Suppression of Prostate Epithelial Proliferation and Intraprostatic Progrowth Signaling in Transgenic Mice by a New Energy Restriction-Mimetic Agent

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Abstract

Cells undergoing malignant transformation often exhibit a shift in cellular metabolism from oxidative phosphorylation to glycolysis. This glycolytic shift, called the Warburg effect, provides a mechanistic basis for targeting glycolysis to suppress carcinogenesis through the use of dietary caloric restriction and energy restriction-mimetic agents (ERMA). We recently reported the development of a novel class of ERMAs that exhibits high potency in eliciting starvation-associated cellular responses and epigenetic changes in cancer cells though glucose uptake inhibition. The lead ERMA in this class, OSU-CG5, decreases the production of ATP and NADH in LNCaP prostate cancer cells. In this study, we examined the effect of OSU-CG5 on the severity of preneoplastic lesions in male transgenic adenocarcinoma of the mouse prostate (TRAMP) mice. Daily oral treatment with OSU-CG5 at 100 mg/kg from 6 to 10 weeks of age resulted in a statistically significant decrease in the weight of urogenital tract and microdissected dorsal, lateral, and anterior prostatic lobes relative to vehicle controls. The suppressive effect of OSU-CG5 was evidenced by marked decreases in Ki67 immunostaining and proliferating cell nuclear antigen (PCNA) expression in the prostate. OSU-CG5 treatment was not associated with evidence of systemic toxicity. Microarray analysis indicated a central role for Akt, and Western blot analysis showed reduced phosphorylation and/or expression levels of Akt, Src, androgen receptor, and insulin-like growth factor-1 receptor in prostate lobes. These findings support further investigation of OSU-CG5 as a potential chemopreventive agent.

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Introduction

In 1924, Otto Warburg reported that cancer cells preferentially metabolize glucose via glycolysis to lactate, even in the presence of adequate oxygen. This phenomenon, termed "aerobic glycolysis," results in the net production of 2 adenosine triphosphate (ATP) molecules per molecule of glucose, in contrast to the approximately 36 molecules produced per molecule of glucose directed into the tricarboxylic acid cycle and used for oxidative phosphorylation (1–7). The metabolic shift toward aerobic glycolysis provides cancer cells with growth advantages (3–7). For example, limiting ATP production to the glycolytic pathway permits diversion of intermediates into anabolic pathways to synthesize the nucleic acids, proteins, and fatty acids needed for extensive cell proliferation (3–7).

Although this metabolic adaptation provides growth advantages to cancer cells, it also presents opportunities to exploit the peculiarities of tumor cell metabolism for therapeutic purposes. The proof-of-concept for targeting energy metabolism for cancer chemoprevention is provided by the fact that inhibition of glycolysis through dietary caloric restriction or treatment with energy restriction-mimetic agents (ERMA) such as 2-deoxyglucose (2-DG) suppresses the growth of tumor xenografts and carcinogenesis in various animal models (8–12). To date, most of the animal studies that have assessed the anti-cancer effects of ERMAs have focused on late stages of cancer development or tumor growth, whereas their effects on the development and progression of preneoplastic conditions, such as prostatic intraepithelial neoplasia (PIN), remain largely undefined.

The prostates of transgenic adenocarcinoma of the mouse prostate (TRAMP) mice undergo a series of pathologic changes that mirror those which occur in men (13–15). Lesions develop progressively following the testosterone-dependent activation of the rat probasin promoter and...
expression of the SV40 large and small T antigens (T Ag) in the prostatic epithelium. Transgene expression results in inhibition of p53 and Rb tumor suppressors and development of prostate tumors (14–17). Prostates from 6-week-old intact TRAMP mice typically exhibit varying degrees of PIN with the development of well-differentiated adenocarcinomas by approximately 18 weeks of age (13, 14, 16, 18) and the emergence of poorly differentiated cancers with neuroendocrine differentiation at later time points. Although controversy exists about the histogenesis of the poorly differentiated carcinomas, that is, whether they originate from epithelial cells within PIN lesions or from a distinct neuroendocrine stem cell population (19, 20), this has little relevance to studies that focus on the PIN lesions in lieu of carcinomas.

Prostate epithelial proliferation in the PIN lesions of TRAMP mice has been shown to be amenable to modulation by dietary energy restriction. Specifically, calorically restricting 7-week-old intact male TRAMP mice for 4 weeks reduces prostate pathology and accessory sex gland weights (21). Therefore, we chose to use TRAMP mice to investigate the in vivo efficacy of the novel ERMA OSU-CG5 in modulating preexisting PIN lesions. Our hypothesis was that ERMA treatment would reduce the severity of lesions in the prostates of 10-week-old TRAMP mice.

OSU-CG5 is a derivative of OSU-CG12 (Fig. 1A), a previously described ERMA with a potency that is 3 orders of magnitude higher than that of 2-DG in inducing cell death (22). Both OSU-CG compounds elicit energy restriction-associated cellular responses by inhibiting glucose transporters in tumor cells (23). OSU-CG5 exhibits an improved potency relative to OSU-CG12 in suppressing the [3H]2-deoxyglucose uptake (IC$_{50}$ 6 vs. 9 μmol/L) and viability of human LNCaP prostate cancer cells (IC$_{50}$, 4.5 vs. 6 μmol/L; refs. 23, 24). Here, we examine the ability of OSU-CG5 to suppress cancer cell energy production in vitro and the in vivo efficacy and safety of OSU-CG5 in the TRAMP mouse model system of prostate neoplasia at the preneoplastic stage.

Figure 1. Evidence that OSU-CG5 reduces energy production. A, chemical structures of OSU-CG12 and OSU-CG5. B, dose-dependent suppressive effects of OSU-CG5 on ATP and NADH levels in LNCaP cells after 24 hours of treatment. The analyses were conducted in triplicate according to procedures described under Materials and Methods.

Materials and Methods

Cell cultures and reagents

Human LNCaP prostate cancer cells were obtained from the American Type Culture Collection and maintained with 10% FBS and 1% penicillin/streptomycin-supplemented RPMI-1640 medium (Invitrogen). No authentication of this cell line was conducted. All cells were cultured at 37°C in a humidified incubator containing 5% CO$_2$. OSU-CG5 was synthesized in our laboratory (data not shown). OSU-CG5 was dissolved in dimethyl sulfoxide (DMSO) and added to the culture medium with a final DMSO concentration of 0.1%.

ATP and NADH bioassays

The EnzyLight ATP Assay Kit (EATP-100) for rapid bioluminescent quantification of ATP and the EnzyChrom NAD+/NADH Assay Kit (E2ND-100) were obtained from BioAssay Systems. LNCaP cells were cultured in the presence of DMSO or OSU-CG5 at the indicated concentrations for 24 hours. Bioassays were conducted according to the manufacturer’s instructions in triplicate.

In vivo study

TRAMP mice (C57BL/6 TRAMP × FvB) were bred and housed, and the presence of the transgene in each mouse was confirmed by PCR, as previously reported (25). Mice received a standard rodent diet and water ad libitum. All procedures were conducted in accordance with protocols approved by the Institutional Animal Care and Use Committee of The Ohio State University (Columbus, OH). At 6 weeks of age, intact male transgenic mice were randomized to 2 groups that received either vehicle (n = 20) or 100 mg/kg/d of OSU-CG5 (n = 19). In addition, 6-week-old intact male wild-type littersmates (n = 6/group) were also randomized into the same two groups. The test article was prepared as a suspension in sterile water containing 0.5% methylcellulose (w/v) and 0.1% Tween-80 (v/v). Suspensions of OSU-CG5 were prepared on a weekly basis and stored at room temperature. Mice received treatments once daily via oral gavage under isoflurane anesthesia for the duration of the study (4 weeks). Body weights were measured weekly and at necropsy. At the termination of the study (age 10 weeks), mice were euthanized via CO$_2$ approximately 2 to 4 hours after they had received their last dose, and complete necropsies were conducted. Urogenital tracts (UGT) were removed from all mice and weighed, after which relative UGT weights (UGT weight/terminal body weight × 100%) were determined. The livers, kidneys, hearts, spleens, and testes of each wild-type mouse were weighed, and relative organ weights were determined as described above for the UGT.

The individual lobes of the prostate were microdissected at necropsy and immersed in either RNAlater (Qiagen; transgenic mice only, n = 9 for vehicle; n = 8 for OSU-CG5) or PBS (n = 11 for each transgenic mouse treatment group, n = 6 for each wild-type mouse treatment group). The dorsal and lateral lobes of prostates microdissected in RNAlater were stored at 4°C overnight in RNAlater and then transferred to empty vials for storage at −80°C for

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subsequent RNA extraction. The dorsal, lateral, ventral, and anterior lobes microdissected in PBS were weighed individually. One lobe of each pair was snap-frozen in liquid nitrogen and stored at −80°C until needed for Western blot analysis of relevant biomarkers, whereas the other was fixed in 10% neutral-buffered formalin (NBF). All other tissues were fixed in NBF.

At necropsy, serum was collected from 5 transgenic mice per group and submitted to Analytics Inc. for determination of free and total testosterone concentrations via radioimmunoassay. Serum was also collected from 5 transgenic mice of the treated group for determination of liver enzyme values, including alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALKP), and γ-glutamyl transpeptidase (GGT), and total bilirubin by the Comparative Pathology and Mouse Phenotyping Shared Resource (CPMPSR) at the Ohio State University. Serum was collected from 3 wild-type mice per group for determination of serum glucose, insulin-like growth factor (IGF)-1, and insulin concentrations. Serum glucose was measured by the CPMPSR. IGF-1 and insulin concentrations were determined using the IGF1 Mouse ELISA Kit (ab100695) from Abcam Inc and Insulin (Mouse) ELISA Kit (80-INSMS-E01) from ALPCO Diagnostics. Each ELISA was conducted according to the manufacturers’ instructions in duplicate.

Histopathology and scoring of prostatic lesions in the TRAMP mice

Five-micrometer-thick, paraffin-embedded sections of the lobes of the prostate (n = 11 transgenic mice/group) as well as selected organs (n = 5 transgenic mice/group: liver, kidney, spleen, lung, heart, small intestine, testes, thymus, brain, eye, and bone marrow) were stained with hematoxylin and eosin (H&E) by standard procedures. All tissues were examined via light microscopy by a board-certified veterinary anatomic pathologist (LDDB) using an Olympus Model CHT research microscope (Olympus). The H&E-stained sections of the four lobes of the prostate of each transgenic mouse were evaluated and scored separately using a grading scheme (19) in which the most severe and most common lesions in each lobe were determined and assigned numerical scores. The sum of the scores of the most severe lesion and the most common lesion, termed the “sum of the adjusted lesion scores” (SALS), was then obtained for each lobe. The average SALS as well as the average most severe and average most common lesion scores of each lobe were compared between groups.

Immunohistochemistry

To evaluate prostate and small intestinal epithelial proliferation, 5-μm-thick, paraffin-embedded tissue sections of the lobes of the prostate and cross-sections of small intestine were immunostained for Ki67 using a commercially available rabbit anti-human monoclonal antibody (clone SP6; catalog#RM-9106-S0; Thermo Fisher Scientific). This primary antibody was applied at a dilution of 1:180. The immunohistochemistry staining protocol used the Avidin-Biotin Complex method with DakoCytomation Target Retrieval Solution and a Decloaking Chamber (Biocare Medical) according to the manufacturer’s instructions, following the application of a protein block (DakoCytomation Serum-Free Protein Block, Dako). For each lobe of the prostate and section of small intestine, the number of Ki67-positive cells in 3 randomly selected 400× (i.e., high-power) fields was counted and divided by the total number of cells (Ki67-positive and -negative) in those fields (n = 5/group) to yield the percentage of Ki67-immunopositive cells.

RNA isolation and microarray analysis

Using a tissue homogenizer (Tissue Tearor, Model 985370-395; BioSpec Products, Inc.) and TRIzol reagent (Invitrogen), total RNA was isolated from the combined dorsal and lateral prostate lobes of control and OSU-CG5–treated transgenic mice (n = 3/group). After purification using the RNAeasy Mini Kit (Qiagen), the RNA was submitted to the Microarray Shared Resource at The Ohio State University Comprehensive Cancer Center (OSU-CCC) for RNA quantification and microarray analysis of gene expression using Affymetrix GeneChip Mouse Genome 430 2.0 Arrays (Affymetrix). Microarray data were deposited in NCBI’s Gene Expression Omnibus (GEO), and can be accessed via accession number GSE32422.

Protein isolation and Western blot analysis

For Western blot analysis of intraprostatic biomarkers, the dorsal and lateral prostate lobes of individual transgenic mice were combined and pulverized in liquid nitrogen (n = 5/group). SDS lysis buffer (1% SDS, 50 mmol/L Tris-HCl, pH 8.0, 10 mmol/L EDTA) containing 1× protease inhibitor cocktail (Sigma) and PhoSTOP phosphatase inhibitor cocktail (Roche) were added to the crushed tissue. The resulting lysates were sonicated until clear and centrifuged at 16,100 × g for 15 minutes. Protein concentrations in the supernatants were determined using the Micro BCA Protein Assay (Pierce Chemical). Proteins were separated by 1-dimensional electrophoresis in 8%–12% SDS-PAGE and transferred onto nitrocellulose membranes. After blocking with 5% nonfat milk, the membranes were incubated with primary antibodies (see below) at 1:1,000 dilution in TBS-Tween 20 overnight at 4°C. The protein bands were developed using horseradish peroxidase–conjugated secondary antibodies at 1:5,000 dilution in the same buffer for 1 hour at room temperature. Bands were visualized on X-ray film using an enhanced chemiluminescence system. Densitometric analysis of protein bands was conducted using ImageJ software to determine the relative intensities of protein expression in drug-treated samples versus those of vehicle-treated controls after normalization to the internal reference protein β-actin.

The target proteins and commercial sources of the antibodies for several biomarkers are given here. Mouse monoclonal antibodies directed against β-actin were from MP Biomedicals, Srl from Calbiochem, or SV40 T Ag from Santa Cruz Biotechnology. Rabbit polyclonal antibodies came from 2 sources. Reagents directed against IGF-1 receptor...
and differences in gene expression profiles were considered.

Analysis of UGT weights

All data were found to be normally distributed except the Ki67 values for the ventral and anterior lobes of the prostate and the small intestine; the SALS for the dorsal, lateral, and anterior lobes of the prostates; the serum concentrations of total and free testosterone, glucose, IGF-1, and insulin; the serum activities of liver enzymes; and the relative weights of the heart, kidney, and spleen. For the normally distributed data, differences between group means were analyzed for statistical significance using the Wilcoxon rank-sum test. Differences were considered to be significant at  \( P < 0.05 \). Microarray data were submitted to The OSU-CCC Biomedical Informatics Shared Resource for statistical analysis, using the Bioconductor microarray analysis package, using the Student \( t \) test. Fold changes and differences in gene expression profiles were considered significant at \( P < 0.05 \). Microarray pathways were analyzed using Ingenuity Pathway Analysis (IPA) software (Ingenuity Systems). Only genes with greater than 1.5-fold up- or downregulation and \( P < 0.05 \) were selected for pathway analysis. All \( P \) values were 2-sided, except for \( P \) values from the Western blots, which were 1-sided.

Results

Suppressive effects of OSU-CG5 on LNCaP cell ATP and NADH production

Previously, we showed that OSU-CG5, a structurally optimized derivative of OSU-CG12, blocked glucose uptake in LNCaP cells by blocking glucose transporters (23, 24). The consequent effect on energy production was manifested by its ability to lower the levels of ATP and NADH in a dose-dependent manner after 24 hours of treatment. At 5 \( \mu \)mol/L, OSU-CG5 reduced ATP and NADH production by 58\% \( \pm \) 1\% \( (P < 0.0001) \) and 28\% \( \pm \) 1\% \( (P < 0.0001) \), respectively, relative to the DMSO control (Fig. 1B).

OSU-CG5 decreased prostate weight and prostate epithelial cell proliferation in transgenic TRAMP mice

To investigate the effect of OSU-CG5 on the progression of preneoplastic lesions, 6-week-old TRAMP mice were treated once daily with OSU-CG5 (100 mg/kg, \textit{per os}, \( n = 19 \)) or vehicle \( (n = 20) \) for 4 weeks. As UGT weight has previously been shown to correlate significantly with prostate lesion severity and progression (13, 26), the average UGT weights of each group were compared. OSU-CG5 treatment resulted in 12\% and 11\% reductions in the absolute UGT weight \( (332 \pm 40 \text{ mg vs. } 377 \pm 37 \text{ mg in controls}; P = 0.001) \) and relative UGT weight \( (1.24\% \pm 0.14\% \text{ vs. } 1.39\% \pm 0.11\% \text{ in controls}; P = 0.001; \text{ Fig. 2A}) \). Moreover, the weights of the individual prostate lobes of the OSU-CG5–treated mice were decreased relative to vehicle controls \( (n = 11 \text{ mice/group}) \): dorsal, 25.7\% \( (P = 0.013) \); lateral, 31.5\% \( (P = 0.003) \); ventral, 10\% \( (P = 0.413) \); anterior, 16.5\% \( (P = 0.026; \text{ Fig. 2B}) \). Importantly, these reductions in weight were not associated

Figure 2. OSU-CG5 reduced prostate weight in TRAMP mice. Six-week-old intact male TRAMP mice were treated orally with OSU-CG5 (100 mg/kg) once daily for 4 weeks. The weights of the UGTs from each mouse were determined, as well as the weights of individual prostate lobes after microdissection. Treatment with OSU-CG5 caused statistically significant reductions in (A) absolute and relative UGT weights and (B) the weights of the dorsal, lateral, and anterior lobes of the prostate. C, drug treatment had no effect on the expression level of the SV40 large T antigen in the combined dorsal and lateral prostates (5 mice/group) as determined by Western blotting. In (A) and (B), bars represent the mean \( \pm \) SD. AP, anterior lobe of the prostate; CG5, OSU-CG5; DP, dorsal lobe of the prostate; LP, lateral lobe of the prostate; VP, ventral lobe of the prostate.
with decreased SV40 large T Ag expression in the prostate (n = 5 mice/group), as Western blot analysis indicated that the amount of SV40 oncoprotein in the combined dorsal and lateral lobes was comparable in both treatment groups (Fig. 2C).

The suppressive effect of OSU-CG5 on the UGT weight was linked to its ability to inhibit the proliferation of prostate epithelial cells. OSU-CG5 treatment led to a marked decrease in the proliferation index, as indicated by Ki67 immunoreactivity, in all 4 lobes of the prostate (P < 0.05 for all lobes; n = 5 mice/group; Fig. 3A and B). Western blot analysis indicated that OSU-CG5 decreased the expression level of PCNA in the combined dorsal and lateral lobes by 32% (P = 0.002) relative to the vehicle (n = 5 mice/group; Fig. 3C).

Histopathologic examination and scoring of each lobe of the prostate was conducted (n = 11 mice/group), and the sum of the adjusted lesion score (SALS) for each lobe was determined. The average SALS of the dorsal, lateral, ventral, and anterior lobes of the prostates from the vehicle-treated mice were 9.5 ± 1.4, 9.0 ± 4.4, 6.0 ± 2.1, and 4.5 ± 3.0, respectively, whereas the average SALS for these lobes in OSU-CG5–treated mice were 9.1 ± 1.8, 7.5 ± 1.0, 5.6 ± 2.5, and 5.5 ± 3.0. No statistical differences were found in the average SALS of each lobe or the average scores for the most severe or most common lesions in each lobe (Supplementary Table S1).

Testosterone radioimmunoassays indicated that the serum concentrations of free and total testosterone were 0.21 ± 0.11 and 0.33 ± 0.09 ng/mL, respectively, for OSU-CG5–treated mice (n = 5 mice/group) versus 3.4 ± 4.6 and 1.6 ± 2.0 ng/mL, respectively, for vehicle-treated mice (n = 5 mice/group). Despite 93.7% and 79.7% decreases in the free and total testosterone, respectively, these reductions were not statistically significant (P > 0.05), in part, due to large variations in the testosterone concentrations of vehicle controls. This wide variation in the serum testosterone level, however, is in line with the reported range of 1 to 90 pg/mL in nude mice (27).

OSU-CG5 treatment was not associated with any evidence of systemic toxicity

No overt toxicity was noted with OSU-CG5, as the drug-treated transgenic group gained a similar amount of weight to that of the control group after 4 weeks of treatment (Table 1). Similarly, there were no significant changes in the absolute or relative weights of the UGT, liver, kidneys, hearts, or testes (Table 1), in the wild-type mice (n = 6 mice/group; P > 0.05). Histologic evaluation of H&E-stained sections of metabolically active organs

Figure 3. OSU-CG5 reduced prostate epithelial proliferation in TRAMP mice. Proliferation within the prostates of TRAMP mice treated with OSU-CG5 (100 mg/kg/d) or vehicle for 4 weeks was assessed by immunohistochemistry for Ki67 (A and B) and Western blotting for PCNA (C). A, photomicrographs showing Ki67 immunoreactivity in the dorsal and lateral lobes of representative vehicle- and OSU-CG5–treated TRAMP mice (×400). B, the percentages of cells that were immunopositive for Ki67 in each prostate lobe were reduced in OSU-CG5–treated TRAMP mice relative to vehicle-treated controls (5 mice/group). C, Western blot analysis shows that OSU-CG5 treatment reduced the expression levels of PCNA in the combined dorsal and lateral lobes prostates of TRAMP mice relative to vehicle-treated controls (5 mice/group). In (B) and (C), bars represent the mean ± SD. AP, anterior lobe of the prostate; Ctl, vehicle control; CG5, OSU-CG5; DP, dorsal lobe of the prostate; LP, lateral lobe of the prostate; VP, ventral lobe of the prostate.
including liver, kidney, spleen, lung, heart, small intestine, testes, thymus, brain, eye, and bone marrow (n = 5 mice/group) did not reveal any lesions consistent with systemic or organ-specific toxicity. Although OSU-CG5 treatment was associated with mild splenomegaly with slight increases in the average absolute and relative weights of the spleen (P < 0.05; Table 1), there were no associated histologic lesions. There was no biochemical evidence of hepatotoxicity as the concentrations of various hepatic biomarkers, including ALT, AST, ALKP, and GGT, and total bilirubin were not affected by 4 weeks of OSU-CG5 treatment (vehicle: 0.1 ± 0.02 mg/dL; OSU-CG5: 0.3 ± 0.02 mg/dL). Together, these findings indicate that OSU-CG5 treatment was not associated with any detectable systemic toxicity.

OSU-CG5–mediated suppression of prostate epithelial proliferation in transgenic TRAMP mice was associated with modulation of gene expression in the combined dorsal and lateral lobes of the prostate

Earlier studies indicate that caloric restriction and 2-DG suppress carcinogenesis by perturbing cellular signaling and/or gene expression profiles (9–10, 12, 28–30). Microarray analysis was conducted using RNA from the combined dorsal and lateral prostate lobes of OSU-CG5– and vehicle-treated animals (n = 3 mice/group). Using IPA software, the top 5 gene networks affected by OSU-CG5 treatment were identified (Supplementary Table S2). Of these, we focused on the "Cellular Assembly and Organization, Cellular Function and Maintenance, Gene Expression" because of the likely role of genes in this network in maintaining cellular homeostasis and suppression of tumorigenesis (Supplementary Table S2). The pathway network is shown in Fig. 4. Akt appeared to have a central role in this constellation, with 5 genes in the network interacting directly with Akt and 5 genes interacting indirectly with Akt. In light of this information and due to the fact that an earlier study showed upregulation of Akt signaling, as manifested by dramatically increased phosphorylation of Akt and GSK3β, in proliferative prostate epithelium in TRAMP mice with PIN (25), we investigated the phosphorylation status of Akt and GSK3β in the combined dorsal and lateral lobes of prostates of OSU-CG5–versus vehicle-treated mice. As shown in Fig. 5, OSU-CG5 treatment significantly reduced phospho-Ser473-Akt and phospho-GSK3β levels by 50% (P = 0.012) and 45% (P = 0.043), respectively, relative to the vehicle-treated controls.

The microarray data also showed changes in the expression of genes encoding proteins of potential therapeutic relevance for prostate cancer. OSU-CG5 exhibited suppressive effects on the expression of the Rous sarcoma virus oncogene (Sv40 T antigen) and the E-cadherin, a cell adhesion molecule important in maintaining epithelial integrity and tumorigenicity. In addition, the expression of the tumor suppressor gene p53 was increased, which is consistent with the reduced tumor burden observed in OSU-CG5-treated mice.

Table 1. Evidence that OSU-CG5 caused no systemic toxicity in young adult mice after 4 weeks of treatment

<table>
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<th>Vehicle-treated</th>
<th>OSU-CG5-treated</th>
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<td>2.2 ± 1.1 (n = 19)</td>
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<td>Wild-type littermates of TRAMP mice: organ weight (n = 6 for both groups), mg</td>
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<td>Liver (% body weight)</td>
<td>1303 ± 124 (4.89 ± 0.31)</td>
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<td>Kidney (% body weight)</td>
<td>408 ± 28 (1.54 ± 0.14)</td>
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<td>Heart (% body weight)</td>
<td>164 ± 19 (0.62 ± 0.07)</td>
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<td>Spleen (% body weight)</td>
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<td>Testes (% body weight)</td>
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<td>221 ± 16 (0.81 ± 0.09)</td>
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<td>Total bilirubin, mg/dL</td>
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<td>TRAMP mice: cell proliferationa in nonneoplastic tissues (n = 5 for both groups)</td>
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<td>Small intestinal epithelium</td>
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NOTE: Statistically equivalent for OSU-CG5– and vehicle-treated mice, P > 0.05, for all values except absolute and relative weights of the spleen.

aProliferation evaluated as percentage of Ki67-immunopositive cells.
oncogene (v-src; −1.44-fold, \( P = 0.007 \)), and its cellular counterpart, the proto-oncogene c-src (−1.36-fold, \( P = 0.012 \)). Downregulation of Src represents a therapeutically relevant target in prostate cancer (31). In addition, OSU-CG5 decreased the expression of the Myc-like oncogene, s-myc (−1.32-fold, \( P = 0.007 \)) and upregulated the expression of PTEN (1.25-fold, \( P = 0.033 \)). Further review of the data revealed a number of other upregulated genes with central roles in suppressing tumorigenesis and downregulated genes with roles in tumor development and progression. These genes, categorized according to mechanism of action, are found in Supplementary Tables S3 and S4.

Considering the importance of Src, myc, and PTEN in prostate cancer pathogenesis, as well as the importance of growth factor signaling through IGF-1R and AR for prostate epithelial proliferation, we assessed the expression levels of these proteins by Western blotting (\( n = 5 \)/group). OSU-CG5 significantly reduced the expression of Src, AR, and IGF-1R by 28% (\( P = 0.001 \)), 26% (\( P = 0.036 \)), and 47% (\( P = 0.001 \)), respectively, whereas no significant changes were noted in the protein levels of PTEN or c-Myc (Fig. 5).

Despite reduced intraprostatic IGF-1R expression, OSU-CG5 did not affect glucose homeostasis, as no significant changes (\( P > 0.05 \)) in the serum levels of glucose, IGF-1, or insulin were noted in drug-treated mice. Concentrations of these serum parameters in vehicle- and OSU-CG5–treated mice were as follows, respectively: glucose, 279 ± 11 and 288 ± 26 mg/dL; IGF-1, 2.7 ± 0 and 2.7 ± 0.1 ng/mL; insulin, 0.92 ± 0.23 and 0.94 ± 0.29 ng/mL. These results suggest that the ERMA activity of OSU-CG5 was restricted to the proliferating cells within the PIN lesions and did not affect whole-body energy metabolism.
Discussion

The adaptation of cancer cells to preferentially use aerobic glycolysis is an early step in carcinogenesis and presents an opportunity for therapeutic exploitation. The efficacy of energy restriction as a chemotherapeutic or chemopreventive strategy has been shown by the finding of AR expression likely contributed, at least in part, to carcinogenesis and tumor progression (35), downregulating expression of the transcription factor Sp1 (22). In addition, ERMA OSU-CG12 suppressed AR expression in LNCaP cells by downregulating expression of the transcription factor Sp1 (22). In this study, we also investigated multiple mechanisms by which OSU-CG5 acts to decrease prostate epithelial proliferation. For example, treatment with OSU-CG5 resulted in decreased AR expression, which accords well with our previous finding that the ERMA OSU-CG12 suppressed AR expression in LNCaP cells by downregulating expression of the transcription factor Sp1 (22). In light of the pivotal role of AR signaling in prostate carcinogenesis and tumor progression (35), downregulation of AR expression likely contributed, at least in part, to the decreased prostate epithelial proliferation observed in this study.

Although chronic dietary restriction (21, 33) or intermittent caloric restriction (34) has been shown to retard prostate lesion development in the TRAMP mouse model, we are aware of only one study that included an evaluation of caloric restriction on the progression of preneoplastic lesions (21). Studies in TRAMP mice have found that 20% caloric restriction initiated at approximately 7 weeks of age had a greater effect on the suppression of lesion development than 20% caloric restriction started at a later time point (20 weeks; refs. 21, 33). This result implies that the age at which the dietary intervention is initiated influences its effectiveness. In addition, intermittent caloric restriction delayed prostate tumor development longer than chronic caloric restriction when both were begun at the same age (34). Given the results from these studies, it seems logical to examine the effect that an ERMA administered beginning at puberty could have on the severity of PIN lesions.

In this study, we showcase the ability of OSU-CG5, a novel glucose transporter inhibitor (23, 24), to modify the early lesions of TRAMP mice. OSU-CG5 reduced the weights of the UGTs as well as the dorsal, lateral, and anterior lobes of prostates by suppressing prostate epithelial proliferation. As the UGT weight correlates with lesion severity and progression in TRAMP mice (13, 26), the effect of OSU-CG5 on UGT weights underscores this ERMA’s ability to reduce the prostate pathology within these mice. The lack of statistically significant changes in the histopathologic lesion scores of the individual prostate lobes in this study likely reflects the fact that the traditional scoring criteria for mouse prostatic cancer models are weighted to the assessment of later-stage neoplastic lesions rather than to the early PIN changes.

Importantly, our data indicate that OSU-CG5 is well tolerated. Chronic oral administration of OSU-CG5 resulted in no untoward (i.e., “toxic”) effects in metabolically active organs with naturally high glycolytic rates, including the liver, kidney, small intestine, brain, and eye, did not impact the proliferation of a nonneoplastic tissue (the small intestine) and did not induce elevations in serum activities of liver enzymes. Taken together, these animal data underscore the translational potential of OSU-CG5.

In this study, we also investigated multiple mechanisms by which OSU-CG5 results in no untoward (i.e., “toxic”) effects in metabolically active organs with naturally high glycolytic rates, including the liver, kidney, small intestine, brain, and eye, did not impact the proliferation of a nonneoplastic tissue (the small intestine) and did not induce elevations in serum activities of liver enzymes. Taken together, these animal data underscore the translational potential of OSU-CG5.

In this study, we also investigated multiple mechanisms by which OSU-CG5 acts to decrease prostate epithelial proliferation. For example, treatment with OSU-CG5 resulted in decreased AR expression, which accords well with our previous finding that the ERMA OSU-CG12 suppressed AR expression in LNCaP cells by downregulating expression of the transcription factor Sp1 (22). In light of the pivotal role of AR signaling in prostate carcinogenesis and tumor progression (35), downregulation of AR expression likely contributed, at least in part, to the decreased prostate epithelial proliferation observed in this study.

Despite decreased AR expression levels in the prostate, there was no evidence of testicular atrophy in OSU-CG5–treated wild-type mice as suggested by lack of histologic
lesions within the testes or changes in testicular weights. Together with the finding that OSU-CG5 did not reduce the wild-type UGT weights, these data imply that OSU-CG5 does not act as an anti-androgen. Although the decrease in serum testosterone concentrations in OSU-CG5–treated mice was not statistically significant due to a high degree of interindividual variability, caloric restriction has been reported to decrease circulating testosterone (36). Therefore, the decreased testosterone level in this study might represent a cellular response to OSU-CG5 as it mimics caloric restriction in vivo. Any decrease in serum testosterone may have potentiated the effect of the decreased AR. Further studies are needed to determine any effect that OSU-CG5 may have had on the hypothalamic–pituitary hormonal axis that control gonadal function, especially after prolonged therapy. Interestingly, lesion progression in TRAMP mice has previously been shown to be relatively resistant to reductions in serum testosterone, with 80% of TRAMP mice castrated by 12 weeks of age developing prostate tumors by 24 weeks of age (37).

Moreover, OSU-CG5 inhibited the IGF-1R/PI3K/Akt signaling axis in the combined dorsal and lateral lobes of prostates. The microarray data suggested a central role for Akt in the top network modulated by OSU-CG5, and we subsequently showed the suppressive effect of OSU-CG5 on phosphorylation of Akt and its target GSK3β. This finding is in accordance with a recent report that caloric restricting Hi-Myc transgenic mice reduces the incidence of prostatic adenocarcinomas and the phosphorylation level of Akt (38).

From a mechanistic perspective, the ability of OSU-CG5 to inhibit Akt signaling might, in part, be attributable to the observed downregulation of intraprostatic IGF-1R expression, which would have led to decreased IGF-1/IGF-1R signaling. Downregulation of IGF-1/IGF-1R signaling has been shown to be a major mechanism underlying the effect of caloric restriction on tumor suppression (39). Despite reduced intraprostatic IGF-1R expression, there was no significant change in the serum level of IGF-1 in the OSU-CG5–treated wild-type cohort. Together with the lack of changes in serum glucose and insulin, this finding lends credence to our hypothesis that OSU-CG5 targets neoplastic epithelial cells in TRAMP mice without evidence of toxicity or modulation of whole-body glucose homeostasis. Microarray and Western blotting data revealed that OSU-CG5 targeted proliferating preneoplastic cells via multiple mechanisms that include the modulation of cell survival and proliferation pathways and interference with cellular energy metabolism. This range of antitumor activities in the absence of toxicity suggested the translational potential of OSU-CG5 as a chemopreventive agent, warranting further investigation in this regard.

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

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