From the Cyclooxygenase-2 Inhibitor Celecoxib to a Novel Class of 3-Phosphoinositide-Dependent Protein Kinase-1 Inhibitors

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ABSTRACT

The blockade of Akt activation through the inhibition of 3-phosphoinositide-dependent kinase-1 (PDK-1) represents a major signaling mechanism whereby celecoxib mediates apoptosis. Celecoxib, however, is a weak PDK-1 inhibitor (IC50, 48 µM), requiring at least 30 µM to exhibit discernable effects on the growth of tumor cells in vitro. Here, we report the structure-based optimization of celecoxib to develop PDK-1 inhibitors with greater potency in enzyme inhibition and growth inhibition. Kinetics of PDK-1 inhibition by celecoxib with respect to ATP suggest that celecoxib derivatives inhibit PDK-1 by competing with ATP for binding, a mechanism reminiscent to that of many kinase inhibitors. Structure-activity analysis together with molecular modeling was used to generate compounds that were tested for their potency in inhibiting PDK-1 kinase activity and in inducing apoptosis in PC-3 prostate cancer cells. Docking of potent compounds into the ATP-binding site of PDK-1 was performed for lead optimization, leading to two compounds, OSU-03012 and OSU-03013, with IC50 values in PDK-1 inhibition and apoptosis induction in the low µM range. Exposure of PC-3 cells to these agents led to Akt dephosphorylation and inhibition of p70 S6 kinase activity. Moreover, overexpression of constitutively active forms of PDK-1 and Akt partially protected OSU-03012-induced apoptosis. Screening in a panel of 60 cell lines and more extensive testing in PC-3 cells indicated that the mean concentration for total growth inhibition was ~3 µM for both agents. Considering the conserved role of PDK-1/Akt signaling in promoting tumorigenesis, these celecoxib analogs are of translational relevance for cancer prevention and therapy.

INTRODUCTION

After demonstration of the efficacy of celecoxib in reducing colorectal polyps in patients with familial adenomatous polyposis (1), use of this cyclooxygenase (COX)-2 inhibitor in the prevention of epithelial malignancies has been the subject of a series of clinical trials. Despite these ongoing clinical investigations, the molecular mechanism underlying celecoxib-mediated in vivo antitumor effects remains elusive. At the cellular level, celecoxib inhibits COX-2 and causes cell cycle arrest and apoptosis in cancer cells. Evidence is accumulating that the in vitro effects of celecoxib on cell cycle progression and apoptosis are mediated through COX-2-independent signaling mechanisms (2–6). Among various putative pathways reported, the blockade of Akt activation through the inhibition of its upstream kinase, 3-phosphoinositide-dependent kinase-1 (PDK-1), is especially noteworthy (2–5, 7, 8). Our data indicate that celecoxib inhibits PDK-1/Akt signaling with moderate potency and that structural modifications allowed the inhibitory effect of celecoxib on Akt activation to be separated from the COX-2 inhibitory activity (4, 5). Together, this paradigm shift provides molecular underpinnings for the pharmacological exploitation of celecoxib to develop a novel class of potent PDK-1/Akt signaling inhibitors, of which the proof of principle has been demonstrated in our previous reports (4, 5).

The phosphatidylinositol 3'-kinase/PDK-1/Akt signaling cascade represents a convergence point for a plethora of receptor tyrosine kinase and cytokine-mediated pathways that regulate cell proliferation and survival and offers a framework to account for the ability of many extracellular trophic factors to maintain cell survival (9–15). Dysregulation of this signaling cascade due to constitutive growth factor-receptor activation and/or PTEN mutations results in Akt up-regulation, which subsequently promotes tumor invasiveness, angiogenesis, and progression (16–19). Thus, PDK-1/Akt signaling inhibitors are of translational relevance for development into useful chemotherapeutic or chemopreventive agents. In this study, we carried out structure-based optimization of celecoxib using an integrated approach combining structure-activity analysis and molecular modeling, leading to two potent PDK-1 inhibitors, OSU-03012 and OSU-03013, with IC50 values at the low µM range. These two PDK-1 inhibitors could achieve total growth inhibition in 60 different human tumor cell lines at a mean concentration of ~3 µM.

MATERIALS AND METHODS

Reagents. Celecoxib was extracted from Celebrex capsules obtained from Amerisource Health (Malvern, PA) with ethyl acetate followed by recrystallization from a mixture of ethyl acetate and hexane. The Cell Death Detection ELISA kit was purchased from Roche Diagnostics (Mannheim, Germany). Rabbit polyclonal antibodies against Akt and phospho-3'-Ser Akt were obtained from Cell Signaling Technologies (Beverly, MA). Mouse monoclonal anti-poly(ADP-ribose) polymerase (PARP) antibody was provided by PharmaMingen (San Diego, CA). The PDK-1 kinase assay kit was purchased from Upstate (Lake Placid, NY). Other chemical and biochemicals were obtained from Sigma-Aldrich (St. Louis, MO) unless otherwise mentioned. Nuclear magnetic resonance spectra (1H NMR) were measured on Bruker 250 MHz. Chemical shifts (δ) are reported in parts per million relative to tetramethylsilane peak with CDCl3, as solvent unless otherwise mentioned. High-resolution electrospray ionization mass spectrometry analyses were performed with a 3-Tesla Finnigan FTMS-2000 Fourier Transform mass spectrometer.

Synthesis of Chemicals. In this article, we discussed 36 compounds, of which the chemical names, proton nuclear magnetic resonance (1H NMR), and high-resolution mass spectrometry data are summarized in Table 1. The procedures used to synthesize compounds 1–36 are described in the Supplementary Data section.

Cell Culture. PC-3 (p53−/−) human androgen-nonresponsive prostate cancer cells were purchased from the American Type Tissue Collection (Manassas, VA). Cells were cultured in RPMI 1640 (Life Technologies, Inc., Grand Island, NY) supplemented with 10% fetal bovine serum (FBS; Life Technologies, Inc.) at 37°C in a humidified incubator containing 5% CO2.

Cell Viability Analysis. The effect of celecoxib and its derivatives on PC-3 cell viability was assessed by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide assay in six replicates. Cells were grown in 10% FBS supplemented RPMI 1640 in 96-well, flat-bottomed plates for 24 h, and exposed to various concentrations of celecoxib derivatives dissolved in DMSO (final concentration ≤0.1%) in 1% serum-containing RPMI 1640 for different time intervals. Controls received DMSO vehicle at a concentration equal to that in drug-treated cells. The medium was removed, replaced by 200
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Table 1 Nomenclature, 1H NMR, and HRMS characterizations of compounds 1–36

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Theoretical mass, actual mass, and HRMS characterizations of compounds 1–36 are provided in the table. The compounds are characterized by 1H NMR and HRMS data, indicating the structure and purity of the synthesized compounds.
μl of 0.5 mg/ml of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide in 10% FBS-containing RPMI 1640, and cells were incubated in the CO₂ incubator at 37°C for 2 h. Supernatants were removed from the wells, and the reduced 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide dye was solubilized in 200 μl/well DMSO. Absorbance at 570 nm was determined on a plate reader.

**Cell Proliferation.** PC-3 cells were seeded into six-well plates at ~75,000 cells/well in 10% FBS-containing RPMI 1640. After a 24-h attachment period, cells were treated in triplicate with the indicated concentration of celexocib derivatives or DMSO vehicle in 10% FBS-containing RPMI 1640. At different time intervals, cells were harvested by trypsinization and numerated using a Coulter counter model Z1 D/T (Beckman Coulter, Fullerton, CA).

**Apoptosis Analysis.** Two methods were used to assess drug-induced apoptotic cell death: detection of DNA fragmentation by the Cell Death Detection ELISA kit (Roche Diagnostics) and Western blot analysis of PARP cleavage. The ELISA was performed according to the manufacturer’s instructions and is based on the quantitative determination of cytoplasmic histone-associated DNA fragments in the form of mononucleosomes or oligonucleosomes generated after induced apoptotic death. In brief, 4 × 10⁶ PC-3 cells were cultured in a T-25 flask for 24 h before treatment. Cells were treated with the DMSO vehicle or the test agent at the indicated concentrations for 6–24 h, collected, and cell lysates equivalent to 2 × 10⁶ PC-3 cells were used in the ELISA. For the PARP cleavage assay, drug-treated cells were collected 4–8 h after treatment, washed with ice-cold PBS, and resuspended in lysis buffer containing 20 mM Tris-HCl (pH 8.0), 137 mM NaCl, 1 mM CaCl₂, 10% glycerol, 1% NP-40, 0.5% deoxycholate, 0.1% SDS, 100 μM 4-(2-aminoethyl)-benzenesulfonyl fluoride, leupeptin at 10 μg/ml, and aprotinin at 10 μg/ml. Soluble cell lysates were collected after centrifugation at 10,000 × g for 5 min. Equivalent amounts of proteins (60–100 μg) from each lysate were resolved in 8% SDS-polyacrylamide gels. Bands were transferred to nitrocellulose membranes and analyzed by immunoblotting with anti-PARP antibody.

**Immunoblotting.** The general procedure for the Western blot analysis of Akt and phospho-Akt is described as follows. Cells were washed in PBS, resuspended in SDS sample buffer, sonicated by an ultrasonic sonicator for 5 s, and boiled for 5 min. After brief centrifugation, equivalent protein concentrations from the soluble fractions were resolved in 10% SDS-polyacrylamide gels on a Mini gel apparatus, and transferred to a nitrocellulose membrane using a semi-dry transfer cell. The transblotted membrane was washed three times from the soluble fractions were resolved in 10% SDS-polyacrylamide gels. Bands were transferred to nitrocellulose membranes and analyzed by immunoblotting with anti-PARP antibody.
Immunocomplex was washed with lysis buffer twice, followed by assay buffer 1 h, followed by incubation with protein A-Sepharose bead for 2 h. The immobilized protein A-Sepharose was washed with lysis buffer containing 0.1% Tween 20 and resuspended in assay buffer. The assay was carried out using a p70 S6K assay kit (Upstate). In some instances, the p70 S6K immunoprecipitation was performed according to a modification of a published method.2

**Immunoprecipitated Akt Kinase Assay.** Akt immunoprecipitation was carried out according to a modified, published procedure (7). PC-3 cells were treated with DMSO vehicle or the test agents at the indicated concentrations for 2 h and then lysed at 4 °C for 1 h in buffer containing 50 mM Tris-HCl (pH 7.5), 1% Triton X-100, 1 mM EDTA, 1 mM EGTA, 50 mM sodium fluoride, 10 mM sodium β-glycerophosphate, 0.1% 2-mercaptoethanol, 0.1 mM phenylmethylsulfonyl fluoride, and 1 μg/ml each of aprotonin, pepstatin, and leupeptin. Cell lysates were centrifuged at 10,000 × g for 5 min, and the supernatant was treated with anti-Akt at 4 °C for 60 min, followed by protein G-agarose beads for additional 60 min. The immunoprecipitate was used to analyze Akt kinase activity using the Akt/serum- and glucocorticoid-regulated kinase-specific peptide substrate RPRAATF as described above. Values represent the means of two independent determinations.

**Immunoprecipitated p70 S6 kinase (p70S6k) Assay.** Immunoprecipitation of p70S6K was carried out according to a modification of a published procedure (20). In brief, PC-3 cells were cultured in T-75 flasks (1.5 × 10^6 flask), and treated with OSU-03012 at the indicated concentrations in 1% FBS-containing RPMI 1640 for 6 h. Both floating and adherent cells were collected and lysed in 1 ml of lysis buffer [50 mM Tris (pH7.4), 150 mM NaCl, 1% NP40, 0.5% sodium deoxycholate, 1 mM EGTA, and 10 μM protease inhibitor mixture (Calbiochem)] for 30 min on ice. Lysates were centrifuged at 10,000 × g at 4 °C for 20 min. Equal amounts of total protein were subjected to immunoprecipitation with anti-p70S6K antibody (sc-8418; Santa Cruz Biotechnology, Santa Cruz, CA). The mixture was incubated on ice with rocking for 1 h, followed by incubation with protein G-Sepharose beads for 2 h. The immunocomplex was washed with lysis buffer twice, followed by assay buffer [20 mM 4-morpholinopropanesulfonic acid (pH 7.2), 25 mM β-glycerol phosphate, 5 mM EGTA, 1 mM sodium orthovanadate, and 1 mM DTT], and resuspended in assay buffer. The assay was carried out using a p70S6K assay kit (Upstate) according to the manufacturer’s instruction.

**Transient Transfection.** The constitutively active Akt construct HA-PKB1308D/S473D and the constitutively active PDK-1 construct pcDNA-PDK1-A280V were kindly provided by Dr. Brain Hemmings (Friedrich Miescher Institute, Basel, Switzerland) and Dr. Feng Liu (University of Texas Health Science Center, San Antonio, TX), respectively. PC-3 cells were seeded into T-75 flasks (1.5 × 10^6/g flask). Aliquots containing 3 μg of each plasmid or a control pcDNA3.1(+)-vector in 750 μl of Opti-MEM (Invitrogen-Life Technologies, Inc.) were incubated with 9 μl of the FuGene 6 reagent (Roche Diagnostics Corp., Indianapolis, IN) for 15 min. Each flask was washed with Opti-MEM and then received the plasmid-FuGene 6 mixture and 4 ml of Opti-MEM. The flask was placed in a CO2 incubator for 4 h, and the transfection medium was replaced with 10% FBS-supplemented RPMI 1640. After 24 h, Mock-, Akt-, and PDK-1-transfected PC-3 cells were seeded into 96-well plates at 5000 cells/well in 10% FBS-supplemented RPMI 1640. On the next day, cells were treated in four replicates with the indicated concentrations of OSU-03012 in 1% FBS- containing medium for 24 h. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide assay was used to determine the cell viability.

**Molecular Modeling.** The crystal structure of PDK-1 in complex with ATP, obtained from the Brookhaven Protein Data Bank (entry code 1H1W), was subject to the deletion of heteroatoms and the addition of polar hydrogens, partial atomic charges, and atomic solvation parameters using the molecular modeling software AutoDock Tools. The three-dimensional structures of small molecules were generated using the software SYBYL 6.9 (Tripos Associate, St. Louis, MO). 2D chemical structures are shown in the Results and Discussion section.

This software is an automated docking package that combines a rapid grid-based method for energy evaluation with a Lamarckian genetic algorithm method of conformation search. All of the molecular modeling calculations and manipulations were performed on Silicon Graphics O2 (Silicon Graphics Inc., Mountain View, CA).

**Statistical Analysis.** Each experiment was performed in triplicate unless otherwise mentioned. All of the experiments were carried out at least twice on different occasions. Where appropriate, the data are presented as the mean ± SD.

**RESULTS**

**Structural Optimization of Celecoxib in PDK-1 Inhibition.** On the basis of structure-activity analysis, we reported previously a working model that defined structural features essential to the apoptotic effect of celecoxib in PC-3 prostate cancer cells (5). This model suggested that the 5-aryl and sulfonamide (-SO2 NH2) moieties were amenable to alterations, whereas the electrostatic potential surrounding the heterocyclic system was integral to the apoptosis-inducing potency. Accordingly, in the first series of structural modifications, the 4-methylphenyl moiety of celecoxib was replaced by a series of aromatic groups with discrete stereochemical properties, whereas the integrity of the pyrazole ring was maintained. The structure and potency in inhibiting PDK-1 kinase activity and PC-3 cell growth of 24 representative derivatives are summarized in Fig. 1.

These compounds, except the indole derivative 8, showed improved PDK-1 inhibitory and antiproliferative activities vis-à-vis celecoxib, whereas none of these derivatives displayed measurable COX-2 inhibitory activity (data not shown). This finding confirmed the structural divergence required for the inhibition of PDK-1 versus COX-2 (5). A general increase in PDK-1 inhibitory activity was noted with increasing bulkiness of the aromatic ring, i.e., tricyclic aromatic rings (21–23) > substituted biphenyl (9–19) > substituted phenyl (1–6). These data, together with the decrease in potency in compound 8, suggested that the aromatic system bound to a large, hydrophobic region of the enzyme pocket. Among the 24 analogs examined, compound 23 (designated as OSU-02067) represented the optimal derivative with IC50 values of 9 μM and 5 μM for inhibiting PDK-1 activity and PC-3 cell viability, respectively (Fig. 2, A and C). These IC50 values corresponded to a 5–6-fold improvement over the activities of celecoxib (48 μM and 30 μM, respectively). However, the OSU-02067 isomer 24 exhibited a precipitous decrease in PDK-1 inhibitory activity, which might be attributable to steric hindrance imposed by an unfavorable orientation of the tricyclic aromatic ring.

There existed a correlation between PDK-1 and PC-3 growth inhibition potency in all of the compounds examined, suggesting the mechanistic relevance of PDK-1 inhibition to the antiproliferative effect. Overall, the IC50 value for inhibiting PC-3 cell proliferation was approximately one half of that of PDK-1 inhibition. This discrepancy might arise from a mechanistic synergy between PDK-1 inhibition and concomitant Akt dephosphorylation by protein phosphatase 2A in drug-treated cells, resulting in augmented Akt deactivation. To examine this premise, PC-3 cells were treated with different concentrations of OSU-02067 for 2 h, and the consequent effect on Akt was assessed by two independent assays: immunoprecipitated Akt kinase activity (Fig. 2B, top panel) and Akt phosphorylation status (Fig. 2B, bottom panel). As shown, both assays gave consistent results.

**A NOVEL CLASS OF PDK-1 INHIBITORS**

1 Internet address: http://www.scripps.edu/pub/olson-web/doc/autodock/tools.html.

2 Internet address: http://www.scripps.edu/pub/olson-web/people/gmm/autodock/obtaining.html.
According to the kinase assay, the IC_{50} of OSU-02067 for inhibiting intracellular Akt activation was 5 μM vis-à-vis 28 μM for celecoxib. Neither OSU-02067 nor other celecoxib derivatives displayed a direct inhibitory effect on immunoprecipitated Akt activity. Meanwhile, Western blot analysis showed that treatment of PC-3 cells with OSU-02067 at ≥5 μM led to significant Akt dephosphorylation.

The inhibition of PDK-1/Akt signaling led to apoptotic death in PC-3 cells in 1% of FBS-containing RPMI 1640 in a dose-dependent manner (Fig. 2C), as evidenced by DNA fragmentation (Fig. 2D, top panel) and PARP cleavage (Fig. 2D, bottom panel). The dose of OSU-02067 required to induce 50% PC-3 cell death at 24 h was 5 μM, as compared with that of ~30 μM for celecoxib (data not shown). The IC_{50} values for both OSU-02067 and celecoxib to induce PC-3 cell death was consistent with that of inhibiting Akt activation in drug-treated cells. Furthermore, the effect of OSU-02067 vis-à-vis celecoxib on PC-3 cell proliferation was examined in 10% FBS-supplemented RPMI 1640 (Fig. 2E). As shown, OSU-02067 at 1 μM showed substantial antiproliferative activity, exceeding that of 30 μM celecoxib. Together, these data clearly indicated the in vitro efficacy of OSU-02067 in PC-3 growth inhibition and prompted us to undertake additional lead optimization via structure-based design.

**Molecular Modeling and Structure-Based Optimization.** Kinetics of PDK-1 inhibition by celecoxib with respect to ATP was examined to shed light onto the mechanistic basis by which these celecoxib derivatives mediated enzyme inhibition. The inhibitory effect of celecoxib on PDK-1 kinase activity was determined in the presence of various concentrations of ATP. Kinetic data revealed an inverse relationship between the degree of celecoxib-exerted PDK-1 inhibition and ATP concentrations. The resulting Lineweaver-Burke plot indicated that celecoxib inhibited PDK-1 through competition with ATP (Fig. 3).

This mode of inhibition (i.e., ATP competition) is common among numerous classes of protein or lipid kinase inhibitors that possess therapeutic potential (22), including those of Bcr-Abl (e.g., Gleevec; Refs. 23–25), epidermal growth factor receptors (e.g., Iressa; Ref. 26), phosphatidylinositol 3'-kinase (e.g., LY294002; Ref. 27), protein kinase C (staurosporine; Ref. 28), cyclin-dependent kinases (29), and vascular endothelial growth factor receptors (30). Structure-activity data indicate that although the ATP-binding site is conserved, architecture in the proximal regions of different protein kinases provides a high degree of diversity (22). Evidence indicates that this diversity allows the design of selective kinase inhibitors.

Together, these findings provided a molecular rationale for structure-based optimization on the basis of the framework generated by the crystal structure of PDK-1-ATP complex (31). OSU-02067 was docked into the ATP-binding domain that is located within a deep cleft between the two lobes of PDK-1 (Fig. 4A). Although OSU-02067 competed with ATP for binding, the mode of binding for OSU-02067 was found to be somewhat different from that of ATP (Fig. 4B).

Although the benzenesulfonamide moiety occupied the adenine-binding motif, the planar pyrazole moiety was perpendicular to the ribose ring. This arrangement positioned the adjacent phenanthrene ring behind the trisphosphate-binding pocket. The phenanthrene ring formed hydrophobic interactions with an apolar region formed by residues 88–96 encompassing part of two adjacent β sheets joined by a glycine-rich loop. This locality, adjoining to the phosphate-binding motif, could be exploited for structural optimization as demonstrated by the >5-fold increase in potency of OSU-02067 (IC_{50}, 9 μM) over celecoxib (IC_{50}, 48 μM). This hydrophobic cleft appeared to favor the binding of a large, planar aromatic system such as the phenanthrene ring. For example, as compared with OSU-02067, compounds 9–19,

![Diagram](image)
kinase activity, intracellular Akt activation, and survival of PC-3 cells. A, dose-dependent inhibition of recombinant PDK-1 kinase activity by OSU-02067 versus celecoxib. B, top, effect of OSU-02067 versus celecoxib on the kinase activity of Akt immunoprecipitated from drug-treated PC-3 cells. Bottom, phosphorylation status of Akt in PC-3 cells treated with increasing concentrations of OSU-02067. Control PC-3 cells received DMSO vehicle. Cell viability was analyzed by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. D, evidence of apoptotic death in OSU-02067-treated PC-3 cells. Top, induction of cytoplasmic nucleosomal DNA in PC-3 cells treated with DMSO vehicle or the indicated concentrations of OSU-02067. DNA fragmentation was quantitatively measured by a cell death detection ELISA kit. O.D. = absorbance. Bottom, poly(ADP-ribose) polymerase cleavage by OSU-02067 in PC-3 cells. Poly(ADP-ribose)polymerase proteolysis to the apoptosis-specific 85-kDa fragment was monitored by Western blotting. E, antiproliferative effect of 1 and 5 μM OSU-02067 versus 30 μM celecoxib in PC-3 cells. PC-3 cells were seeded into six-well plates (50,000 cells/well) and exposed to the test agent at the indicated concentration in 10% fetal bovine serum-supplemented RPMI 1640. At different time intervals, cells were harvested, and counted using a Coulter counter. Values were obtained from triplicates for the aforementioned plots except cell viability (C) in which the means of six replicates were used; bars, ±SD. Western blots shown in B and D are representatives of three independent experiments.

containing conformationally more flexible biphenyl systems, were less effective in enzyme inhibition. The discrepancy in potency between celecoxib and OSU-02067 was likely attributable to the desolvation of the hydrophobic amino acid residues upon binding of the methylphenyl versus phenanthrenyl moiety (29). Assuming that the measured IC₅₀ values were proportional to the Kᵦᵣ of the protein-ligand interaction, the relative free energy difference between celecoxib and OSU-02067 was approximately −4 kJ/mol. The upper limit for the free energy associated with the desolvation of hydrophobic groups has been estimated to be −136 J/molÅ² (32). Considering that the binding of the phenanthrene ring of OSU-02067 covers 32 Å² more apolar surface than the methylphenyl moiety does, the corresponding free energy change was estimated to be −4.35 kJ/mol, which is consistent with the experimentally measured free energy difference.

The sulfonamide group (either oxygen or nitrogen) formed hydrogen bonds with the backbone amide of Ala162 in the hinge region. This bond lattice mimicked one of the two hydrogen bonds formed by the adenosine of ATP in the same microdomain, in which 6-NH donates a hydrogen bond to carbonyl of Ser160 and 1-N accepts a hydrogen bond from NH of Ala162 (31). No hydrogen bond was formed between the pyrazole ring and Glu166, but the ribose of ATP accepts a hydrogen bond from Glu166. In light of the importance of hydrogen bonding with Ala 162 and Ser160 in ligand anchoring, we carried out structure optimization of OSU-02067 by replacing its sulfonamide moiety with a series of heteroatom-rich functional groups that could potentially form hydrogen bonding with the backbone amide and/or carbonyl of these two residues. Structures of 13 representative derivatives, their potency against PDK-1, and their ability to cause apoptotic death in PC-3 cells are summarized in Fig. 5.

Among these derivatives, compounds 34 (designated as OSU-03012) and 35 (designated as OSU-03013) exhibited IC₅₀ values for PDK-1 inhibition of 5 and 2 μM, respectively, which represented 2- and 5-fold increases in potency over OSU-02067 (Fig. 6A).

Compounds 34 and 35 contained side chains of 2-aminoacetamide and guanidine, respectively. This improvement in potency reflected a strengthening of the hydrogen bonding in the protein-ligand interactions for these derivatives. This premise was supported by the modeled docking of OSU-03013 into the ATP-binding site (Fig. 6C). The guanidino group of OSU-03013 resembled the partial structure of the purine ring of ATP, which allowed the formation of hydrogen bonds with Ser160 and Ala162 as depicted by the docking model.

Like OSU-02067, they exhibited no appreciable direct inhibition on immunoprecipitated Akt kinase activity (data not shown) nor was any measurable COX-2 inhibitory activity detected at concentrations up to 50 μM. As shown in Fig. 6B, exposure of PC-3 cells to either agent, even at 1 μM, resulted in a substantial decrease in the phospho-Akt level. Because PDK-1 also phosphorylates other members of the AGS protein kinase family such as p70S6K (20, 33), we assessed the kinase activity of immunoprecipitated p70S6K in drug-treated PC-3 cells. As indicated in Fig. 6C, the activity of immunoprecipitated p70S6K was reduced in a dose-dependent manner after exposing PC-3 cells to OSU-03012 at the indicated concentrations for 6 h.

Cellular Effects of PDK-1/Akt Signaling Inhibitors. Both OSU-03012 and OSU-03013 induced apoptotic death in PC-3 cells in 1% FBS-containing medium in a dose-dependent manner, as demon-

Fig. 2. Effects of OSU-02067 on 3-phosphoinositide-dependent kinase-1 (PDK-1) kinase activity, intracellular Akt activation, and survival of PC-3 cells. A, dose-dependent inhibition of recombinant PDK-1 kinase activity by OSU-02067 versus celecoxib. B, top, effect of OSU-02067 versus celecoxib on the kinase activity of Akt immunoprecipitated from drug-treated PC-3 cells. Bottom, phosphorylation status of Akt in PC-3 cells treated with increasing concentrations of OSU-02067. Control PC-3 cells received DMSO vehicle. Cell viability was analyzed by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. D, evidence of apoptotic death in OSU-02067-treated PC-3 cells. Top, formation of cytoplasmic nucleosomal DNA in PC-3 cells treated with DMSO vehicle or the indicated concentrations of OSU-02067. DNA fragmentation was quantitatively measured by a cell death detection ELISA kit. O.D. = absorbance. Bottom, induction of poly(ADP-ribose) polymerase cleavage by OSU-02067 in PC-3 cells. Poly(ADP-ribose)polymerase proteolysis to the apoptosis-specific 85-kDa fragment was monitored by Western blotting. E, antiproliferative effect of 1 and 5 μM OSU-02067 versus 30 μM celecoxib in PC-3 cells. PC-3 cells were seeded into six-well plates (50,000 cells/well) and exposed to the test agent at the indicated concentration in 10% fetal bovine serum-supplemented RPMI 1640. At different time intervals, cells were harvested, and counted using a Coulter counter. Values were obtained from triplicates for the aforementioned plots except cell viability (C) in which the means of six replicates were used; bars, ±SD. Western blots shown in B and D are representatives of three independent experiments.

Fig. 3. Lineweaver-Burke plots of the competition of celecoxib with ATP in 3-phosphoinositide-dependent kinase-1 kinase activity. Activity of the recombinant in 3-phosphoinositide-dependent kinase-1 kinase toward the peptide substrate was determined using 1–100 μM ATP in the presence of 0, 25, and 50 μM celecoxib, as described in “Materials and Methods.”
strated by DNA fragmentation and PARP cleavage (Fig. 7, A and B). These agents exhibited higher potency than OSU-02067 in apoptosis induction at concentrations >2.5 \mu M.

The effects of OSU-03012 and OSU-03013 on PC-3 cell proliferation in 10% FBS-supplemented medium were also examined (Fig. 7C). High levels of serum might affect the efficacy of these PDK-1 inhibitors due to several factors. First, these celecoxib derivatives might display high serum-binding affinity, thereby sequestering them from entering cells. Second, continuous stimulation of phosphatidylinositol 3'-kinase/Akt signaling through various growth factor receptors counters the inhibitory effect of these agents on Akt. Third, serum could up-regulate Bcl-xL in PC-3 cells, which enhances the threshold to apoptotic signals emanating from phosphatidylinositol 3'-kinase/Akt inhibition (36). As shown, OSU-03012 was effective in suppressing PC-3 cell proliferation at sub-\mu M, consistent with that noted in 1% serum. In contrast, OSU-03013 required higher concentrations than OSU-03012 to achieve the same degree of antiproliferative effects although it had higher PDK-1 inhibitory potency.

To confirm that inhibition of PDK-1/Akt signaling represented the underlying antitumor mechanism for these agents, we assessed the protective effect of the transient expression of the constitutively active forms of PDK-1 and Akt [PDK-1^{A280V} (37) and Akt^{T308D/S473D} (38), respectively] on the drug-induced PC-3 cell death. Western blot analysis using antibodies against PDK-1, Akt, and the respective myc and hemagglutinin tags confirmed that transient transfection of PDK-1^{A280V} and Akt^{T308D/S473D} into PC-3 cells led to increase in the expression levels of the respective kinases (Fig. 8A). These transient transfectants were exposed to 1–10 \mu M OSU-03012 in 1% FBS-containing medium for 24 h to examine the susceptibility to OSU-03012-induced cell death vis-à-vis transfectants with an empty pcDNA vector. As shown, both PDK-1^{A280V} and Akt^{T308D/S473D} expression gave partial, yet significant, protection against OSU-03012-induced apoptotic death (Fig. 8B), which underscores the involvement of both kinases in the antitumor effects of these celecoxib derivatives.

Moreover, these derivatives were submitted to the Developmental Therapeutic Program at the National Cancer Institute for screening against 60 human tumor cell lines, representing leukemia, melanoma, and cancers of the lung, colon, brain, ovary, breast, prostate, and kidney. Dose-response data of one representative cell line from each class of tumor cells after 2-day exposure in 5% FBS-containing medium are shown in Fig. 9, which include: (a) RPMI-8226 leukemia cells; (b) NCI-H322M non-small cell lung cancer cells; (c) HT29 colon cancer cells; (d) U251 glioblastoma cancer cells; (e) SK-MEL-28 melanoma cancer cells; (f) SK-OV-3 ovarian cancer cells; (g) RXF 393 renal cancer cells; (h) PC-3 prostate cancer cells; and (i) MDA-MB-231 breast cancer cells. Many of these cell lines were responsive to the growth inhibitory effect of both agents at concentrations as low as 0.1 \mu M.

\[ \text{Internet address: http://dtp.nci.nih.gov/index.html.} \]
In the 60 cell line assay, three dose-response parameters for each cell line were calculated based on growth inhibition curves. These parameters include concentration resulting in 50% growth inhibition, concentration resulting in total growth inhibition, and concentration resulting in a 50% reduction in the measured protein level at the end of drug treatment as compared with that at the beginning. The means of these parameters among the 60 different cell lines for OSU-03012 and OSU-03013 after 2-day treatment were as follows, respectively, concentration resulting in 50% growth inhibition, 1.1 and 1.2 \( \mu M \); concentration resulting in total growth inhibition, 3.2 and 2.9 \( \mu M \); and concentration resulting in a 50% reduction in the measured protein level at the end of drug treatment as compared with that at the beginning, 6.8 and 8.5 \( \mu M \). In contrast, for OSU-02067, the corresponding values were 3.0, 19, and 66 \( \mu M \), respectively. These data clearly demonstrate the in vitro efficacy of OSU-03012 and OSU-03013. Both agents were able to completely suppress cell growth in a diverse range of tumor cell lines at the 3–5 \( \mu M \) therapeutic range, as compared with the concentration of at least 50 \( \mu M \) required for celecoxib.

DISCUSSION

In light of the conserved role of PDK-1/Akt signaling in cancer cell survival and proliferation (9, 10, 12–15), this pathway represents a therapeutically relevant target for developing orally bioavailable, small-molecule inhibitors. Our previous structure-activity analysis provided a proof of principle that the apoptosis-inducing activity of celecoxib could be separated from its COX-2 inhibitory activity via structural modifications (5). Furthermore, these data allowed us to establish a working model depicting the structural attributes essential to the PDK-1 versus COX-2 inhibitory activity of celecoxib. This model, in conjunction with the crystal structure of PDK-1-ATP complexes reported recently (31), provided a molecular foundation for the present structure-based optimization. Kinetic and molecular modeling data indicate that celecoxib derivatives exerted PDK-1 inhibition by competing with ATP for binding, a mechanism shared by many types of PDK-1 inhibitors.
of kinase inhibitors. Structural optimization of celecoxib by altering the terminal phenyl ring led to OSU-02067 with a 5-fold improvement in PDK-1 inhibitory potency. This improvement was attributable to the terminal phenyl ring and guanidine moiety and the backbone oxygen of Ser160. The effect of these substituents on ligand binding, however, was subtle, as illustrated by the structure-activity relationship summarized in Fig. 5.

The high potency of OSU-03012 and OSU-03013 in PDK-1 inhibition was reflected in their abilities to effectively block the activation of Akt and p70S6K (Fig. 6) and to induce apoptotic cell death in PC-3 cells at low μM concentrations (Fig. 7). More importantly, due to the conserved role of PDK-1/Akt signaling in cell proliferation and survival, these agents were potent in inhibiting cell growth in serum-containing medium in all 60 of the human tumor cell lines examined, with mean concentration resulting in 50% growth inhibition values of 1.2 μM and 1.3 μM, respectively (Fig. 6A). Docking of OSU-03013 into the ATP binding site revealed increased hydrogen bonding between the guanidine moiety and the backbone oxygen of Ser160. The effect of these substituents on ligand binding, however, was subtle, as illustrated by the structure-activity relationship summarized in Fig. 5.

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tively, and concentration resulting in total growth inhibition (total growth inhibition) values of 3.2 and 2.9 μM, respectively.

In summary, the present study has clearly demonstrated the successful development of a new class of PDK-1/Akt signaling inhibitors via structure-based optimization of celecoxib. In light of the prominent role of PDK-1/Akt signaling in different stages of tumorigenesis, these molecules have translational potential to be developed into antitumor agents for the prevention and/or therapy of cancers alone or in combination with other treatments. Testing of in vivo efficacy against different tumor xenografts in nude mice is currently under way in this laboratory.

ACKNOWLEDGMENTS

We thank Drs. Rao Vishvukajala and Robert Schultz in the Developmental Therapeutics Program at National Cancer Institute for critical comments on this manuscript. The in vitro 60-cell line screening was carried out by the Developmental Therapeutic Program anticancer drug discovery program.

REFERENCES

Editor’s Note: From the Cyclooxygenase-2 Inhibitor Celecoxib to a Novel Class of 3-Phosphoinositide-Dependent Protein Kinase-1 Inhibitors

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The editors are publishing this note to alert the readers to a concern about this article (1). The editors were made aware of apparent splicing between Western blot lanes 3 and 4 in Fig. 7B. Ohio State University (Columbus, OH) conducted an investigation, but was not able to determine whether the splicing of the image had any impact on the reported results of the experiment described in Fig. 7B because the authors were unable to provide the original research records related to the figure.

Reference

Published online April 1, 2019.
doi: 10.1158/0008-5472.CAN-19-0502
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