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Bioactivity and Prostate Tissue Distribution of Metformin in a Pre-prostatectomy Prostate Cancer Cohort

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Abstract

Metformin has recently been shown to have potential to reduce prostate cancer risk. We conducted a randomized, double-blind, placebo-controlled trial to determine the modulating effects of metformin on tissue and systemic biomarkers of drug activity and its distribution into the prostate tissue. Twenty patients with prostate cancer scheduled to undergo prostatectomy were randomly assigned to receive either extended-release metformin or placebo for a median of 34 days before surgery. Prostatectomy and serum samples were analyzed for metformin concentrations, serum biomarkers of drug activity (prostate specific antigen, insulin, insulin-like growth factor-1, IGF binding protein 3, sex hormone binding globulin, and testosterone) and tissue biomarkers of proliferation, apoptosis, cell cycle regulation, and mTOR inhibition.

RESULTS: For participants in the metformin arm, the prostate tissue and serum metformin concentrations ranged from 0.88 to 51.2 μg/g tissue and from not detectable to 3.6 μg/ml, respectively. There were no differences between the two groups in either the post-intervention tissue biomarker expression in the prostatectomy tissue or pre to post-intervention changes in serum biomarkers. We conclude that metformin distributes to human prostate tissue, suggesting that metformin could exert its effects directly on tissue targets. However, there was no difference in tissue and systemic drug effect biomarkers between the two treatment arms. Future studies with longer intervention duration and larger sample size should be considered in order to evaluate the potential of metformin for prostate cancer prevention.

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Keywords

Metformin; Prostate; Clinical Trial; Cancer Prevention; Biomarkers

Introduction

An aging society predicts an ever-increasing burden of prostate cancer. Primary chemoprevention of prostate cancer development and secondary chemoprevention of prostate cancer progression represent important potential strategies to help reduce this anticipated burden.

Metformin is a biguanide initially introduced in the 1970s for treating individuals with type 2 diabetes that has recently been shown to also have potential chemopreventive effects. Preclinical studies have shown that metformin can significantly reduce cell proliferation in several prostate cancer cell lines and the tumor growth in xenograft models (Ben Sahra et al., 2008, Kato et al., 2015, Loubiere et al., 2015). Mechanistically, metformin may act through direct means by activation of AMP-activated protein kinase (AMPK) or other molecular targets at the prostate tissue or through indirect means by modulating systemic hormones, cytokines, and growth factors through its action on the liver and adipose tissue. Although, the direct effects on tissue targets would require the presence of drug transporter(s) because metformin is present in the circulation as a cationic molecule and its cellular uptake is dependent on organic cation transporters (Quinn et al., 2013).

Case control and cohort studies indicate that diabetics on metformin may have a decreased risk of cancer incidence compared to those taking other antidiabetic medications (Rizos and Elisaf, 2013). This includes a risk reduction of prostate cancer in some (Wright and Stanford, 2009, Preston et al., 2014), but not all studies (Azoulay et al., 2011, Lehman et al., 2012, Margel et al., 2013). The effect of metformin on mortality following prostate cancer diagnosis is also mixed, with cohort studies demonstrating both improved (Margel et al., 2013) and no change in survival (Kaushik et al., 2014, Lega et al., 2014). Given the retrospective nature of these studies and the possibility that the comparison treatments may increase risk, randomized, placebo-controlled intervention trials are clearly needed to assess the potential of metformin for prostate cancer prevention.

We conducted a randomized, double-blind, and placebo-controlled trial of metformin in prostate cancer patients scheduled to undergo radical prostatectomy to evaluate its potential for prostate cancer prevention. The study aimed to determine the modulating effects of metformin on tissue and systemic drug effect biomarkers and the distribution of metformin to the prostate tissue.

Methods

Study Design

The study was a randomized, double-blind, placebo controlled trial. Patients with a diagnosis of prostate cancer scheduled to undergo radical prostatectomy were randomly assigned to receive either metformin or placebo for 4–12 weeks before surgery. The study

was conducted at the University of Arizona and the University of Southern California. The study was approved by the Institutional Review Board at each institution.

Study Drugs

The drug product and placebo were supplied to the study site by the Division of Cancer Prevention, National Cancer Institute. The drug product was commercially available metformin hydrochloride extended-release tablets, USP, 500 mg, manufactured by Watson Laboratories, Inc. (Corona, CA). Each tablet contained 500 mg metformin hydrochloride as the active ingredient, and hypromellose 2208, colloidal silicon dioxide, and magnesium stearate as inactive ingredients. Extended release tablets were comprised of a monohydrophilic polymer matrix system in which metformin hydrochloride was combined with a drug release controlling polymer. The placebo tablets, manufactured by Pharm Ops, Inc. (Phillipsburg, NJ), contain anhydrous lactose, microcrystalline cellulose, crospovidone, and magnesium stearate.

Study Population

Participants were required to have a histologically confirmed prostate carcinoma electing prostatectomy as their primary treatment, have a current prostate specific antigen (PSA) less than 50 ng/ml, have not received chemotherapy and/or radiation for any malignancy (excluding non-melanoma skin cancer and cancers confined to organs with removal as only treatment) in the past 5 years, be over the age of 18, have good performance status, and have normal renal, hepatic, and marrow function to participate. Patients were excluded if they were on treatment with any drug for type I or II diabetes, had fasting glucose levels compatible with a diagnosis of diabetes, had uncontrolled intercurrent illness, were receiving other investigational agents, had a history of lactic acidosis or risk factors for lactic acidosis, had renal disease or dysfunction, had hepatic disease, had high alcohol consumption, had a history of allergic reactions to metformin or similar drugs, or had a history of acute or chronic metabolic acidosis. Study participants were instructed not to use non-study biguanides and cationic drugs (e.g., amiloride, digoxin, morphine, procainamide, quinidine, quinine, ranitidine, triamterene, trimethoprim, or vancomycin) while on study. Written informed consent was obtained from all participants.

Study Procedures

During the initial visit, participants underwent an interview and brief physical examination to obtain medical history, performance status, height, weight, blood pressure, pulse, and temperature measurements. A fasting blood sample was collected for complete blood count (CBC), comprehensive metabolic panel (CMP), serum biomarkers, and measurement of serum metformin concentrations. Upon determination of eligibility, participants were randomized (1:1) to receive metformin or placebo. Extended-release metformin was selected for this study to increase compliance and reduce gastrointestinal side effects. Participants took one 500 mg metformin extended-release tablet or one placebo tablet daily with the evening meal for the first week, then two tablets daily with the evening meal for the second week, then three tablets daily with the evening meal until the evening before surgery. Participants were required to keep an intake calendar and adverse event diary throughout the study participation. Participants were contacted in the interim for evaluation of safety and

adherence. Following 4–12 weeks of daily metformin/placebo dosing, participants returned for an end-of-intervention blood draw within three days prior to surgery for CBC, CMP, serum biomarkers and measurement of serum metformin concentrations.

At surgery, a prostate tissue sample was collected, when feasible, snap-frozen, and stored at −80°C for measurement of metformin concentrations. Paraffin-embedded tissue blocks or slides from prostatectomy were requested from each institution's pathology department for measurement of tissue biomarkers.

Safety of metformin intervention was assessed by reported adverse events and clinical labs. Adverse events were graded using NCI Common Terminology Criteria for Adverse Events (CTCAE) version 4.0.

Analysis of Serum and Tissue Metformin Concentrations

Serum and tissue metformin concentrations were measured by a published high performance liquid chromatography-tandem mass spectrometry (HPLC-MS) assay (Wang et al., 2004) with minor modifications. Briefly, aliquot of serum (25 μl) was mixed with cold acetonitrile to precipitate plasma protein. The supernatant was injected onto the HPLC-MS system. A piece of frozen prostate tissue was cut (30–70 mg) and weighed and homogenized in acidified 50:50 acetonitrile:water containing the internal standard (phenformin). The supernatant was extracted with dichloromethane. An aliquot of the aqueous layer was injected onto the HPLC-MS system. Chromatographic separation was achieved by reverse phase chromatography. The mass spectrometric analysis was performed using atmospheric pressure chemical ionization operated in the positive ion mode. The analytes were detected by multiple reaction monitoring.

Analysis of Serum Biomarkers

Serum concentrations of prostate specific antigen (PSA), insulin, insulin-like growth factor (IGF)-1, IGF binding protein (IGFBP)-3, and sex hormone binding globulin (SHBG) were assessed by commercially available ELISA assays (PSA from GenWay, insulin from Calbiotech, IGF-1/IGFBP-3 from R&D Systems, and SHBG from GenWay). Serum testosterone concentrations were measured by a sensitive and specific liquid chromatography-tandem mass spectrometry assay (Moal et al., 2007) with minor modifications to improve assay specificity.

Immunohistochemistry (IHC) for Tissue Biomarkers

IHC assays were used to assess markers of cell proliferation (Ki67), apoptosis (cleaved caspase 3, CC3), cell cycle regulation (cyclin D1), and mTOR inhibition (phospho-S6 ribosomal protein, pS6) in prostatectomy tissue sections. An expert pathologist (RN) reviewed the pathology reports and H&E slides to select three tissue blocks from each participant that consisted of adequate tumor tissue. A precision microtome was used to prepare tissue sections on coated slides for each tissue block. The expression of each marker was determined on a tissue section from each of the selected blocks. The IHC was performed on a Discovery XT Automated Immunostainer (VMSI - Ventana Medical Systems, Tucson, Arizona) using VMSI validated reagents, including deparaffinization,

antigen retrieval with a borate-EDTA buffer, primary antibody staining, detection and amplification and hematoxylin counterstaining. A biotin-free DAB (diaminobenzidine) detection system was used for CC3, cyclin D1, and pS6 and a biotinylated-streptavidin-HRP and DAB system was used for Ki67.

For Ki67 staining, mouse monoclonal antibody (clone: MIB-1, Dako) was diluted 1:100. For CC3 staining, anti-CC3 rabbit polyclonal antibody (Cell Signaling Technology #9661) was diluted 1:8,000. For cyclin D1 staining, rabbit monoclonal antibody (clone:SR4-R, VMSI) in a pre-diluted dispenser was used. For pS6 staining, rabbit monoclonal antibody (clone D57.2.2E, XP, Cell Signaling Technology #4858) was diluted 1:30. Human tonsil carcinoma was used as a positive control for all the IHCs.

For the IHC analysis, a single expert pathologist (RN) scored the percent of positively stained nuclei in the tumor regions for Ki67 and Cyclin D1, the percent of positively stained cells in the tumor regions for pS6, and the average number of positive stained cells that exhibited nuclear fragmentation from 5 randomly selected high power fields (40×) in the tumor regions for CC3. The marker expression was averaged from the selected tissue blocks for each participant.

Statistical Analysis

The primary study endpoint was initially the distribution of metformin to the prostate tissue. However, because fresh frozen surgical tissue may not be available from all study sites, the protocol was amended to list cell proliferation in the prostatectomy tissue as the primary endpoint and the tissue disposition of metformin as the principal secondary endpoint. Other secondary endpoints include assessment of the effects of metformin on systemic and tissue biomarkers related to drug activity or prostate cancer carcinogenesis. We had initially planned to randomize 50 eligible participants to have, at least 46 participants (23 per group) evaluable for tissue endpoints and for serum biomarkers. A sample size of 23 participants per group would achieve 90% power for detecting a difference between the two intervention groups equal to an effect size (mean divided by standard deviation) of 1.0, using a two-group t-test at a two-sided 0.05 level of significance. However, the study was closed prior to reaching the accrual target due to slow accrual.

Wilcoxon rank sum test was performed to compare the drug levels between the two study groups. Tissue biomarker data and changes in serum biomarkers (from baseline to post-intervention) between the two study groups were compared also using Wilcoxon rank sum test. No correction for multiple comparisons was performed because the comparisons were considered as exploratory. Multiple comparisons were accounted for only when interpreting the results. Spearman correlation of coefficient was calculated between the plasma and tissue metformin concentrations.

Results

The study opened for recruitment on 11/30/11 and closed on 4/15/14 prior to reaching the accrual target due to slow accrual. Sixty-seven men were prescreened for eligibility. Twenty-one met initial eligibility evaluation and subsequently consented. One did not meet all

eligibility evaluation. Twenty were randomized to receive metformin or placebo. Nineteen completed the agent intervention, one participant in the metformin arm discontinued after one dose due to an unrelated AE (influenza). A total of 17 AEs from 8 participants and 12 AEs from 6 participants were reported in the metformin and placebo arm, respectively. Possibly related AEs included nausea (1 in metformin and 1 in placebo arm), diarrhea (2 in metformin arm), constipation (1 in metformin arm), and bloating (1 in placebo arm). These were all grade 1 events.

Demographics of randomized participants are shown in Table 1. The average age was similar between the two arms, 65±10 and 61±6 yrs for the metformin and placebo arm, respectively. The average BMI was 30.1±4.5 and 30.3±5.6 kg/m^2 for the metformin and placebo arm, respectively. The duration of intervention was governed by the surgery schedule. It ranged from 28 to 80 days with the median duration of 34 days.

Table 2 summarizes the post-intervention prostate tissue and serum metformin concentrations. Flash frozen prostate tissue was available from 8 and 7 participants in the metformin and placebo arm, respectively, for tissue metformin concentration measurements. Serum sample was not collected from one of the participants in the metformin arm. For participants in the metformin arm, the prostate tissue and serum metformin concentrations ranged from 0.88 to 51.2 μg/g tissue and from not detectable to 3.6 μg/ml, respectively. Tissue and serum metformin concentrations did not correlate with the duration of intervention. Metformin was not detectable in the prostate tissue or serum in the placebo arm. We explored the relationship between tissue and serum metformin concentrations, recognizing that these samples were not matched in the sample collection time. The Spearman correlation of coefficient between the tissue and serum metformin concentrations is 0.67 ($p = 0.07$).

Table 3 summarizes the expression of markers of proliferation, apoptosis, cell cycle regulation, and mTOR inhibition in the available prostatectomy tissue. Prostatectomy tissue sections were available from 8 and 9 participants in the metformin and placebo arm, respectively. Due to the concern of the small sample size, we selected multiple tissue blocks from each participant and averaged the tissue marker expression to allow for a more representative assessment. There was no difference in the marker expression between the two groups.

Table 4 summarizes the change (from baseline to post-intervention) in fasting serum PSA, insulin, IGF axis, testosterone, and SHBG. There was no difference in the change in these systemic hormones and growth factors between the two treatment arms.

Discussion

Metformin possesses several features that would make it an attractive candidate agent for chemoprevention. It is a widely used antidiabetic drug now prescribed to almost 120 million of people worldwide. Because of its documented safety profile, metformin may have a higher level of acceptance and a higher proportion of uptake and compliance than dutasteride and finasteride for prostate cancer prevention, if it were shown effective in

controlled clinical trials. Furthermore, it could have pleiotropic cancer preventive benefits for multiple organ sites.

We showed that the extended-release metformin product was well tolerated in non-diabetic men with prostate cancer. Our study also showed that metformin distributes to the human prostate tissue. Prostate tissue metformin concentrations ranged between 0.88 – 51.2 μg/g tissue (7 – 400 μM) in eight participants in the metformin arm. The prostate tissue to serum metformin concentration ratio ranged between 2 – 32. Even though the tissue and plasma samples were not matched in sample collection time, differences in sample collection time would not substantially affect the tissue to serum metformin concentration ratio of the extended release metformin product. This finding corroborates the results of a prior trial where metformin was detected in prostate tissue after metformin treatment at 500 mg TID for a medium duration of 41 days (Joshua et al., 2014). Metformin-mediated activation of AMPK and its downstream molecular targets is dependent on cellular uptake of metformin which is controlled by the membrane transporters (Quinn et al., 2013). The observed drug distribution to the prostate tissue with a prostate tissue-to-serum metformin concentration ratio greater than unity suggests that metformin could exert its effects directly on the tissue targets.

Our study assessed the effects of metformin on post-intervention tissue drug effect biomarkers, including markers of proliferation (Ki67), apoptosis (CC3), cell cycle regulation (cyclin D1), and mTOR inhibition (pS6). The expression of these tissue drug effect biomarkers in the prostatectomy tissue sections was not different between the two treatment arms, although an unfavorable trend of higher cyclin D1 expression was observed in the metformin-treated arm. Multiple window-of-opportunity trials of metformin have been conducted in patients with breast and endometrial cancer (Hadad et al., 2011, Bonanni et al., 2012, Niraula et al., 2012, Laskov et al., 2014, Sivalingam et al., 2016). Favorable changes in tissue biomarkers of carcinogenesis such as Ki67 have been observed in some but not all studies (Hadad et al., 2011, Bonanni et al., 2012, Niraula et al., 2012, Laskov et al., 2014, Sivalingam et al., 2016). In addition, modulation of the molecular targets of metformin have not always been consistent in these trials (Hadad et al., 2011, Bonanni et al., 2012, Niraula et al., 2012, Laskov et al., 2014, Sivalingam et al., 2016). There was one window-of-opportunity trial of metformin reported in prostate cancer patients (Joshua et al., 2014). This trial was conducted as a single-arm trial and showed that metformin treatment (500 mg TID) reduced the Ki67 expression from a median of 4.7% in the pre-intervention needle biopsies to 2.8% in the prostatectomy tissue (Joshua et al., 2014). The study also showed a significant decrease in p-4EBP1 staining, a significant increase in pGSK3B staining but no change in p-AMPK or p-ACC (Joshua et al., 2014). The authors acknowledged that comparison between tissue biomarkers between needle biopsy and prostatectomy tissue could be confounded by tissue heterogeneity and preservation of phospho-proteins in the prostatectomy tissue. Participants in the prior trial and our study were exposed to a similar median treatment duration. However, the prior trial escalated to 1,500 mg dose of metformin by day 5 whereas our study implemented a slower escalation schedule to minimize GI side effects. In addition, the prior study compared the changes in tissue biomarkers between the pre-intervention needle biopsy and prostatectomy tissue in metformin-treated participants whereas our study

compared the expression of the tissue biomarkers in the prostatectomy tissue between metformin-treated and placebo groups.

There was also no change in circulating drug effects biomarkers with short-term metformin intervention in our study. Metformin taken at 850 mg BID for 3 months decreased free testosterone levels in non-diabetic obese men, with a corresponding increase in sex hormone binding globulin (Ozata et al., 2001). The prior single-arm trial of metformin in the pre-prostatectomy cohort showed that metformin treatment (500 mg TID) resulted in a significant decrease in IGF-1 and fasting glucose (Joshua et al., 2014). The lack of change in circulating drug effects biomarkers in our study may be attributed to the short exposure duration to the full dose and/or the small sample size.

We conclude that metformin was well tolerated in non-diabetic men with prostate cancer. Metformin distributes to human prostate tissue, suggesting that metformin could exert its effects directly on tissue targets. Future studies with longer intervention duration and larger sample size such as the ongoing metformin active surveillance trial (NCT01864096) being conducted across Canada would help define specific roles for metformin for prostate cancer prevention.

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Table 1

Demographics of randomized study subjects.

	Metformin (n =10)	Placebo (n =10)	P-value[a]
Age, y, n (%)			
Mean ± SD	65±10	61±6	0.27
< 65	4 (40)	6 (60)	0.66
≥ 65	6 (60)	4 (40)	
Race, n (%)			
White	9 (90)	9 (90)	1.00
Black or African American	1 (10)	1 (10)	
Ethnicity, n (%)			
Hispanic or Latino	1 (10)	1 (10)	1.00
Not Hispanic or Latino	9 (90)	9 (90)	
Body Mass Index, kg/m^2			
Mean ± SD	30.1±4.5	30.3±5.6	0.95
<25, n (%)	2 (20)	2 (20)	1.00
25–29.9 n (%),	3 (30)	4 (40)	
≥ 30, n (%)	5 (50)	4 (40)	

[a] derived from two-sample t-test with unequal variances for continuous variables and Fisher's exact test for categorical variables.

Table 2

Post-intervention prostate tissue and serum metformin concentrations.

	Metformin	Placebo	P-value[b]
Prostate tissue concentration (μg/g tissue)	5.71 (13.56)[a] (n=8)	0 (0) (n=7)	<0.01
Serum concentration (μg/ml)	1.02 (0.82) (n=9)	0 (0) (n=10)	<0.01

[a] median (interquartile range)

[b] derived from Wilcoxon rank sum test

Table 3

Expression of tissue biomarkers in prostatectomy specimens (post-intervention).

	Metformin (n = 8)	Placebo (n = 9)	P-value[b]
Ki67 (% positive)	6.50 (6.00)[a]	3.67 (4.00)	0.23
Cleaved caspase 3 (average number from five 40× fields)	0.07 (0.27)	0.10 (0.27)	0.77
Cyclin D1 (% positive)	37.5 (38.3)	13.3 (15.0)	0.09
pS6 (% positive)	66.7 (51.7)	63.3 (40.8)	0.72

[a] median (interquartile range)

[b] derived from Wilcoxon rank sum test

Table 4

Intervention-induced changes in serum biomarkers[a].

	Metformin (n=8)	Placebo (n=10)	P-value[b]
PSA			
Baseline, ng/ml	6.90 (10.45)[c]	7.23 (5.22)	0.76
% change	−6.53 (26.21)	5.98 (29.89)	0.63
Insulin			
Baseline, ng/ml	5.63 (7.02)	5.27 (4.33)	0.93
% change	−11.42 (50.14)	5.66 (33.14)	0.25
IGF-1			
Baseline, ng/ml	61.77 (34.22)	63.93 (22.16)	0.66
% change	−5.10 (15.36)	3.44 (13.74)	0.32
IGFBP-3			
Baseline, ng/ml	1503 (1455)	1586 (499)	0.83
% change	−9.18 (17.77)	12.38 (45.63)	0.36
IGF-1/IGFBP-3 ratio			
Baseline, ng/ml	0.04 (0.02)	0.04 (0.01)	0.69
% change	7.07 (30.28)	−2.24 (43.91)	0.46
Testosterone, ng/ml			
Baseline, ng/ml	3.01 (1.31)	2.68 (2.40)	0.83
% change	−16.12 (23.53)	2.04 (45.47)	0.46
SHBG, nmol/L			
Baseline, ng/ml	42.63 (29.21)	44.58 (27.41)	0.83
% change	0.48 (13.83)	−6.49 (16.76)	0.36

[a] only fasting data are included.

[b] derived from Wilcoxon rank sum test

[c] median (interquartile range)