x. Anti–β-C1.2 L-1-nitrosourea was used for the detection of mammary tumors twice weekly starting from 21 days after carcinogen. At necropsy, rats were skinned and the skin to which mammary gland chains were attached was examined under translucent light for detectable mammary pathologies at 5× magnification. All detectable mammary gland pathologies were excised and prepared for histologic classification according to published criteria (26, 27). Only confirmed mammary carcinomas are reported. The experimental protocols were reviewed and approved by the Institutional Animal Care and Use Committee and conducted according to the committee guidelines.

**Blood collection and plasma biomarker analyses**

**Blood collection.** Following an overnight fast, rats were euthanized over a 3-hour time interval, between 8 and 11 am, via inhalation of gaseous carbon dioxide. The sequence in which rats were euthanized was stratified across groups to minimize the likelihood that order effects would masquerade as treatment-associated effects. After the rat lost consciousness, blood was directly obtained from the retro-orbital sinus and gravity fed through heparinized capillary tubes (Fisher Scientific) into EDTA-coated tubes (Becton Dickinson) for plasma. The bleeding procedure took approximately 1 minute/rat. Thereafter, the unconscious rat was euthanized by cervical dislocation. Plasma was isolated by centrifugation at 1,000 × g for 10 minutes at room temperature.

**Assessment of circulating molecules.** Insulin-like growth factor 1 (IGF1), IGF-binding protein 3 (IGFBP-3), adiponectin, insulin, leptin, and glucagon in plasma or cAMP in tissue lysate were determined using commercially available ELISA assays as previously described. Glucose in plasma was determined enzymatically using a commercially available kit (Pointe Scientific, Inc.).

**Western blotting.** Each mammary carcinoma was homogenized in lysis buffer (40 mmol/L Tris-HCl [pH 7.5], 1% Triton X-100, 0.25 mol/L sucrose, 3 mmol/L EGTA, 3 mmol/L EDTA, 50 μmol/L L-mercaptoethanol, 1 mmol/L phenyl-methylsulfonyl fluoride, and complete protease inhibitor cocktail (Calbiochem)). The lysates were centrifuged at 7,500 × g for 10 minutes at 4°C and supernatant fractions collected and stored at −80°C. Supematant protein concentrations were determined by the Bio-Rad protein assay (Bio-Rad). Western blotting was performed as described previously. Briefly, 40 μg of protein lysate per sample was subjected to 8% to 16% SDS-PAGE after being denatured by boiling with SDS sample buffer [63 mmol/L Tris-HCl (pH 6.8), 2% SDS, 10% glycerol, 50 mmol/L DTT, and 0.01% bromophenol blue] for 5 minutes. After electrophoresis, proteins were transferred to a nitrocellulose membrane. The levels of pPKA Thr198/PKA, pCREB Ser133/CREB, pSTAT3 Tyr705/STAT3, pSrc Ser127/137/Src, pACC Ser79/ACC, pAKT Ser473/Akt, pAMPK Thr172/AMPK, p4EBP1 Thr37/4EBP1, p70S6K Thr389/p70S6K, EPAC-1, Gfiren, PI3K110, and β-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 1,300 × 1,030. Quantity One software was used for image analysis.

**HPLC analysis of biguanides.** Diet and tissue were homogenized (20% w/v) using ultrasound and then extracted with acetonitrile/methanol (2/1 v/v) for 30 minutes at room temperature. Follow ing extraction, samples were centrifuged at 17,000 × g for 10 minutes and the supernatant fraction was directly analyzed via HPLC using a Waters C18 reverse phase column (3.9 mm column, 3.9 mm, 5 μm, Bondapak C18, 10 mm column, 3.9 mm, 5 μm, Bondapak C18, 300 mm, equipped with a diode array detector). The HPLC system consisted of a Waters 2487 UV detector, a 590 manual injector, and a 515 series pump. The mobile phase was 40 mmol/L KH2PO4/K2HPO4 pH 6.8: ACN (37:63) at a flow rate of 1.5 mL/minute, the injection volume was 20 μL and the run time was 8 minutes.
Table 1. Effect of biguanides on final mammary cancer incidence and multiplicity

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Palpable carcinomas*</th>
<th>Microcarcinomas</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Incidence %</td>
<td>Multiplicity</td>
<td>Incidence %</td>
</tr>
<tr>
<td>Control</td>
<td>56.7c</td>
<td>1.2 ± 0.3d</td>
<td>80.0c</td>
</tr>
<tr>
<td>Metformin</td>
<td>76.7c</td>
<td>2.0 ± 0.5</td>
<td>86.7c</td>
</tr>
<tr>
<td>Buformin</td>
<td>16.7c</td>
<td>0.2 ± 0.1c</td>
<td>36.7c</td>
</tr>
<tr>
<td>Phenformin</td>
<td>46.7c</td>
<td>0.6 ± 0.1c</td>
<td>63.5c</td>
</tr>
<tr>
<td>Overall P</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

NOTE: Values are mean ± SEM for cancer multiplicity, n = 30 rats/group. Cancer incidence was analyzed by the Fisher exact test and cancer multiplicity by Poisson regression. Values within a column with different superscripts (c–e) were statistically different by post hoc multiple comparisons, P < 0.008.

*Eleven (9%) of these carcinomas were detected at necropsy. This 9% error rate was not statistically different among groups (P = 0.26).

Biguanide was provided in the diet at concentrations of: 9.3 mmol metformin/kg diet, 7.6 mmol buformin/kg diet, or 5.0 mmol phenformin/kg diet.

(Bio-Rad) was used in the analysis. The Quantity One software has a warning algorithm that notifies the user if pixel density is approaching saturation so that all signals used for analysis are in the linear range. All Western blot signals were within a range where the signal was linearly related to the mass of protein and actin-normalized scanning density data were used for analysis.

Statistical analyses

Differences among groups were evaluated as follows: incidence of mammary carcinomas by the Fischer exact test, the number of mammary carcinomas per rat (multiplicity) by Poisson regression, and cancer burden by the nonparametric Kruskal–Wallis test. Palpable cancer latency was evaluated by survival analysis using the Mantel–Haenszel method. P values were adjusted for multiple comparisons using a Bonferroni correction. Differences in final body weight and circulating analytes were evaluated by ANOVA with post hoc comparisons by the method of Tukey (28). For Western blot analyses, the data were either the actin-normalized scanning data for proteins or the ratio of the actual scanning units derived from the densitometry analysis of each Western blot analysis for the phospho-proteins. For statistical analyses, the actin-normalized scanning density data obtained from the ChemiDoc scanner using Quantity One (Bio-Rad) were rank transformed, an approach that is particularly suitable for semiquantitative measurements that are collected as continuously distributed data, as is the case with Western blot analyses (29). Ratio data were computed from the scanning units derived from the densitometry analysis, that is, the arbitrary units of optical density, and then the ratios were rank transformed and medians ± the interquartile ranges were computed and shown using scatter plots (Supplementary Fig. S1). All analyses were performed using Systat statistical analysis software, version 13 (Systat Software, Inc.) or Prism for Windows, version 6 (GraphPad Software Inc.). All P values are two sided and statistical significance was set a priori at P < 0.05.

Multivariate analysis

Supervised and unsupervised multivariate techniques were used to evaluate and visualize the data per our previously published approach (30–33).

Unsupervised analysis. Principal components analysis (PCA) was used to analyze plasma analytes and Western data (34). The PCA model can be written as follows:

\[ X = Xbar + TP' + E, \]  

where \( X \) is the matrix of measured variables, \( Xbar \) is a vector of means (all 0 when the data are centered), \( T \) is a matrix of scores that summarize the \( X \) variables, \( P \) is a matrix of loadings, and \( E \) is a matrix of residuals.

Supervised multivariate analysis

Orthogonal projections to latent structures for discriminant analysis (OPLD-DA) was used as a supervised, class-based method (30–32, 35) to study plasma analytes and Western data. The OPLS-DA model can be written as follows:

\[ Y = T_y P_p' + E, \]  

where the interpretation of equation B is similar to that for the PCA model; however, an additional rotation has been applied using the class information to partition \( T_y \) into a predictive, \( T_p P_p' \), and an orthogonal, \( T_o P_o' \), component. The number of predictive and orthogonal components in the models was determined by cross-validation.

Three key statistics describe the fit of each model: \( R^2_X \) (cum), the total amount of explained variation in \( X \); \( R^2_Y \) (cum), the total amount of variation explained in \( Y \); and \( Q^2 \) (cum), the total amount of predicted variability in \( Y \), estimated by 7-fold cross-validation. The contribution of each component partitioned into between (predictive) and within (orthogonal) class is also estimated, and summarized as \( R^2_X \) and \( R^2_Y \), respectively. \( R^2_X \) and \( R^2_X \) were drawn, along with 95% confidence ellipses based on Hotelling’s multivariate \( T^2 \), to identify outliers that might bias the results of OPLS-DA. For OPLS-DA, class separation was shown in several ways. The first predictive score was plotted against the first orthogonal score to visualize the within- and between-class variability associated with the first principal component, and dendrograms were drawn using the first (or first and second) predictive scores, by the single linkage method, and sorted by size. In the single linkage method, observations were merged by proximity to neighbors based on Euclidean distance, building the hierarchy from individual observations by progressively merging clusters until all observations are merged into one parent cluster (36).

Scatter plots of the first two score vectors for the PCA models were drawn, along with 95% confidence ellipses based on Hotelling’s multivariate \( T^2 \), to identify outliers that might bias the results of OPLS-DA. For OPLS-DA, class separation was shown in several ways. The first predictive score was plotted against the first orthogonal score to visualize the within- and between-class variability associated with the first principal component, and dendrograms were drawn using the first (or first and second) predictive scores, by the single linkage method, and sorted by size. In the single linkage method, observations were merged by proximity to neighbors based on Euclidean distance, building the hierarchy from individual observations by progressively merging clusters until all observations are merged into one parent cluster (36).
upper right and lower left tails of the feature cloud within the model space (31, 32). All multivariate analyses were done using SIMCA-P+ v.12.0.1 (Umetrics).

Results

Effect of biguanides on the carcinogenic response

A total of 106 histopathologically confirmed mammary carcinomas were detected by palpation in this study and 11 additional carcinomas with a mass > 100 mg were found at necropsy. The effects of biguanide treatment were determined not only on palpable mammary pathologies, but also small pathologies that were identified by 5× magnification at necropsy and that we refer to as microcarcinomas (<100 mg in size; Table 1). In comparison with the control group, only buformin treatment reduced the incidence and multiplicity of palpable carcinomas and microcarcinomas at levels of statistical significance that were significant with adjustment for multiple comparisons (P < 0.008). In addition, the carcinogenic response was lower in buformin-treated rats in comparison with rats treated with metformin or phenformin, although the magnitude and statistical significance of these differences varied by cancer endpoint (Supplementary Table S1 for detailed statistical summary).

The emergence of carcinomas over time (cancer latency, Fig. 1) was most rapid in the metformin group whether the endpoint was the occurrence of the first carcinoma in an animal (cancer incidence, Fig. 1A) or the average number of cancers per rat (cancer multiplicity, Fig. 1B). In comparison with the control group, metformin shortened cancer latency (P = 0.025), whereas buformin prolonged cancer latency (P = 0.003). Phenformin was without effect on cancer latency relative to the control group (P = 0.265). Buformin prolonged cancer latency relative to either metformin (P < 0.001) or phenformin (P = 0.015). However, these P values are not significant after adjustment for multiple comparisons. Thus, only buformin significantly affected cancer latency. Figure 1C shows the effect of biguanide treatment on cancer mass per rat, with only buformin reducing cancer burden.

Tissue concentrations of biguanides. Biguanide concentrations were determined in blood and tissue obtained at necropsy following an overnight fast (Supplementary Table S2). Plasma and tissue concentrations of metformin and buformin were similar and not statistically different with the exception of skeletal muscle in which metformin concentration was three times higher for metformin than buformin. Phenformin was only detected in skeletal muscle and mammary gland following the overnight fast. In mammary gland, the concentrations of the three biguanides were similar. In mammary carcinomas, metformin, and buformin were found to have similar concentrations.

Effects of biguanides on circulating analytes

Blood from overnight fasted rats was collected at necropsy and all plasma samples were evaluated for the analytes shown in Table 2. Of the analytes measured, only the plasma concentrations of IGFI (P < 0.001), glucagon (P = 0.002), and adiponectin (P = 0.003) were affected. In the buformin- and phenformin-treated groups, IGFI was 30.4% and 22.8% lower than the control group and glucagon was 63.0% and 57.5%, lower than the control group, respectively. In the buformin group, adiponectin was 19.1% higher than in the control group.
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Table 2. Effect of biguanides on plasma analytes

<table>
<thead>
<tr>
<th>Analyte (mg/dL)</th>
<th>Control</th>
<th>Metformin*</th>
<th>Phenformin</th>
<th>Buformin</th>
<th>Overall P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose (mg/dL)</td>
<td>125.4 ± 12.9</td>
<td>128.5 ± 11.7</td>
<td>128.7 ± 17.4</td>
<td>130.7 ± 23.9</td>
<td>0.697</td>
</tr>
<tr>
<td>Insulin (ng/mL)</td>
<td>1.2 ± 0.3</td>
<td>1.1 ± 0.2</td>
<td>1.1 ± 0.3</td>
<td>1.1 ± 0.4</td>
<td>0.879</td>
</tr>
<tr>
<td>IGF-I (ng/mL)</td>
<td>328.7 ± 94.8&lt;sup&gt;b&lt;/sup&gt;</td>
<td>332.5 ± 74.4&lt;sup&gt;b&lt;/sup&gt;</td>
<td>254.2 ± 62.4&lt;sup&gt;c&lt;/sup&gt;</td>
<td>229.4 ± 61.5&lt;sup&gt;c&lt;/sup&gt;</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>IGFBP3 (ng/mL)</td>
<td>112.9 ± 29.9</td>
<td>113.0 ± 18.8</td>
<td>107.2 ± 24.3</td>
<td>106.3 ± 18.5</td>
<td>0.542</td>
</tr>
<tr>
<td>Glucagon (pg/mL)</td>
<td>73.5 ± 59.9</td>
<td>65.9 ± 40.7&lt;sup&gt;d&lt;/sup&gt;</td>
<td>26.8 ± 12.1&lt;sup&gt;c&lt;/sup&gt;</td>
<td>31.1 ± 9.5&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.002</td>
</tr>
<tr>
<td>Leptin (ng/mL)</td>
<td>1.9 ± 0.7</td>
<td>1.8 ± 0.7</td>
<td>1.5 ± 0.4</td>
<td>1.5 ± 0.6</td>
<td>0.087</td>
</tr>
<tr>
<td>Adiponectin (ng/mL)</td>
<td>18.8 ± 3.9&lt;sup&gt;b&lt;/sup&gt;</td>
<td>18.6 ± 4.0&lt;sup&gt;b&lt;/sup&gt;</td>
<td>20.8 ± 3.3&lt;sup&gt;c&lt;/sup&gt;</td>
<td>22.4 ± 6.2&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.003</td>
</tr>
<tr>
<td>Total cholesterol (mg/dL)</td>
<td>101.7 ± 8.1</td>
<td>104.5 ± 14.9</td>
<td>100.2 ± 16.5</td>
<td>100.3 ± 13.0</td>
<td>0.564</td>
</tr>
<tr>
<td>HDL cholesterol (mg/dL)</td>
<td>50.6 ± 9.8&lt;sup&gt;b&lt;/sup&gt;</td>
<td>55.2 ± 10.0&lt;sup&gt;c&lt;/sup&gt;</td>
<td>55.5 ± 11.1&lt;sup&gt;c&lt;/sup&gt;</td>
<td>60.4 ± 12.0&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.0068</td>
</tr>
<tr>
<td>LDL cholesterol (mg/dL)</td>
<td>31.6 ± 11.4&lt;sup&gt;c&lt;/sup&gt;</td>
<td>30.6 ± 8.2&lt;sup&gt;c&lt;/sup&gt;</td>
<td>25.9 ± 9.3&lt;sup&gt;d&lt;/sup&gt;</td>
<td>21.1 ± 9.1&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.0001</td>
</tr>
<tr>
<td>Triglyceride (mg/dL)</td>
<td>97.6 ± 71.1</td>
<td>93.7 ± 20.6</td>
<td>94.0 ± 18.1</td>
<td>94.2 ± 19.9</td>
<td>0.7973</td>
</tr>
</tbody>
</table>

NOTE: Values are mean ± SD, n = 30/group. Data were evaluated by ANOVA with post hoc comparison by the method of Tukey. Values within a row with different superscripts (b–d) were statistically different by post hoc multiple comparisons, P < 0.05.

*Biguanide was provided in the diet at concentrations of: 9.3 mmol metformin/kg diet, 7.6 mmol buformin/kg diet, or 5.0 mmol phenformin/kg diet.

For hypothesis generation concerning the role of host systemic factors in accounting for the effects of biguanide treatment, plasma analyte data were subjected to multivariate analysis of variance using unsupervised and supervised clustering techniques as implemented in SIMCA-P. Those analyses (Fig. 2) failed to reveal a pattern of analytes that distinguished among treatment groups (Fig. 2A) using unsupervised principle components analyses (Fig. 2A) or a supervised technique, OPLS-DA (Fig. 2B). The groups (Fig. 2A) using unsupervised principle components analysis occurring between the control group and the metformin group. This approach also determined whether the plasma analyte profile could distinguish between cancer-bearing and cancer-free rats irrespective of the treatment group in which they occurred. Neither unsupervised (Fig. 2D) or supervised (Fig. 2E) analyses distinguished between these classes as illustrated in the dendrogram from the supervised analysis (Fig. 2F).

Identification of influential proteins

To determine the proteins responsible for the distinctions observed among treatment groups and provide insight into differences in the carcinogenic response, two-class OPLS-DA modeling was performed. Because buformin and metformin were in similar concentrations in mammary carcinomas and mammary gland, but metformin was ineffective in inhibiting the carcinogenic process, these two treatments were compared. A two-class OPLS-DA model comparing buformin and metformin (Fig. 4A) was used to generate the S-plot shown in Fig. 4B with magnitude and variation in each protein shown in Fig. 4C. Among the most influential proteins for separation are those involved in the regulation of de novo lipid synthesis (pACC and FASN) and known to be regulated at least in part by components of the mTOR signaling pathway, which also contributed significantly to observed separation between groups.

Discussion

Many compounds exist that have biguanide-related activity, and two of these compounds, buformin and phenformin, were synthesized in the same era that metformin was developed (9). In general, they are not used in the treatment of diabetes because they have a greater potential to induce lactic acidosis. Nonetheless, the question has been raised about whether metformin is the best biguanide with which to answer questions about the anticancer activity of this class of compounds (10). To address this question relative to breast cancer, the effects of metformin, buformin, and phenformin were evaluated. Butorfin, but not metformin or phenformin, was found to have strong cancer inhibitory activity. In this regard, the biguanide concentration data (Supplementary Table S2) merit comment. Butoformin and metformin were present in tissue at similar concentrations yet butorfin was highly effective in inhibiting mammary cancer whereas, metformin was not. This situation established a ratio of de novo lipid synthesis (pACC and FASN) and known to be regulated at least in part by components of the mTOR signaling pathway, which also contributed significantly to observed separation between groups.
Multivariate discriminant analysis was used to determine whether plasma analyte data could distinguish among treat groups (A–C) or whether an animal was cancer bearing versus cancer free (D–F). A, to visualize inherent clustering patterns, the scatter plot represents unsupervised analysis through the PCA 4-class model. Poor separation of treatment groups is observed. Model fit: $R^2_{X(cum)} = 0.437$, and $Q^2_{(cum)} = 0.093$. B, to determine contributing sources of variation, the scatter plot represents supervised analysis of the 4-class OPLS-DA model, which rotates the model plane to maximize separation due to class assignment. Separation is still poor with an overall misclassification rate of 45%. Model fit: $R^2_{Y(cum)} = 0.199$, $Q^2_{Y(cum)} = 0.132$. C, to visualize the misclassification rate, the dendrogram depicts hierarchical clustering patterns among treatment groups using single linkage and size. D, to visualize inherent clustering patterns, the scatter plot represents unsupervised analysis through the PCA 2-class model. Poor separation of treatment groups is observed for the categories: cancer free $= 0$ versus cancer bearing $= 1$. E, to determine contributing sources of variation, the scatter plot represents supervised analysis of the two-class OPLS-DA model, which rotates the model plane to maximize separation due to class assignment. Separation is still poor with an overall misclassification rate of 55%. Model fit: $R^2_{Y(cum)} = 0.124$, $Q^2_{Y(cum)} = 0.080$. F, to visualize the misclassification rate, the dendrogram depicts hierarchical clustering patterns among treatment groups using single linkage and size.
in order to achieve effective doses of the compound in target tissue(s).

The main finding for metformin was a lack of effectiveness against the carcinogenic response, compared with control and other biguanides. The results of this study showed that administration of a clinically relevant dose of metformin in the diet, based on allometric dose conversion, may have accelerated the carcinogenic response in this nondiabetic model for breast cancer. However, the effects of metformin on multiple cancer endpoints were judged not to be statistically different from the control group following adjustment for multiple comparisons (Supplementary Table S1). Metformin was detected in liver, mammary gland, and mammary carcinomas suggesting that absence of the compound in potential target tissues is unlikely to account for lack of cancer inhibitory activity (Supplementary Table S2). In addition, the effect of metformin compared with control was qualitatively in the wrong direction. Though existing literature has supported an indirect action by metformin through its activity in the liver, differences in plasma analytes, associated with hepatic metabolism and thought to play a role in breast carcinogenesis, were not detected in metformin versus the control group (Table 2).

**Studies of mechanism**

The understanding of how biguanides exert protective activity against cancer *in vivo* is limited. One question of interest is whether anticancer activity is driven indirectly through effects on host systemic factors that impact cellular activity in the breast or whether the effects are direct. In order to generate further hypotheses about this question for investigation in future experiments, we used multivariate analysis of plasma analytes (Fig. 2) and mammary carcinoma Western blot data (Figs. 3 and 4) to determine whether either dataset would distinguish among treatment groups. The hypothesis generating experiments on plasma analytes were insufficient to distinguish among treatment groups or cancer-bearing versus cancer-free animals (Fig. 2); whereas, the patterns of protein expression in carcinomas resulted in 100% classification accuracy by treatment group (Fig. 3).

The multivariate analyses that resulted in high classification accuracy were based on the evaluation of the effects of biguanide treatment on cell signaling events in mammary carcinomas. The measured proteins represent multiple mechanisms proposed to explain the antidiabetic effects of biguanides. In terms of anticancer activity, these proteins are associated with energy sensing.

![Figure 3](image_url)

Multivariate discriminant analysis was used to determine whether Western blot data for 26 proteins assessed in mammary carcinomas (Supplementary Figs. S1 and S2) could distinguish among treatment groups. A, to visualize inherent clustering patterns, the scatter plot represents unsupervised analysis through the PCA 4-class model. Separation of treatment groups is observed. Model fit: \( R^2_X(cum) = 0.747 \), with 5 components, and \( Q^2(cum) = 0.155 \). B, to determine contributing sources of variation, the scatter plot represents supervised analysis of the 4-class OPLS-DA model, which rotates the model plane to maximize separation due to class assignment. Complete separation of the 4 classes was observed. Model fit: \( R^2_Y(cum) = 0.984 \), \( Q^2_Y(cum) = 0.963 \). C, to visualize the misclassification rate, the dendrogram depicts hierarchical clustering patterns among the treatment groups using single linkage and size. Two main clusters comprise (i) buformin versus (ii) phenformin, control, metformin.
pathways, cell proliferation, and cell death. Western blot data (Supplementary Fig. S1) were subject to unsupervised and supervised multivariate clustering analyses. This approach revealed that mammary carcinoma patterns of protein expression distinguished among treatment groups with the groups ordering in a manner consistent with treatment response (Fig. 4 dendrogram; Figure 4.

To determine the proteins responsible for class separation, multivariate analysis was extended to identify influential proteins responsible for the separation between classes. A, a supervised OPLS-DA model was created to compare buformin with metformin; complete separation was observed. B, an S-plot was constructed by plotting modeled correlation in the first predictive principal component against the modeled covariance in the first predictive component. Top right and bottom left regions of the S-plots contain candidate proteins with both high reliability and high magnitude. C, to determine the statistical reliability of the proteins shown in 4B, jack-knifed confidence intervals (JKCI) were created on the magnitude of covariance in the first component for the 26 proteins and sorted in ascending order based on expression in the buformin group; proteins with JKCIIs including 0 were not considered to account for separation.
than other biguanides. Though nondiabetic animal models are animal model in which metformin had less anticancer activity from buformin-treated rats. These findings, which are considered hypothesis generating, will permit further experiments to be designed to identify causal mechanisms.

Limitations
The studies carried out in this work used a nondiabetic animal model in which metformin had less anticancer activity than other biguanides. Though nondiabetic animal models are relevant to anticipating outcomes in ongoing clinical trials that exclude diabetics and individuals taking metformin, the biguanides buformin and phenformin are no longer used clinically due to risk for lactic acidosis. Though buformin and phenformin are unlikely to see clinical use, understanding biguanide drug targets in preclinical animal models will help develop new agents.

Human subjects are generally administered metformin once or twice a day; whereas, it was administered in the diet in this study. This could result in differences in tissue concentrations that might impact target protein activity. This underscores a critical need for more data on the pharmacokinetics and pharmacodynamics of metformin if its current indications are extended to cancer prevention at various organ sites.

Implications
A lack of effect for metformin in nondiabetic preclinical rodent models of breast cancer has been recently reported (37). These findings are confirmed and extended in this work by evaluation of additional biguanides. These preclinical studies raise many questions about the use of biguanides such as metformin in cancer prevention and control. In the context of having an impact in vivo, this study indicates that in nondiabetic individuals, the effects of an antidiabetic therapeutic dose of metformin may have limited benefit. In contrast, other biguanides outperform metformin in the preclinical model. As the mechanisms that account for the observed protection are established, the goal will be to identify agents that render protection in the absence of dose-limiting toxicities, for example, lactic acidosis, and such agents would represent the next generation of compounds with biguanide-related anticancer activity.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): Z. Zhu, W. Jiang, M.D. Thompson, J.N. McGinley, H.J. Thompson
Writing, review, and/or revision of the manuscript: Z. Zhu, W. Jiang, M.D. Thompson, J.N. McGinley, H.J. Thompson
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): Z. Zhu, H.J. Thompson
Study supervision: Z. Zhu, H.J. Thompson

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