Targeting Energy Metabolic and Oncogenic Signaling Pathways in Triple-negative Breast Cancer by a Novel Adenosine Monophosphate-activated Protein Kinase (AMPK) Activator*

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The antitumor activities of the novel adenosine monophosphate-activated protein kinase (AMPK) activator, OSU-53, were assessed in vitro and in vivo models of triple-negative breast cancer. OSU-53 directly stimulated recombinant AMPK kinase activity (EC_{50}, 0.3 µM) and inhibited the viability and clonogenic growth of MDA-MB-231 and MDA-MB-468 cells with equal potency (IC_{50}, 5 and 2 µM, respectively) despite lack of LKB1 expression in MDA-MB-231 cells. Nonmalignant MCF-10A cells, however, were unaffected. Beyond AMPK-mediated effects on mammalian target of rapamycin signaling and lipogenesis, OSU-53 also targeted multiple AMPK downstream pathways. Among these, the protein phosphatase 2A-dependent dephosphorylation of Akt is noteworthy because it circumvents the feedback activation of Akt that results from mammalian target of rapamycin inhibition. OSU-53 also modulated energy homeostasis by suppressing fatty acid biosynthesis and shifting the metabolism to oxidation by up-regulating the expression of key regulators of mitochondrial biogenesis, such as a peroxisome proliferator-activated receptor γ coactivator 1α and the transcription factor nuclear respiratory factor 1. Moreover, OSU-53 suppressed LPS-induced IL-6 production, thereby blocking subsequent Stat3 activation, and inhibited hypoxia-induced epithelial-mesenchymal transition in association with the silencing of hypoxia-inducible factor 1α and the E-cadherin repressor Snail. In MDA-MB-231 tumor-bearing mice, daily oral administration of OSU-53 (50 and 100 mg/kg) suppressed tumor growth by 47–49% and modulated relevant intratumoral biomarkers of drug activity. However, OSU-53 also induced protective autophagy that attenuated its antiproliferative potency. Accordingly, cotreatment with the autophagy inhibitor chloroquine increased the in vivo tumor-suppressive activity of OSU-53. OSU-53 is a potent, orally bioavailable AMPK activator that acts through a broad spectrum of antitumor activities.

The functional role of adenosine monophosphate-activated protein kinase (AMPK)2 in regulating energy homeostasis at both cellular and whole body levels is well recognized (1–4). In response to stimuli such as exercise, cellular stress, and adipokines, this cell energy-sensing enzyme induces a series of metabolic changes to balance energy consumption via multiple downstream signaling pathways controlling nutrient uptake and energy metabolism (5). More recently, accumulating evidence suggests a link between AMPK and cancer cell growth and survival in light of its ability to activate tuberous sclerosis complex 2, a tumor suppressor that negatively regulates protein synthesis by inhibiting mammalian target of rapamycin (mTOR) (6). Thus, AMPK integrates growth factor signaling with cellular metabolism through the negative regulation of mTOR (5). From a therapeutic perspective, considering the feedback activation of Akt that occurs in response to rapamycin-based mTOR inhibitors, targeting AMPK activation represents a promising therapeutic strategy in cancer (7, 8). This premise is supported by recent preclinical findings that metformin and the AMP analog 5-aminimidazole-4-carboxamide ribose (AICAR), both pharmacological activators of AMPK, exhibit in vivo efficacy in blocking carcinogen-induced tumorigenesis and/or suppressing tumor growth in animal models (9).

* This work was supported, in whole or in part, by National Institutes of Health Grants R01CA112250 and R21CA158807 (USPHS) from NCI (to C. L. S.).
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2 The abbreviations used are: AMPK, adenosine monophosphate-activated protein kinase; mTOR, mammalian target of rapamycin; AICAR, 5-aminimidazole-4-carboxamide ribose; PPARγ, peroxisome proliferator-activated receptor-γ; TNBC, triple-negative breast cancer; EMT, epithelial-to-mesenchymal transition; ACC, acetyl-CoA carboxylase; FASN, fatty-acid synthase; PARP, poly(ADP-ribose) polymerase; LC3, microtubule-associated light chain 3; HMGR, hydroxymethylglutaryl-CoA reductase; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; mtTFA, mitochondrial transcription factor A; CQ, chloroquine; KD, kinase domain; AID, autoinhibitory domain.
Antitumor Effects of a Novel AMPK Activator in TNBC Cells

10. Moreover, epidemiologic data of population-based cohort studies indicate a significantly reduced risk of breast cancer in patients with type 2 diabetes who are taking metformin on a long term basis compared with those taking thiourea, suggesting metformin as a potential candidate for breast cancer prevention (11–14). Despite this strong rationale, drawbacks associated with metformin as a chemopreventive agent include low in vivo efficacy with IC50 in the millimolar range (15), dependence on the tumor suppressor liver kinase B1 (LKB1) to mediate AMPK activation (16, 17), and untoward gastrointestinal side effects (2). Thus, there is an urgency to develop potent activators of this fuel-sensing enzyme with a distinct mode of action. Based on our finding that thiazolidinediones activated AMPK, in part, via a peroxisome proliferator-activated receptor (PPARγ)-independent mechanism, we used ciglitazone as a scaffold to develop a lead AMPK-activating agent, OSU-53 (18). OSU-53, a PPARγ-inactive derivative, stimulates AMPK kinase activity through direct activation with high potency, a mechanism distinct from that of metformin (16) or AICAR (19). For example, although AICAR requires intracellular phosphorylation to form an AMP analog to induce AMPK activation, the inhibition of complex 1 of the mitochondrial respiratory chain with the consequent increase in cytosolic AMP concentrations has been implicated in the mode of action of metformin (20). Moreover, the effects of both agents have also been linked to AMPK-independent mechanisms (21, 22).

In this study, we investigated the translational potential of OSU-53 as a therapeutic agent for triple-negative breast cancer (TNBC), a subtype of breast cancer characterized by a lack of estrogen receptor, progesterone receptor, and human EGF receptor (HER2) expression (23). Unlike estrogen receptor-positive and HER2-overexpressing breast cancers, the only available therapeutic options for TNBC patients are chemotherapy, which are associated with poorer overall prognosis (24). Thus, relevant target(s) and optimal treatments remain to be defined in TNBC.

We obtained evidence that OSU-53 at low micromolar concentrations effectively inhibited TNBC cell proliferation by concurrently blocking multiple oncogenic signaling pathways and energy metabolism through AMPK activation. Also noteworthy is the ability of OSU-53 to inhibit hypoxia-induced epithelial-mesenchymal transition (EMT) by reducing hypoxia-inducible factor (HIF)-1α expression. Moreover, oral OSU-53 suppressed TNBC xenograft tumor growth in vivo. This in vivo efficacy, along with the broad spectrum of antitumor activities of OSU-53, provides a proof-of-concept that targeting AMPK activation with small molecule agents represents a therapeutically relevant strategy for TNBC.

EXPERIMENTAL PROCEDURES

Cell Culture—The TNBC cell lines, MDA-MB-231 and MDA-MB-468, were obtained from American Type Culture Collection (Manassas, VA). The nonmalignant MCF-10A breast epithelial cells were a kind gift from Dr. Robert Bruegge-meier, Ohio State University. MDA-MB-231 and MDA-MB-468 cells were cultured in DMEM supplemented with 10% FBS (Invitrogen), and MCF10A cells were maintained in DMEM/F-12 medium supplemented with 5% horse serum (Invitrogen), 0.5 μg/ml hydrocortisone, 100 ng/ml cholera toxin, 10 μg/ml insulin, and 20 ng/ml recombinant human EGF. All cells were cultured in a humidified incubator containing 5% CO2 at 37 °C. For experiments requiring hypoxic conditions, cells were first cultured under normoxic conditions to obtain the desired level of confluence before experimentation under strictly controlled hypoxic conditions (0.3% O2) using the Proox model C21 O2/CO2 controller and C-Chamber (BioSperix, Lacona, NY).

Reagents and Antibodies—OSU-53 was synthesized according to a published procedure (18). For in vitro experiments, OSU-53 was dissolved in DMSO, diluted in culture medium, and added to cells at a final DMSO concentration of 0.1%. For in vivo studies, OSU-53 was prepared as a suspension in vehicle (0.5% methylcellulose, 0.1% Tween 80 in sterile water) for oral administration to tumor-bearing immunocompromised mice.

Mouse monoclonal antibodies against lysosome-associated membrane protein (LAMP)2 and β-actin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA) and MP Biomedicals (Irvine, CA), respectively. Rabbit antibodies against Thr(P)172-AMPK, AMPK, Ser(P)2448-mTOR, mTOR, Thr(P)389/p70S6K, p70S6K, Ser(P)79-acetyl-CoA carboxylase (ACC), ACC, fatty acid synthase (FASN), Myc tag, Ser(P)9-glycerogen synthase kinase (GSK)3β, GSK3β, Ser(P)473-Akt, Akt, Thr(P)308-Akt, Akt, Tyr(P)705-Stat3, Stat3, Tyr(P)1007/1008-Jak2, Jak2, HIF-1α, E-cadherin, vimentin, poly(ADP-ribose) polymerase (PARP), and microtubule-associated light chain (LC)3 were obtained from Cell Signaling (Beverly, MA); Ser(P)872-hydroxymethylglutaric-acid-CoA reductase (HMGCAR), HMGCAR, and PPARγ coactivator (PGC)-1α from Millipore (Billerica, MA); cyclin D1, Twist, and Slug from Santa Cruz Biotechnology; and Snail from Abcam (Cambridge, MA). Alexa Fluor dye-conjugated phalloidin (Alexa Fluor 488) and secondary antibodies (Alexa Fluor 488 and 555) were purchased from Invitrogen.

Cell Viability and Colony Formation Assays—Cell viability was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assays as reported previously (18). Cells were seeded at 3 × 104 cells/well 24 h prior to treatment. For colony formation assays, cells were seeded at a density of 1 × 104 cells per 6-cm dish. After 24 h, cells were exposed to different concentrations of OSU-53 for 10–12 days with changes of drug-containing medium every 3 days. Cells were fixed with 4% formaldehyde in PBS and stained with a 0.5% crystal violet solution in 25% methanol. Colonies of more than 15 cells were counted.

Cell Cycle Analysis—Cells were plated in 6-cm plates (4 × 105 cells/well) and were exposed to OSU-53 at the indicated concentrations for 48 h. Cell cycle distribution was analyzed by propidium iodide staining, followed by flow cytometry (FACScan; BD Biosciences).

Radiometric in Vitro AMPK Kinase Assay—The kinase activity of the recombinant AMPK holoenzyme α1β1γ2 was assessed in an in vitro radiometric assay using the SAMS peptide (HMRSAMSGLHVKKR) as substrate and [γ-32P]ATP as phosphate donor. Twenty ng of the recombinant AMPK (Cell Signaling, catalog no. 7381) was incubated with different concentrations of OSU-53 or AMP in 15 μl of AMPK kinase assay buffer (Cell Signaling, catalog no. 9802) at room temperature.
for 20 min. The reaction was initiated by addition of 5 µl of SAMS peptide (1.0 µg/µl) and 5 µl of [γ-32P]ATP solution (0.16 µCi/µl), and after incubation at 30 °C for 30 min, the reaction was terminated by addition of 10 µl of 1% phosphoric acid. The reaction mixtures were spotted onto P81 phosphocellulose paper, followed by washes in 75 mM phosphoric acid. The 32P-labeled peptides were measured by scintillation counting.

**Transient Transfection**—Cells were transfected with 3 µg of plasmid encoding the K45R kinase-dead dominant-negative AMPK (Addgene, Cambridge, MA) or empty vector by electroporation using the Amaxa Nucleofector system (Lonza, Walkersville, MD) according to the manufacturer’s protocol. Treatments were initiated 48 h after transfection. Expression of dominant-negative AMPK was confirmed by immunoblotting of the Myc tag, phosphorylated AMPK, and downstream targets of AMPK.

**RT-PCR Analysis**—Total RNA was isolated from drug-treated cells with TRIzol (Invitrogen) and then reverse-transcribed to cDNA using the Omniscript RT kit (Qiagen, Valencia, CA). The PCR products were resolved by electrophoresis on 1% agarose gels and visualized by ethidium bromide staining. The sequences of the primers used in this study are listed as follows: FASN, forward 5’TCTACGGCTCAGCTCT-3’ and reverse 5’AGGCGTATAGACCCGTGACG-3’; PGC-1α, forward 5’CAAGCCAAACCAACACTTTATCT-3’ and reverse 5’CTGCCAATCAGGAGGACATC-3’; nuclear respiratory factor (NRF)-1, forward 5’CCACGTTACAGGGAGGTGAG-3’ and reverse 5’TGTAGCTCCCTGCTGATCT-3’; mitochondrial transcription factor A (mtTFA), forward 5’TATCAAGTGGTATAGGGC-3’ and reverse 5’ACTCCTAGGACCATATTT-3’; IL-6, forward 5’AGAAAGGACATGTGAACAGAGT-3’ and reverse 5’GCGGAGATGAGTGGTTT-3’; GAPDH, forward 5’AGGGTCTTACATGGCAACTG-3’ and reverse 5’CGACACTTTGTCAAGCCTA-3’. The cycle numbers for the RT-PCR of each target gene were as follows: FASN, 30; PGC-1α, 31; NRF-1, 33; mtTFA, 33; IL-6, 32; GAPDH, 26.

**Immunoblotting**—Cells were suspended in SDS sample buffer, sonicated, and boiled for 10 min. After brief centrifugation, equivalent amounts of proteins from the soluble fractions of cell lysates were resolved by SDS-PAGE, transferred to nitrocellulose membranes, and immunoblotted as described previously (18).

**MitoTracker Assay**—Mitochondria mass was determined by MitoTracker Green FM staining (Invitrogen). Cells were plated in 6-well plates (2 × 10^5 cells/well), incubated with OSU-53 at the indicated concentrations for 48 h, washed with serum-free DMEM, and stained with 100 nM MitoTracker Green FM in 6-well plates (2 × 10^5 cells/well), incubated with OSU-53 at the indicated concentrations for 48 h, washed with serum-free DMEM, and stained with 100 nM MitoTracker Green FM in 6-well plates containing 10% FBS-supplemented medium. After 3 h, the cells were fixed with 100% methanol and stained with Giemsa. Unmigrated cells remaining in the upper chambers were removed by wiping the top of the insert membranes with a damp cotton swab leaving only those cells that had migrated to the underside of the membranes. The membranes were mounted on glass slides, and the numbers of cells in three randomly chosen high power fields were counted. Experiments were performed three times.

**Immunofluorescence**—Treated cells were washed with cold PBS, fixed with 4% formaldehyde in PBS for 10 min at 37 °C, permeabilized with 0.5% Triton X-100 in PBS for 5 min at room temperature, and then blocked with 3% BSA in PBS overnight at 4 °C. After washing with PBS, the cells were incubated with primary antibody in PBS containing 1% BSA for 1 h at room temperature and then with secondary antibody conjugated to Alexa Fluor 488 (for LC3) or 555 (for LAMP2) for 1 h at room temperature. Nuclei were stained with DAPI contained in the Vectashield mounting medium (Vector Laboratories, Burlingame, CA). Confocal images were obtained with an Olympus FV1000 confocal microscope (Olympus Corp., Japan) using the 40× oil immersion lens. To visualize actin cytoskeletal structure, treated cells were fixed, permeabilized, and blocked as described above and then incubated with Alexa Fluor 488-conjugated phalloidin in the presence of 1% BSA for 30 min.

**MDA-MB-231 Xenograft Tumor Model**—Xenograft tumors were established in female NCr athymic nude mice (5–7 weeks of age; NCI, Frederick, MD) by subcutaneous injection of 1 × 10^6 MDA-MB-231 cells in a total volume of 0.1 ml of PBS containing 50% Matrigel (BD Biosciences). To test the activity of OSU-53 as a single agent, mice with established tumors (46.8 ± 8.4 mm^3) were randomized to three groups (n = 7) receiving single daily treatments of OSU-53 at 50 or 100 mg/kg or vehicle (0.5% methylcellulose, 0.1% Tween 80 in water) for 28 days by oral gavage. In a separate study to investigate the effect of combining OSU-53 with the autophagy inhibitor chloroquine (CQ), mice with established tumors (53.6 ± 11.6 mm^3) were randomized to four groups (n = 7) receiving the following treatments once daily: (a) CQ alone (50 mg/kg) plus methylcellulose/...
Antitumor Effects of a Novel AMPK Activator in TNBC Cells

Twee 80 vehicle; (b) OSU-53 alone (100 mg/kg) plus PBS vehicle; (c) CQ (50 mg/kg) plus OSU-53 (100 mg/kg), and (d) both vehicles. OSU-53 and its vehicle (methylcellulose/Tween 80) were administered by oral gavage, and CQ and the PBS vehicle were delivered by intraperitoneal injection at a volume of 10 µl/g body weight. Tumor volumes were calculated from weekly caliper measurements using a standard formula (volume = width² x length x 0.52). Body weights were measured weekly. At terminal sacrifice, tumors were harvested, snap-frozen in liquid nitrogen, and stored at −80 °C until used for biomarker analysis as described in the text. All experimental procedures using live animals were conducted in accordance with protocols approved by Ohio State University Institutional Animal Care and Use Committee.

Homology Modeling and Molecular Docking—The primary sequence of human AMPK was retrieved from NCBI (no. 94557301), and the crystal structure of the kinase domain (KD)-autoinhibitory domain (AID) of Schizosaccharomyces pombe AMPK (Protein Data Bank code 3H4]) was used as a template for the homology target of human AMPK α-subunit structure. Sequence similarity and alignment were analyzed by using Discovery Studio 2.1 (Accelrys Inc., San Diego). The construction of the three-dimensional model was carried out by means of homology modeling that was geometry-optimized with CHARMM forced field calculation and further refined by molecular dynamics simulations. The Profiles-3D and Ramachandran plot programs were used to check the validity of human AMPK α-subunit three-dimensional structure by measuring the compatibility of that structure with the sequence of the protein. The structure of OSU-53 was constructed by geometry optimization with CHARMM force field calculation. Docking of OSU-53 into the KD-AID cleft was carried out using the CHARMM-based molecular docking algorithm implemented in the Discovery Studio 2.1 program. Flexibility of OSU-53 was taken into account by including different orientations and its rotatable torsion angles in the docking procedure. Accordingly, 10⁸ conformation structures were generated, among which representatives of 10⁵ stable conformations were obtained for analysis of the OSU-53-docked human KD-AID structure. The remaining results were minimized, and the lowest energy docking result was used as a starting point for further molecular dynamics simulations.

Statistical Analysis—Quantitative data from in vitro experiments are presented as means ± S.D. Data from in vivo experiments are expressed as means ± S.E. Differences among group means were analyzed using one-way analysis of variance or unpaired Student’s t test. Differences were considered significant at p < 0.05. Statistical analyses were performed using SPSS software (SPSS Inc., Chicago).

RESULTS

OSU-53, a Direct Activator of AMPK, Exhibits High Potency in Inhibiting Cell Proliferation in TNBC Cells Irrespective of the Functional Status of LKB1—Radiometric kinase assays to assess the activity of the recombinant AMPK α1β1γ2 showed that OSU-53 (Fig. 1A, left panel) directly stimulated kinase activity with an EC₅₀ of 0.3 µM vis à vis 8 µM for AMP (Fig. 1A, right panel). This finding indicates that the mode of action for OSU-53 in AMPK activation is distinctly different from those of metformin and AICAR. The results of MTT and clonogenic assays show a concentration- and time-dependent suppressive effect of OSU-53 on the viability (Fig. 1B) and survival (Fig. 1C) of LKB1-deficient MDA-MB-231 and LKB1-functional MDA-MB-468 cells with nearly equal potency (IC₅₀, 5 and 2 µM for MTT and clonogenic assays, respectively). This antiproliferative potency is 3–4 orders of magnitude higher than that reported for metformin (15) and AICAR (25) in breast cancer cells. Moreover, this LKB1-independent activity of OSU-53 contrasts with the reported insensitivity of MDA-MB-231 cells to metformin because of the lack of LKB1 expression in this cell line (26). Notably, OSU-53 had no apparent effect on the viability of nonmalignant MCF-10A cells over the same concentration range (Fig. 1B, right panel), indicating the selectivity of OSU-53 for malignant cells.

The suppressive effect of OSU-53 on cancer cell viability was, at least in part, attributable to apoptosis, as evidenced by PARP cleavage (Fig. 1D) and increases in the sub-G₁ apoptotic population (Fig. 1E). In addition, it is well recognized that AMPK activation promotes autophagy in cancer cells via mTOR inhibition (27). The ability of OSU-53 to induce autophagy concomitantly with apoptosis was manifested by the parallel conversion of LC3-I to LC3-II in MDA-MB-231 cells (Fig. 1D). Furthermore, immunocytochemical analysis revealed the accumulation of LC3-positive puncta following OSU-53 (5 µM) treatment, which colocalized with the lysosomal marker LAMP2, indicative of the formation of autophagosome vacuoles (Fig. 1F). In contrast, MCF-10A cells were not susceptible to the effect of the drug on apoptosis and autophagy, as indicated by lack of PARP cleavage and LC3-II conversion (Fig. 1D).

The basis of this discriminative effect of OSU-53 between tumor and nonmalignant cells may lie in differences in the activation status of signaling kinases associated with AMPK and Akt pathways. In principle, cancer cells evade genomic instability-induced apoptosis and acquire aggressive phenotype by up-regulating survival signaling pathways, the so-called oncogenic addiction (28), which is manifested by sharp differences in the activation status of mTOR, p70S6K, Akt, and GSK3β between MDA-MB-231 cells and MCF-10A cells (Fig. 2A). Relative to MDA-MB-231 cells, MCF-10A cells exhibited extremely low basal phosphorylation levels of these kinases, indicating that these signaling pathways are not up-regulated.

Mechanistic Validation of the Role of AMPK Activation in OSU-53-mediated Antitumor Effects—It has been reported that the ability of metformin and AICAR to suppress cancer cell proliferation is attributable to the suppressive effect of AMPK activation on mTOR-p70S6K signaling (26) and lipogenesis (9). Thus, we assessed the effects of OSU-53 on biomarkers pertinent to these signaling pathways to verify its mode of antiproliferative action.

mTOR-p70S6K Signaling—Western blot analysis indicates that even at 1 µM, OSU-53 was able to induce concentration- and time-dependent increases in the phosphorylation levels of AMPK accompanied by parallel decreases in those of mTOR and its downstream target p70S6K in MDA-MB-231 cells (Fig.
In contrast, although OSU-53 facilitated dose-dependent activation of AMPK in MCF-10A cells, changes in the already low levels of phosphorylation of mTOR and p70S6K were imperceptible (Fig. 2B). Considering the lack of LKB1 expression in MDA-MB-231 cells, the modulation of the AMPK-mTOR-p70S6K pathway by OSU-53 refutes the involvement of LKB1 as the upstream kinase mediating AMPK activation in response to OSU-53 treatment, which supports the notion that OSU-53 is a direct AMPK activator.

To validate the causal relationship between AMPK activation and the cytotoxicity of the drug, we examined the effect of the ectopic expression of dominant-negative AMPK, specifically a K45R kinase-dead α1-subunit (29), on OSU-53-mediated inhibition of cell viability. As shown, dominant-negative inhibition of AMPK, as evidenced by the abrogation of the effect of OSU-53 on biomarkers relevant to the AMPK-mTOR signaling pathway (Fig. 2C, right panel), protected cells from the antiproliferative activity of the drug (left panel).
Lipogenesis and Mitochondrial Biogenesis—Reminiscent of the reported effects of metformin and AICAR on lipogenesis (2, 8, 9, 30), OSU-53 inactivated or repressed key enzymes involved in the de novo synthesis of fatty acids and cholesterol in MDA-MB-231 cells. As shown in Fig. 3A, OSU-53 treatment led to increased phosphorylation and consequent inactivation of two AMPK downstream targets, ACC and HMGCR, as well as reduced mRNA and protein expression levels of FASN in time- and concentration-dependent manners. Moreover, AMPK has been reported to play a key role in regulating mitochondrial biogenesis in muscle and neuronal cells (31), in part by up-regulating the expression of key regulators of mitochondrial functions, including PGC-1α, the transcription factor NRF1, and the NRF1 target mtTFA (32). The stimulatory effect of OSU-53 on the PGC-1α-NRF1 pathway was also noted in treated MDA-MB-231 cells as drug treatment led to concentration- and time-dependent increases in the protein and/or mRNA levels of these three key regulators (Fig. 3A). However, despite up-regulation of these mitochondrial biogenesis markers, MitoTracker Green FM staining indicates that OSU-53 did not cause significant increases in mitochondrial mass (Fig. 3B). This discrepancy might be due to the mitochondrial dysfunction reported in MDA-MB-231 cells (33). OSU-53 also interferes with a series of signaling pathways governing the survival and aggressive phenotype of TNBC cells through AMPK activation, which are delineated as follows.

Akt Signaling—It has been reported previously that adiponectin-induced AMPK activation facilitated the dephosphorylation of Akt by stimulating PP2A activity (34). Pursuant to this finding, we demonstrated that OSU-53 significantly increased PP2A activity in MDA-MB-231 cells in a dose-dependent manner (Fig. 4A), which was abolished by pretreatment of cells with the PP2A inhibitor okadaic acid. This PP2A activation led to the dephosphorylation of Akt at Thr308 to a greater

FIGURE 3. OSU-53 modulates biomarkers associated with lipogenesis and mitochondrial biogenesis. A, concentration- (left panel) and time- (right panel) dependent effects of OSU-53 on the phosphorylation and expression levels of markers of lipogenesis and mitochondrial biogenesis in MDA-MB-231 cells, including the phosphorylation levels of ACC and HMGCR, and protein levels of FASN and PGC-1α (upper panels, Western blotting), as well as the mRNA levels of FASN, PGC-1α, NRF1, and mtTFA (bottom panels, RT-PCR). B, MitoTracker Green FM staining analysis of the dose-dependent effect of OSU-53 on mitochondrial mass. Cells were treated with the indicated concentration of OSU-53 for 48 h and then stained with MitoTracker Green FM as described under “Experimental Procedures.” Data are presented as means ± S.D. of three independent experiments.
ext to that at Ser473 (Fig. 4B), which is characteristic of PP2A. This reduction in p-Akt levels was accompanied by parallel decreases in GSK3β phosphorylation and cyclin D1 expression, indicative of the blockade of the Akt signaling cascade. Moreover, okadaic acid abrogated the suppressive effect of OSU-53 on Akt signaling, supporting the functional role of PP2A. The ability of OSU-53 to inactivate Akt is noteworthy because it represents an advantage over current mTOR inhibitors, which cause a compensatory activation of Akt. Relative to MDA-MB-231 cells, MCF-10A cells exhibited very low phosphorylation and cyclin D1 expression, indicative of the blockade of the Akt signaling cascade (Fig. 2A).

**IL-6 Signaling**—AMPK activation has been shown to suppress the expression of the inflammatory cytokine IL-6 (36–38), which plays a key role in promoting breast cancer progression via Jak2/Stat3 signaling (39). We obtained evidence that OSU-53 at low micromolar concentrations was effective in blunting LPS-mediated activation of the IL-6/Jak2/Stat3 pathway through AMPK activation. As shown, relative to DMSO control, LPS stimulated the mRNA expression of IL-6 (Fig. 5, left, upper panel), accompanied by higher levels of Jak2 and Stat3 phosphorylation (Fig. 5, left, lower panel), which, however, could be blocked by OSU-53 in a concentration-dependent manner. This suppressive effect of OSU-53 on LPS-induced IL-6 production and the subsequent activation of Jak/Stat3 signaling was attributable to AMPK activation as it could be abolished by the pharmacological inhibition of AMPK by compound C (Fig. 5, left, lower panel, and B, right panel) and/or the ectopic expression of dominant-negative AMPK (Fig. 5A, right panel).

**Hypoxia-induced EMT**—Substantial evidence indicates that EMT, as characterized by reduced expression of the epithelial cell adhesion marker E-cadherin and aberrant induction of the mesenchymal marker vimentin, underlies enhanced metastasis and unfavorable clinical outcome in breast cancer (40). EMT is triggered by many types of stimuli in the tumor microenvironment, including growth factors, inflammatory cytokines, cell-cell interactions, and hypoxia (41, 42). In this study, we assessed the effect of OSU-53 on hypoxia-induced EMT, in which HIF-1α plays a crucial role (43). We rationalized that OSU-53 could inhibit hypoxia-induced HIF-1α expression through two distinct AMPK-dependent mechanisms as follows: inhibition of mTOR-mediated HIF-1α translation (44) and induction of
Antitumor Effects of a Novel AMPK Activator in TNBC Cells

GSK3β-stimulated HIF-1α degradation (45). As MDA-MB-231 cells lack E-cadherin expression (46), we examined the drug effect in MDA-MB-468 cells. As expected, relative to the normoxic control, hypoxia stimulated HIF-1α protein expression, which was accompanied by changes in EMT markers, including reduced E-cadherin expression and increased vimentin expression (Fig. 6A). In support of our premise, these hypoxia-induced changes to HIF-1α and both EMT markers were abrogated by OSU-53 in a concentration-dependent manner (Fig. 6A). The results also suggest that this inhibition of EMT might be attributable to the suppressed expression of Snail and, to a much lesser extent, Twist, both of which are known repressors of E-cadherin (47, 48) under HIF-1α regulation (49).

Furthermore, OSU-53 was able to suppress processes characteristic of the EMT-associated aggressive phenotype, such as hypoxia-stimulated actin cytoskeletal rearrangement and cell migration, in MDA-MB-468 cells. Relative to normoxia, hypoxia stimulated stress fiber assembly at the cell edge, as manifested by filopodial and lamellipodial protrusions (Fig. 6B, upper panels). These hypoxia-induced changes in F-actin structure, however, could be blocked by OSU-53 at 2.5 and 5 μM (Fig. 6B, lower panels). As these cytoskeletal rearrangements play a central role to cell migration, we assessed the effect of OSU-53 on MDA-MB-468 cell migration in the Boyden chamber system. As shown, hypoxia increased the migration of MDA-MB-468 cells by 2-fold after 48 h of treatment, which was blocked by OSU-53 in a dose-dependent manner (Fig. 6C). As the extent of OSU-53-mediated inhibition of cell migration was substantially greater than that of cell viability, this inhibition was not solely attributable to increased cell death.

Suppressive Effect of OSU-53 on MDA-MB-231 Xenograft Tumor Growth—The in vivo antitumor efficacy of OSU-53 was evaluated in ectopic MDA-MB-231 tumor xenograft models. Athymic nude mice bearing established subcutaneous MDA-MB-231 tumors were treated orally with OSU-53 once daily at 50 or 100 mg/kg versus vehicle control (n = 7 for each group). OSU-53 at both doses was well tolerated as the mice showed no overt signs of toxicity or loss of body weight (data not shown). Although treatment with oral OSU-53 at either dose resulted in significant suppression of tumor growth relative to the vehicle control (p < 0.05) after 28 days of treatment, no dose dependence in the tumor-suppressive response was noted (49 and 48% suppression for 50 and 100 mg/kg, respectively; Fig. 7A). However, examination of intratumoral markers associated with drug activity showed a higher degree of AMPK phosphorylation and parallel decreases in the phosphorylation levels of mTOR, p70S6K, and Akt in the 100 mg/kg group than in the 50 mg/kg group (Fig. 7B), suggesting a dose-related delivery of drug to the tumor. Also noted was a greater extent of apoptosis and autophagy, as indicated by increased PARP cleavage and LC3-II conversion, in the higher dose group.

CQ Enhances Antiproliferative Activity of OSU-53—Although autophagy can mediate either protective or destructive cellular response to metabolic stress or therapeutic agents (50), evidence indicates that autophagy acts as a survival signal in response to inhibitors of the PI3K-Akt-mTOR signaling axis (51). In this context, we examined the effect of CQ, a lysosomotropic inhibitor of autophagy, on antiproliferative activity in vitro of the OSU-53. As shown, CQ at 10 μM significantly increased (*, p < 0.05) the suppressive effect of OSU-53 on the viability of MDA-MB-231 cells (Fig. 8A). Western blot analysis indicates that this increase was attributable to a higher extent of drug-induced apoptosis as a result of autophagy inhibition (Fig. 8B).

This drug combination was also tested in vivo. As shown, CQ (50 mg/kg daily, intraperitoneal injection) was able to enhance the suppressive effect of daily oral OSU-53 at 100 mg/kg on MDA-MB-231 xenograft tumor growth (60% suppression for the combination versus 47% for OSU-53 alone), although CQ alone exhibited no appreciable tumor inhibitory activity (Fig. 8C; n = 7 in each group).

Mechanism by Which OSU-53 Activates AMPK—Our data indicate that OSU-53 is a direct activator of AMPK. In the literature, two other direct, small molecule AMPK activators have been reported, PT1 (52) and A-769662 (53), each of which exhibit a distinct mode of activation. Evidence suggests that PT1 antagonizes AMPK autoinhibition by binding to the α-subunit near the autoinhibitory domain (AID) (52) and that A-769662 stabilizes the active conformation of AMPK through
allosteric binding to the $\gamma$-subunit (53). As the electrostatic potential map of OSU-53 exhibited a high degree of similarity to that of PT1 (Fig. 9A), we conducted molecular simulation by docking OSU-53 into the putative binding site formed between the KD and the AID of the $\alpha$-subunit through homology modeling (Fig. 9B). As shown, OSU-53 bound to the AID domain through electrostatic interactions with His$^{152}$, Lys$^{156}$, and Tyr$^{277}$ in a manner reminiscent of that of PT1. Mutational analysis to confirm this mode of ligand recognition is currently under way.

DISCUSSION

In this study, we obtained evidence of the translational potential of OSU-53, a novel thiazolidinedione-derived AMPK activator, as a therapeutic agent for TNBC. OSU-53 exhibits 3 orders of magnitude higher antiproliferative potency than metformin (IC$_{50}$, low micromolar versus millimolar) and, more importantly, directly activates AMPK (EC$_{50}$, 0.3 $\mu$M) independent of its upstream kinase LKB1. This finding may have important therapeutic implications as LKB1 expression is often lost or down-regulated in breast tumors (54). Moreover, considering the pivotal role of LKB1 in mediating metformin-induced AMPK activation (17), LKB1-deficient breast tumors may be resistant to the chemopreventive and therapeutic effects of metformin (55), which represents a potential therapeutic advantage of OSU-53.

Our findings also show that OSU-53 is a potent antitumor agent that exhibits in vitro and in vivo efficacy in suppressing TNBC cell proliferation via diverse AMPK-dependent mechanisms. In addition to the AMPK-induced down-regulation of mTOR signaling and lipogenesis, which have been shown to underlie the antiproliferative activities of metformin (26) and AICAR (9), respectively, OSU-53 also modulates a series of pathways downstream of the AMPK cascade that govern survival, mitochondrial biogenesis, cytokine production, and EMT, as manifested by effects on the phosphorylation/expresssion levels of Akt, PGC-1$\alpha$, IL-6, and HIF-1$\alpha$ (Fig. 9C).

The suppressive effect of OSU-53 on Akt phosphorylation (Fig. 4) is particularly noteworthy, because it circumvents the feedback activation of Akt that results from mTOR inhibition, a drawback to the use of early generation mTOR inhibitors. The
Antitumor Effects of a Novel AMPK Activator in TNBC Cells

ability of OSU-53 to concurrently block signaling through both kinases, reminiscent of that of the second generation mTOR inhibitors and dual PI3K-mTOR inhibitors (56), provides therapeutic advantages over rapamycin. Moreover, OSU-53-facilitated Akt dephosphorylation in MDA-MB-231 cells was mediated through a PP2A-dependent mechanism, which is consistent with that described for adiponectin-induced AMPK activation in the same cell line (34). However, this result contrasts with recent reports that metformin facilitated Ser473 Akt dephosphorylation in MCF-7 cells via an insulin receptor substrate 1-dependent pathway (15) and that AICAR stimulated Akt phosphorylation through insulin-like growth factor 1 receptor-dependent and -independent mechanisms (57). This discrepancy might reflect the differences in the modes of action among these AMPK activators, which warrants investigation.

The effects of OSU-53 on energy homeostasis in TNBC cells, which parallel those reported for metformin and AICAR in skeletal muscle and neuronal cells (32, 58, 59), were characterized by the blocked activation or expression of key enzymes involved in fatty acid biosynthesis (ACC, HMGCR, and FASN)
and enhanced expression of regulators of mitochondrial biogenesis (PCG-1α, NRF1, and mtTFA) (Fig. 3A). Jointly, these effects on energy homeostasis can contribute to the antiproliferative activity of OSU-53 by inhibiting fatty acid synthesis and shifting cellular metabolism toward oxidation. Such changes in lipid metabolism have been shown experimentally, by use of the fatty-acid synthase inhibitor C75, to induce apoptotic death in breast cancer cells (60).

The ability of OSU-53 to suppress IL-6 production is clinically relevant in light of the major role of this cytokine in driving Stat3 activation in breast cancer (61). Stat3 represents an important therapeutic target in breast cancer because constitutively activated Stat3, which occurs in over 50% of primary breast tumors and is associated with a poor prognosis, endows tumor cells with chemoresistance and angiogenic potential (62). Moreover, the silencing of expression of HIF-1α and the E-cadherin repressor Snail may underlie the suppressive effect of OSU-53 on hypoxia-induced EMT and migratory activity in TNBC cells (Fig. 6) suggesting the ability to repress the aggressive phenotype associated with EMT.

Despite this broad spectrum of antitumor activities, nonmalignant MCF-10A mammary epithelial cells were unaffected by OSU-53, in part, due to the low basal activation levels of Akt and mTOR (Fig. 2). This low cytotoxicity is consistent with the tumor-suppressive effects of OSU-53, in part, due to the low basal activation levels of Akt and mTOR (Fig. 2). This low cytotoxicity is consistent with the low basal activation levels of Akt and mTOR (Fig. 2). This low cytotoxicity is consistent with the low basal activation levels of Akt and mTOR (Fig. 2). This low cytotoxicity is consistent with the low basal activation levels of Akt and mTOR (Fig. 2). This low cytotoxicity is consistent with the low basal activation levels of Akt and mTOR (Fig. 2). This low cytotoxicity is consistent with the low basal activation levels of Akt and mTOR (Fig. 2). This low cytotoxicity is consistent with the low basal activation levels of Akt and mTOR (Fig. 2). 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Antitumor Effects of a Novel AMPK Activator in TNBC Cells

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