# 3-Phosphoinositide-Dependent Protein Kinase-1/Akt Signaling Represents a Major Cyclooxygenase-2-Independent Target for Celecoxib in Prostate Cancer Cells

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## ABSTRACT

Regarding the involvement of cyclooxygenase-2 (COX-2)-independent pathways in celecoxib-mediated antineoplastic effects, the following two issues remain outstanding: identity of the non-COX-2 targets and relative contributions of COX-2-dependent *versus* -independent mechanisms. We use a close celecoxib analog deficient in COX-2-inhibitory activity, DMC {4-[5-(2,5-dimethylphenyl)-3-(trifluoromethyl)-1*H*-pyrazol-1-yl]benzene-sulfonamide}, to examine the premise that Akt signaling represents a major non-COX-2 target. Celecoxib and DMC block Akt activation in PC-3 cells through the inhibition of phosphoinositide-dependent kinase-1 (PDK-1) with $IC_{50}$ of 48 and 38 $\mu$M, respectively. The consequent effect on Akt activation is more pronounced ($IC_{50}$ values of 28 and 20 $\mu$M, respectively), which might be attributed to the concomitant dephosphorylation by protein phosphatase 2A. In serum-supplemented medium, celecoxib and DMC cause $G_1$ arrest, and at higher concentrations, they induce apoptosis with relative potency comparable with that in blocking Akt activation. Moreover, the effect of daily oral celecoxib and DMC at 100 and 200 mg/kg on established PC-3 xenograft tumors is assessed. Celecoxib at both doses and DMC at 100 mg/kg had marginal impacts. However, a correlation exists between the *in vitro* potency of DMC and its ability at 200 mg/kg to inhibit xenograft tumor growth through the inhibition of Akt activation. Analysis of the tumor samples indicates that a differential reduction in the phospho-Akt/Akt ratio was noted in celecoxib- and DMC-treated groups *vis-à-vis* the control group. Together, these data underscore the role of 3-phosphoinositide-dependent protein kinase-1/Akt signaling in celecoxib-mediated *in vitro* antiproliferative effects in prostate cancer cells.

## INTRODUCTION

In the past decade, substantial evidence from epidemiological and animal model studies has suggested the clinical use of nonsteroidal anti-inflammatory drugs in chemoprevention, especially against colon cancer (1–3). This premise is strengthened by two recent large randomized, placebo-controlled clinical trials, demonstrating that aspirin might be effective in primary and secondary prevention of colorectal cancer by lowering the incidence of adenomas (4, 5). The fact that all nonsteroidal anti-inflammatory drugs in clinical use are cyclooxygenase (COX) inhibitors provides a putative link between the inhibition of COX activity and the antitumor effect of nonsteroidal anti-inflammatory drugs. Two isoforms of COX have been characterized, each of which displays a distinct physiological profile. COX-1 is constitutively expressed in nearly all tissues, of which the inhibition underlies the gastrointestinal toxicities of nonselective nonsteroidal anti-inflammatory drugs. In contrast, COX-2, an inducible isozyme, is dramatically up-regulated during pathological conditions such as inflammation and cancer (6). Increased expression of COX-2 has been demonstrated in various human malignancies (7–12). Furthermore,

evidence suggests that COX-2 overexpression leads to increased resistance to apoptotic signals and alterations in extracellular matrix adhesion (13–15). The most compelling evidence for a role of COX-2 in carcinogenesis was obtained by an *in vivo* study showing that knockout of the COX-2 gene could suppress tumorigenesis in mice that had a genetic predisposition to form intestinal polyps (16). Consequently, selective inhibition of COX-2 has emerged as a viable target for cancer prevention and therapy (17, 18). Among various selective COX-2 inhibitors examined, celecoxib has received the most attention (19), in part, because of its demonstrated effectiveness in reducing colorectal polyps in patients with familial adenomatous polyposis (20). In addition to the recent approval by the United States Food and Drug Administration for the adjuvant treatment of familial adenomatous polyposis, celecoxib is currently undergoing a series of clinical trials for its chemopreventive effect on various epithelial malignancies.

In light of the potential use of celecoxib in the prevention of human tumors (19, 21), the underlying mechanism has been the focus of many recent investigations. At the cellular level, celecoxib inhibits COX-2, causes cell cycle arrest, and induces apoptosis in cancer cells. However, there exists a disparity by several orders of magnitude between the concentration needed to inhibit COX-2 ($IC_{50}$, 0.04 $\mu$M) and that for causing cell cycle arrest and apoptosis *in vitro* (>20 $\mu$M; Refs. 22 and 23). In addition, evidence from this and other laboratories has demonstrated the involvement of molecular targets other than COX-2 in celecoxib-mediated *in vitro* antiproliferative effects in prostate (23–26) and colon (27) cancer cells. Among various putative pathways reported in the literature, blockade of Akt signaling is especially noteworthy (23–26). Together, these data suggest that celecoxib may use both COX-2 and non-COX-2 targets to mediate its antitumor activities, although their relative contribution toward the *in vivo* effects remains undefined. Consequently, a better understanding of the COX-2-independent pathways may help optimize the chemopreventive potential of celecoxib. In this study, we used celecoxib and its COX-2-inactive derivative, 4-[5-(2,5-dimethylphenyl)-3-(trifluoromethyl)-1*H*-pyrazol-1-yl]benzene-sulfonamide (DMC; Ref. 25; Fig. 1A), in PC-3 prostate cancer cells to examine the hypothesis that the 3-phosphoinositide-dependent protein kinase-1 (PDK-1)/Akt signaling pathway represents a major COX-2-independent mechanism by which celecoxib exerts *in vivo* antiproliferative effects against prostate tumors.

## MATERIALS AND METHODS

**Reagents.** DMC is a close structural analog of celecoxib, of which the 5-aryl moiety is altered by replacing the 4-methylphenyl with a 2,5-dimethylphenyl function (Fig. 1A). This slight modification abrogated the activity of DMC in COX-2 inhibition ($IC_{50}$ > 100 $\mu$M; Ref. 28); however, its ability to block Akt phosphorylation and to induce apoptosis in cancer cells was retained (25). The synthesis of DMC has been described previously (25, 28). Celecoxib was prepared from commercial Celebrex capsules by solvent extraction followed by recrystallization. For *in vitro* studies, these agents at various concentrations were dissolved in DMSO and were then added to the cells in 10% fetal bovine serum (FBS)-containing RPMI 1640. The final DMSO concentration was kept at 0.1% after addition to medium. For *in vivo* studies,
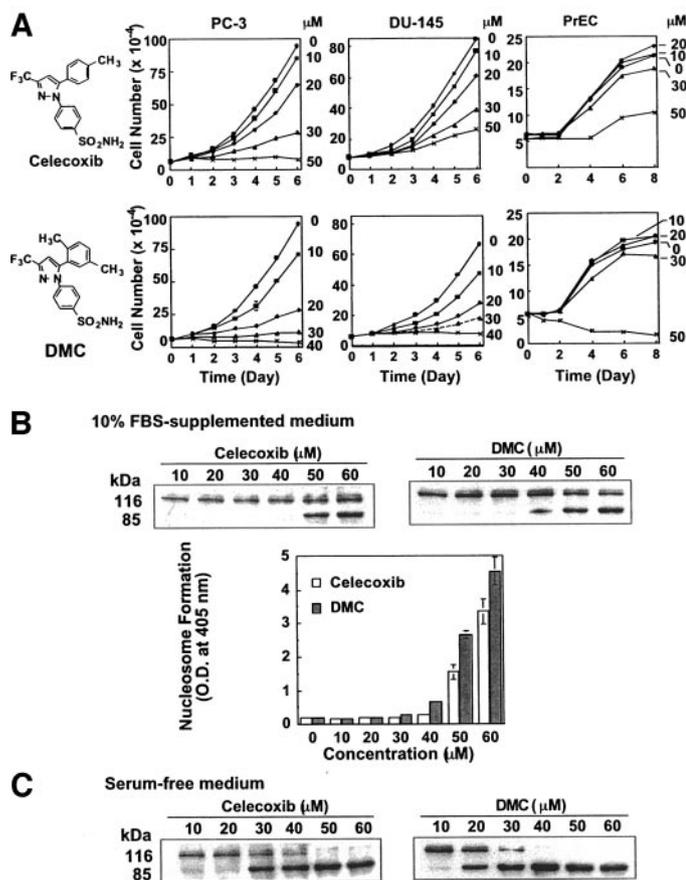
Fig. 1. *A,* time and dose dependency of the antiproliferative effect of celecoxib (*top*) and DMC {4-[5-(2,5-dimethylphenyl)-3(trifluoromethyl)-1*H*-pyrazol-1-yl]-benzene-sulfonamide; *bottom panel*} in PC-3 and DU-145 prostate cancer cells and normal prostate epithelial cells (PrECs). Structures of celecoxib and DMC are compared. Cells were seeded into 6-well plates (50,000 cells/well) and exposed to the test agents at the indicated concentration in 10% fetal bovine serum-supplemented medium. At different time intervals, cells were harvested and counted using a Coulter counter. Each data point represents the mean of three-independent determinations. *B,* apoptosis analysis of drug-treated cells in 10% fetal bovine serum-containing RPMI 1640 at 48 h after treatment. *Top,* induction of poly(ADP-ribose) polymerase (PARP) cleavage. PARP proteolysis to the apoptosis-specific 85-kDa fragment was monitored by Western blotting. *Bottom,* quantitative measurement of the formation of nucleosomes by cell death detection ELISA. O.D., absorbance. Values are means ± SD (*n* = 3). *C,* apoptosis analysis, as measured by PARP cleavage, of drug-treated cells in serum-free medium 48 h after treatment.

celecoxib and DMC were prepared as suspensions in vehicle consisting of 0.5% methylcellulose and 0.1% polysorbate 80 in sterile water (29).

**Cell Culture.** PC-3 and DU-145 human androgen-nonresponsive prostate cancer cells were purchased from the American Type Tissue Collection (Manassas, VA). It is noteworthy that PC-3 cells are devoid of PTEN (phosphatase and tensin homologue deleted from chromosome 10) expression whereas DU-145 cells contain functional PTEN. These prostate cancer cells were cultured in RPMI 1640 supplemented with 10% FBS at 37°C in a humidified $CO_2$ incubator. Normal prostate epithelial cells (PrECs) were obtained from Cambrex Bioscience Walkersville, Inc. (Walkersville, MD). Cells were maintained in the manufacturer's recommended defined prostate epithelial growth medium, which consisted of basal medium supplemented with bovine pituitary extract, hydrocortisone, human epidermal growth factor, epinephrine, insulin, triiodothyronine, transferrin, retinoic acid and antibiotics.

**Cell Proliferation.** PC-3 and DU-145 cells were seeded into 6-well plates at 50,000 cells/well. PrECs were seeded at 35,000/well. Cells were treated in triplicate with DMSO vehicle or the indicated concentration of celecoxib or DMC in 10% FBS-containing RPMI 1640 or 10% FBS-prostate epithelial growth medium. At different time intervals, cells were harvested and numerated using a Coulter counter model Z1 D/T (Beckman Coulter, Fullerton, CA).

**Apoptosis Analysis.** The following two methods were used to assess drug-induced apoptotic cell death: detection of DNA fragmentation by the Cell

Death Detection ELISA kit (Roche Diagnostics, Mannheim, Germany) and Western blot analysis of poly(ADP-ribose)polymerase 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, $2.5 \times 10^6$ PC-3 cells were cultured in a T-75 flask for 24 h before treatment. Cells were treated with the DMSO vehicle or the test agent at the indicated concentrations for 2 days, collected, and cell lysates equivalent to $10^4$ cells were used in the ELISA. For the poly(ADP-ribose) polymerase cleavage assay, drug-treated cells were collected, washed with ice-cold PBS, and resuspended in lysis buffer containing 20 mM Tris-HCl (pH 8), 137 mM NaCl, 1 mM $CaCl_2$, 10% glycerol, 1% NP40, 0.5% deoxycholate, 0.1% SDS, 100 $\mu$M 4-(2-aminoethyl)benzenesulfonyl fluoride, leupeptin at 10 $\mu$g/ml, and aprotinin at 10 $\mu$g/ml. Soluble cell lysates were collected after centrifugation at 10,000 × *g* for 5 min. Equivalent amounts of proteins (60–100 $\mu$g) from each lysate were resolved in 10% SDS-polyacrylamide gels. Bands were transferred to nitrocellulose membranes and analyzed by immunoblotting with anti-poly(ADP-ribose) polymerase antibodies.

**Flow Cytometry for Cell Cycle Analysis.** A detergent-trypsin method was used for the preparation of nuclei for flow cytometric DNA analysis (30). In brief, PC-3 cells were treated with DMSO or the test agent at the indicated concentration for 48 h. The harvested cells ($1 \times 10^6$) were suspended in 500 $\mu$l of 40 mM citrate buffer (pH 7.6), containing 250 mM sucrose and 10% DMSO, and stored at $-80$°C until analysis. The cells were centrifuged, resuspended in 500 $\mu$l of solution A [3.4 mM trisodium citrate, 0.5 mM Tris, 0.1% NP40, and 1.5 mM spermine tetrahydrochloride (with final pH of 7.4)] containing 15 $\mu$g/ml trypsin and 10 $\mu$g/ml EDTA. After incubating at 37°C for 30 min, 500 $\mu$l of solution A containing 0.5 mg/ml trypsin inhibitor and 0.1 mg/ml DNase-free RNase A was added. After another incubation at 37°C for 30 min, 500 $\mu$l of solution A containing 0.05 mg/ml propidium iodide and 1.2 mg/ml spermine tetrahydrochloride was added and incubated on ice for 1 h. Cell cycle phase distributions were determined on a FACScan flow cytometer (Beckman-Coulter, Mountain View, CA).

**Immunoprecipitated Akt Kinase Assay.** Akt immunoprecipitation was carried out according to a modified published procedure (31). PC-3 cells were treated with DMSO vehicle or the test agents at the indicated concentrations for 2 h, then lysed at 4°C for 1 h in buffer A 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 $\beta$-glycerophosphate, 0.1% 2-mercaptoethanol, 0.1 mM phenylmethylsulfonyl fluoride, and 1 $\mu$g/ml each of aprotinin, 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 an additional 60 min. The immunoprecipitate was used to analyze Akt kinase activity by using the Akt/SGK-specific peptide substrate RPRAATF as described below.

**PDK-1 Kinase Assay.** This *in vitro* assay was performed using a PDK-1 kinase assay kit (Upstate, Lake Placid, NY) according to the vendor's instructions. This cell-free assay is based on the ability of recombinant PDK-1, in the presence of DMSO vehicle or the test agent, to activate its downstream kinase, serum- and glucocorticoid-regulated kinase (SGK), which in turn phosphorylates the Akt/SGK-specific peptide substrate RPRAATF with $[\gamma$-$^{32}P]$ATP. The $^{32}P$-phosphorylated peptide substrate was then separated from the residual $[\gamma$-$^{32}P]$ATP using P81 phosphocellulose paper and quantitated by a scintillation counter after three washes with 0.75% phosphoric acid and two washes with acetone. Values represent the means of three-independent determinations.

**Immunoprecipitated $p70^{S6K}$ Assay.** Immunoprecipitation of p70 S6 kinase ($p70^{S6K}$) was carried out according to the modification of a published procedure (32). In brief, PC-3 cells were cultured in T-75 flasks ($2 \times 10^6$/flask), and treated with celecoxib or DMC at the indicated concentrations in serum-free RPMI 1640 for 2 h. Both floating and adherent cells were collected and lysed in 1 ml lysis buffer [50 mM Tris (pH 7.4), containing 150 mM NaCl, 1% NP40, 0.5% sodium deoxycholate, 1 mM EGTA, and 10% 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 subject to immunoprecipitation with anti-$p70^{S6K}$ 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 A Sepharose beads for 2 h. The immunocomplex was washed with lysis buffer twice, followed by assay buffer [20 mM 4-morpholinepropanesulfonic acid (pH 7.2), containing 25 mM $\beta$-

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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 p70$^{S6K}$ assay kit (Upstate) according to the manufacturer's instruction.

**Transient Transfection.** The constitutively active Akt construct HA-PKB-T308D/S473D and the constitutively active PDK-1 construct pcDNA-PDK1-A280V were kindly provided by Dr. Brian Hemmings (Friedrich Miescher Institute, Basel, Switzerland) and Dr. Feng Liu (University of Texas Health Science Center, San Antonio, TX). PC-3 cells were seeded into T-25 flasks ($3 \times 10^5$/flask). Aliquots containing 0.5 $\mu$g of each plasmid or a control pCMV vector in 400 $\mu$l of Opti-MEM (Invitrogen-Life Technologies, Inc.) was incubated with 16 $\mu$l of the LipofectAMINE 2000 reagent (Invitrogen) for 30 min. Each flask was washed with serum-free Opti-MEM and then received the plasmid-Lipofectamine mixture and 4 ml of serum-free Opti-MEM. The flask was placed in a $CO_2$ 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 12-well plates at $10^5$ cells/well in 10% serum-supplemented RPMI 1640. On the next day, cells were treated in triplicate with the indicated concentrations of DMC in serum-free medium. At the indicated time intervals, both floating and adherent cells were harvested and then combined for the assessment of cell viability by the trypan blue dye exclusion method.

**Xenograft Tumor Growth.** Male NCr athymic nude mice (5–7 weeks of age) were obtained from the National Cancer Institute (Frederick, MD). The mice were group-housed under conditions of a constant 12-h photoperiod with *ad libitum* access to sterilized food and water. All experimental procedures using these mice were performed in accordance with protocols approved by the Institutional Laboratory Animal Care and Use Committee of The Ohio State University.

Each mouse was inoculated s.c. in the dorsal flank with $5 \times 10^5$ PC-3 cells suspended in 0.1 ml of serum-free medium containing 50% Matrigel (BD Biosciences, Bedford, MA) under isoflurane anesthesia. When tumors reached a mean volume of $81.4 \pm 16.3$ mm$^3$, mice received single daily oral treatments of celecoxib or DMC at 100 and 200 mg/kg body weight/day for the duration of the study. Controls received vehicle. Tumors were measured weekly using calipers and their volumes calculated using the following standard formula: width$^2$ × length × 0.52. Body weights were measured weekly.

**Serum Concentrations of Celecoxib and DMC.** At the 35th day of treatment, mice were sacrificed at 2, 6, 12, and 24 h after the final administration of compound, and blood was collected immediately thereafter by cardiac puncture. Serum concentrations of celecoxib and DMC were determined by high-performance liquid chromatography using modifications of published procedures for the measurement of celecoxib (33). Briefly, aliquots of serum (0.2 ml) were combined with internal standards (3.6 $\mu$g of DMC for celecoxib-treated mice; 2 $\mu$g of celecoxib for DMC-treated mice) and then extracted with ethyl acetate. The organic phase was collected, dried under a stream of $N_2$, and reconstituted with 150 $\mu$l of the mobile phase (47.5% aqueous acetonitrile). Aliquots were applied to a reverse-phase $C_8$ column ($4.6 \times 150$ mm) and analyzed with an isocratic mobile phase at a flow rate of 1.2 ml/min. Standard curves ranged from 0 to 36 $\mu$g/ml for celecoxib and from 0 to 28 $\mu$g/ml for DMC. The retention times for celecoxib and DMC were 9.5 and 12 min, respectively. Mean serum drug levels at steady state were determined by calculating the area under the serum drug level *versus* time curve for the 24-h dosing period and dividing by the dosing interval (24 h).

**Western Blot Analysis of Phospho (P)-Akt Status in PC-3 Xenograft Tumors.** At sacrifice, tumors were harvested from mice, and a portion of each tumor was snap-frozen in liquid nitrogen and stored at −80°C until analysis. Tumor tissue homogenates were prepared in SDS lysis buffer [50 mM Tris-HCl (pH 7.4), 2% SDS and protease inhibitor mixture] as described previously (34). Western blot analysis was performed as we reported previously (26). The immunoblots were scanned by a Photodyne image system and quantitated using a FOTO/Analyst PC image program (version 3.0) to determine the ratios of the levels of P-Akt to Akt.

**Statistical Analysis.** Tumor growth data points are reported as mean tumor volumes ± SE. Intratumoral P-Akt/Akt ratios are expressed as means ± SD. Comparisons of mean values were performed using the independent samples *t* test in SPSS for Windows 11.5 software (SPSS, Inc., Chicago, IL).

## RESULTS

**Celecoxib and DMC Suppress Prostate Cancer Cell Proliferation in Serum-Containing Medium by Causing G$_1$ Arrest and Apoptosis, Irrespective of COX-2 Inhibitory Activity.** To investigate the mechanism underlying celecoxib-mediated *in vivo* antitumor effects, we assessed the growth-inhibitory effect of celecoxib and DMC in PC-3 and DU-145 prostate cancer cells and normal PrECs in 10% FBS-supplemented medium. Fig. 1*A* indicates that celecoxib (*top*) and DMC (*bottom*) exhibited differential effects on the proliferation of these three types of cells. Relatively, PrECs were less susceptible to the antiproliferative effect of celecoxib and DMC as compared with PC-3 and DU-145 cells. No appreciable effect was noted in PrECs with either agent until the concentration reached approximately 30 $\mu$M. In contrast, the sensitivity of PC-3 and DU-145 cells to the antiproliferative effect of these two agents was comparable. The concentrations required to inhibit 50% PC-3 or DU-145 cell growth were approximately 25 and 15 $\mu$M for celecoxib and DMC, respectively.

Evidence indicates that this growth inhibition was caused by cell cycle arrest and apoptosis induction. Analyses of poly(ADP-ribose) polymerase cleavage in PC-3 cells revealed that apoptotic death in celecoxib-treated cells occurred at $\geq$50 $\mu$M, whereas DMC was able to trigger apoptosis with a threshold of 40 $\mu$M (Fig. 1*B*; *top*). This dose-dependent induction of apoptosis was confirmed by a DNA fragmentation ELISA assay (Fig. 1*B*; *bottom*). In line with our previous reports (24, 25), celecoxib and DMC could induce apoptosis at lower concentrations in serum-free milieu (Fig. 1*C*), with the thresholds of approximately 30 and 20 $\mu$M, respectively. The protective effect of serum from drug-induced apoptosis might be attributable to several factors. First, serum proteins display high binding affinities with celecoxib (35) and, possibly, DMC. This sequestration results in lower intracellular concentrations of these agents, thereby attenuating their apoptosis-inducing potency. 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-x$_L$, which enhances the threshold to apoptotic signals emanating from phosphatidylinositol 3′-kinase/Akt inhibition (36).

Cell cycle analyses of PC-3 cells treated with celecoxib or DMC in 10% FBS-containing medium indicated that these agents caused G$_1$ arrest in a dose-dependent manner (Table 1). Exposure to increasing concentrations of individual agents resulted in a gradual accumulation of cells in the G$_0$/G$_1$ phase (from 48% to 70%), accompanied by a

Table 1 *Cell cycle phase distribution of PC-3 cells treated with celecoxib or DMC at the indicated concentration in 10% FBS-supplemented RPMI-1640 medium for 48 h*

Control cells received DMSO vehicle. Each tabulated percentage represents the average of two independent experiments.

| | Cells in cell cycle phases (%) | | |
| --- | --- | --- | --- |
| | G$_0$/G$_1$ | S | G$_2$/M |
| DMSO vehicle | 48.5 | 27.7 | 23.8 |
| Celecoxib | | | |
| 15 $\mu$M | 54.3 | 24.6 | 21.2 |
| 20 $\mu$M | 56.0 | 23.0 | 20.9 |
| 30 $\mu$M | 61.3 | 15.9 | 22.5 |
| 40 $\mu$M | 67.9 | 10.6 | 21.6 |
| 50 $\mu$M | 70.6 | 6.5 | 22.9 |
| DMC$^a$ | | | |
| 15 $\mu$M | 59.0 | 22.4 | 18.6 |
| 20 $\mu$M | 61.5 | 19.0 | 19.5 |
| 30 $\mu$M | 70.9 | 8.3 | 20.9 |
| 40 $\mu$M | 71.2 | 5.2 | 23.6 |
| 50 $\mu$M | Not determined due to extensive apoptosis | | |

$^a$ DMC, 4-[5-(2,5-dimethylphenyl)-3(trifluoromethyl)-1H-pyrazol-1-yl]benzene-sulfonamide.
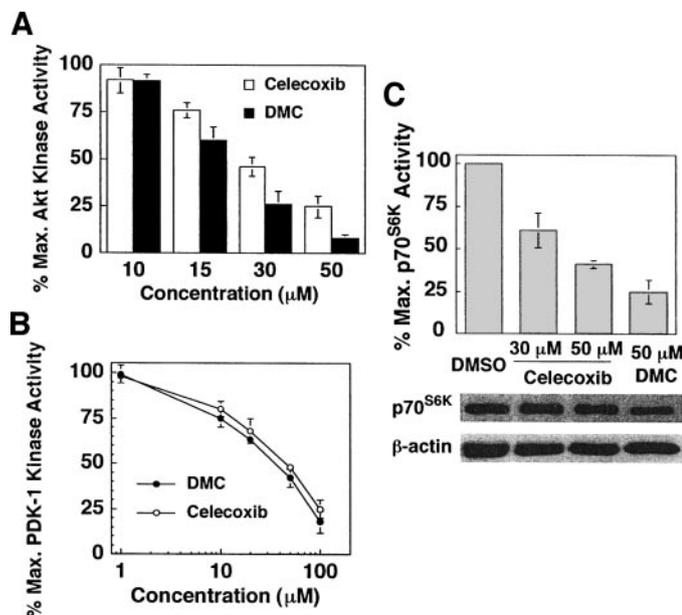
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Fig. 2. *A*, dose-dependent effects of celecoxib and DMC {4-[5-(2,5-dimethylphenyl)-3(trifluoromethyl)-1*H*-pyrazol-1-yl]-benzene-sulfonamide} on the kinase activity of immunoprecipitated Akt in drug-treated PC-3 cells. PC-3 cells were treated with the test agents at the indicated concentration for 2 h, and the kinase activity of immunoprecipitated Akt was analyzed as described in "Materials and Methods." Values are means ± SD ($n = 3$). *B*, dose-dependent effect of celecoxib and DMC on the kinase activity of recombinant 3-phosphoinositide-dependent protein kinase-1 (PDK-1). This *in vitro* assay was performed using a PDK-1 kinase assay kit as described in "Materials and Methods." Values are means ± SD ($n = 3$). *C*, effects of celecoxib and DMC on the kinase activity of immunoprecipitated p70$^{S6K}$ in drug-treated PC-3 cells. PC-3 cells were treated with the test agents at the indicated concentration for 2 h. p70$^{S6K}$ was immunoprecipitated from equal amounts of total protein, and the kinase activity was analyzed as described in "Materials and Methods" (*top*). Values are means ± SD ($n = 3$). A portion of the cell lysates was separated by SDS-PAGE and Western blotted using antibodies specific for β-actin and p70$^{S6K}$ (*bottom*).

comparative decrease in the S fraction (from 28% to 6%). Relatively, the potency of DMC in causing $G_1$ arrest was higher than that of celecoxib, which is consistent with that observed in cell proliferation. Together, these data indicate that celecoxib and DMC at concentrations lower than the respective apoptosis thresholds mediated the antiproliferative effect through the inhibition of cell cycle progression.

**Celecoxib and DMC Block Akt Signaling by Inhibiting PDK-1 Kinase Activity.** Previously, we demonstrated that the proapoptotic activity of celecoxib in PC-3 cells in serum-free media was, in part, because of the inhibition of Akt activation (24). To provide a link between Akt and $G_1$ arrest, we examined the kinase activity of immunoprecipitated Akt from PC-3 cells treated with various levels of individual agents in 10% FBS-supplemented medium (Fig. 2A). The Akt kinase activity in drug-treated cells was attenuated in a dose-dependent manner with IC$_{50}$ values of 28 and 20 $\mu$M for celecoxib and DMC, respectively, which were correlative with the respective concentrations required for suppressing 50% PC-3 cell proliferation.

It is noteworthy that neither compound exhibited any direct-inhibitory effect on immunoprecipitated Akt kinase activity (data not shown). In cells, Akt activity is regulated through a dynamic balance between phosphorylation and dephosphorylation by PDK-1 and protein phosphatase 2A (PP2A), respectively. Previously, we showed that the dephosphorylation of Akt could not be rescued by okadaic acid, a PP2A inhibitor (24). In light of the report by Arico *et al.* (31) that celecoxib inhibited PDK-1, an upstream kinase of Akt, with high potency (IC$_{50}$, 3.5 $\mu$M), we examined the effect of each agent on the kinase activity of recombinant PDK-1 using a commercial assay kit (Fig. 2B). This assay is based on the ability of recombinant PDK-1, in the presence of either agent, to activate its downstream kinase, SGK,

which in turn phosphorylates the peptide substrate RPRAATF with [$\gamma$-$^{32}$P]ATP. Our data show that celecoxib and DMC displayed moderate-inhibitory activities against PDK-1 with IC$_{50}$ values of 48 and 38 $\mu$M, respectively. To exclude the possibility that celecoxib and DMC might interfere with the PDK-1 assay by inhibiting SGK activity, we preincubated recombinant PDK-1 with inactive SGK for 2 h to ensure full SGK activation before adding the inhibitor. Under such conditions, neither celecoxib nor DMC exhibited an inhibitory effect on the peptide phosphorylation (data not shown), indicating that these agents were not SGK inhibitors.

It is especially noteworthy that the estimated IC$_{50}$ of 48 $\mu$M for celecoxib could not reconcile with that of 3.5 $\mu$M reported previously by Arico *et al.* (31). The cause for this discrepancy, however, remains unclear.

In addition, the IC$_{50}$ values of both agents for Akt inhibition in drug-treated cells was substantially lower than those for recombinant PDK-1. We hypothesized that this discrepancy might, in part, arise from the concomitant deactivation of Akt by PP2A. Presumably, partial inhibition of PDK-1 in concert with the concurrent action by an uninhibited PP2A could culminate in a greater extent of inhibition of the Akt kinase activity in drug-treated cells.

**Target Validation.** Two experiments were carried out to validate PDK-1/Akt signaling as a major target responsible for the *in vitro* antiproliferative effect of celecoxib and DMC. In addition to Akt, PDK-1 can also phosphorylate other members of the AGC protein kinase family such as p70$^{S6K}$ (32, 37–39). We thus examined the activity of immunoprecipitated p70$^{S6K}$ in drug-treated PC-3 cells. As indicated in Fig. 2C, the activity of immunoprecipitated p70$^{S6K}$ was significantly reduced in PC-3 cells exposed to celecoxib and DMC at the indicated concentrations for 2 h. In addition, we assessed the protective effect of the transient expression of the constitutively active forms of PDK-1 and Akt (PDK-1$^{A280V}$, Ref. 40; Akt$^{T308D/S473D}$, Ref. 41; respectively) on drug-induced PC-3 cell death. Western blot analysis confirmed that transient transfection of PDK-1$^{A280V}$ and Akt$^{T308D/S473D}$ into PC-3 cells led to a several-fold increase in the expression of the respective kinases (Fig. 3A). These transient transfectants were exposed to 10–50 $\mu$M celecoxib or DMC for 24 h in serum-free medium to examine their susceptibility to drug-induced cell death *vis-à-vis* transfectants with an empty pCMV vector. As shown, Akt$^{T308D/S473D}$ gave partial yet significant protection against either agent before complete apoptotic death took place at higher concentrations (Fig. 3B; celecoxib, *left*; DMC, *right*). In contrast, PDK-1$^{A280V}$ provided only a marginal protection, suggesting that even constitutively active PDK-1 could not overcome the direct inhibition by these agents. At high concentrations of celecoxib or DMC, cells underwent rapid apoptotic death. Fig. 3C indicates the partial protective effect of Akt$^{T308D/S473D}$ against 40 $\mu$M DMC before complete apoptotic death occurred at 6-h posttreatment.

***In Vivo* Effect of Celecoxib and DMC on PC-3 Xenograft Tumor Growth.** Oral celecoxib and DMC at two different doses, 100 and 200 mg/kg, were administered daily to nude mice bearing established PC-3 xenograft tumors. All animals tolerated the treatments well without observable sign of toxicity and were characterized by stable body weights throughout the course of study. No gross pathological abnormalities were noted at necropsy after 35 days of treatment.

The effects of individual treatments on tumor growth were assessed (Fig. 4). In addition, serum concentrations of celecoxib and DMC were measured at different time points during the 24-h period after the final administration of test agents (Fig. 5A). Accordingly, the respective pharmacokinetic parameters in PC-3 tumor-bearing mice are summarized in Fig. 5B.

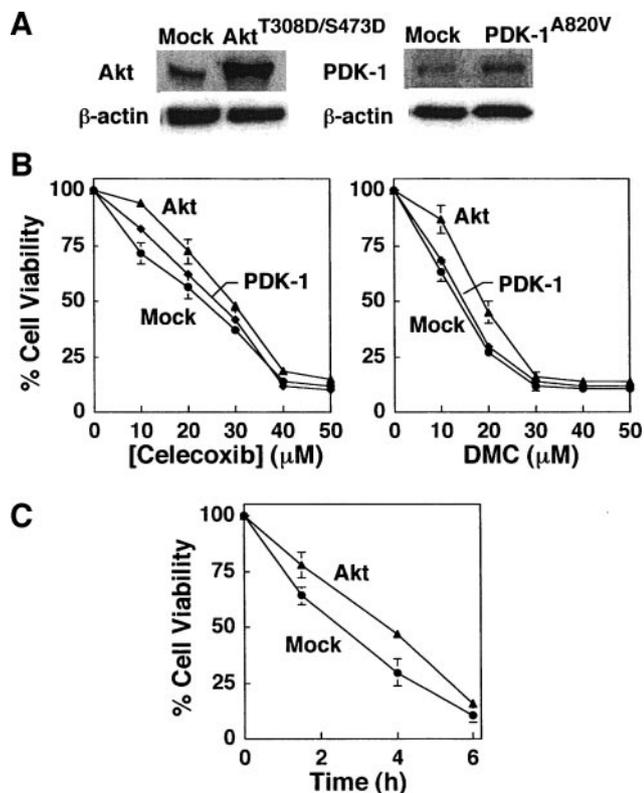Among the four treatments, only the group receiving 200 mg/kg/

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Fig. 3. Protective effect of constitutively active Akt and 3-phosphoinositide-dependent protein kinase-1 (PDK-1) on celecoxib and DMC {4-[5-(2,5-dimethylphenyl)-3(trifluoromethyl)-1H-pyrazol-1-yl]-benzene-sulfonamide}-induced apoptotic death in PC-3 cells. *A,* expression of Akt[T308D/S473D] (*left*) and PDK-1[A820V] (*right*) in PC-3-transient transfectants. *B,* viability of PC-3 cells overexpressing Akt[T308D/S473D] or PDK-1[A820V] *vis-à-vis* PC-3 cells transfected with empty pCMV vector (mock) in the presence of the indicated amount of celecoxib (*left*) or DMC (*right*) in serum-free medium for 24 h. Values are the means ± SD ($n = 3$). *C,* viability of PC-3 cells overexpressing Akt[T308D/S473D] *versus* PC-3 cells transfected with pCMV vector (mock) in the presence of 40 $\mu$M DMC at the indicated time. Values are the means ± SD ($n = 3$).

day DMC displayed a significant effect on the PC-3 tumor growth ($P < 0.1$). Although treatment with celecoxib at 200 mg/kg/day and DMC at 100 mg/kg/day could marginally suppress the proliferation of the xenograft, these effects were not statistically significant. Pharmacokinetic analysis indicates that the peak serum concentrations achieved during the 24-h dosing interval were approximately 20 and 14 $\mu$M for celecoxib and DMC, respectively, at 200 mg/kg/day. The respective average serum concentrations ($C_{average}$), defined as area under the curve/time interval, were calculated to be 11.3 and 5.4 $\mu$M. High-performance liquid chromatography data suggest that DMC underwent oxidative metabolism to a greater extent than celecoxib in mice (not shown). Consequently, the serum concentrations of DMC were significantly lower than those of celecoxib. The major oxidative metabolite extracted from serum was identified by high-resolution mass spectrometry to be the dicarboxylate counterpart of DMC (Fig. 5C). This metabolic profile is analogous to that of celecoxib. However, it remains unclear whether this oxidative metabolite retained any antiproliferative activity.

To correlate biological response with the mechanism of action identified *in vitro,* the effect of orally administered celecoxib and DMC at 200 mg/kg/day on P-Akt in PC-3 tumors was examined by immunoblotting. Fig. 6A depicts Western blots of Akt and P-Akt in the homogenates of three representative PC-3 tumors with different volumes from tumor-bearing mice treated with vehicle, celecoxib, or DMC for 35 days. These immunoblots were scanned and quantitated to determine the ratios of the levels of P-Akt to Akt (in arbitrary units). Overall, a differential reduction in the P-Akt/Akt ratio was

noted in celecoxib- and DMC-treated groups *vis-à-vis* the control group (Fig. 6B).

In line with the *in vivo* data on tumor growth, celecoxib displayed a marginal effect ($P = 0.112$) on P-Akt/Akt ratios, whereas DMC could significantly suppress *in vivo* Akt phosphorylation at $P = 0.056$. However, there existed no direct correlation between the tumor volume and P-Akt status within the same group. Characteristic of rapidly growing and poorly differentiated tumors, these PC-3 xenografts were heterogeneous with respect to the distribution of viable and necrotic regions within the tumors. This heterogeneity in the quality of tumor tissues sampled could account for the lack of correlation.

## DISCUSSION

This study used DMC, a close structural analog of celecoxib, to discern the relative involvement of COX-2-dependent *versus* independent pathways in the antiproliferative effect of celecoxib in prostate cancer. Even deficient in COX-2-inhibitory activity, DMC was able to inhibit PC-3 cell proliferation in a manner qualitatively similar to celecoxib, suggesting that COX-2 inhibition alone could not account for the *in vitro* antiproliferative effect of celecoxib. In drug-treated cells, Akt activity was attenuated as a result of the partial inhibition of PDK-1 in concert with the concomitant Akt dephosphorylation by PP2A. It is well documented that PDK-1/Akt signaling plays a central role in regulating cancer cell survival and proliferation by affecting the phosphorylation status of a plethora of downstream effectors that are either PDK-1 or Akt substrates (42–45). Consequently, blockade of this signaling cascade leads to growth inhibition by cell cycle arrest and/or apoptosis in malignant cells. In 10% FBS-supplemented medium, both compounds caused $G_1$ arrest and, at
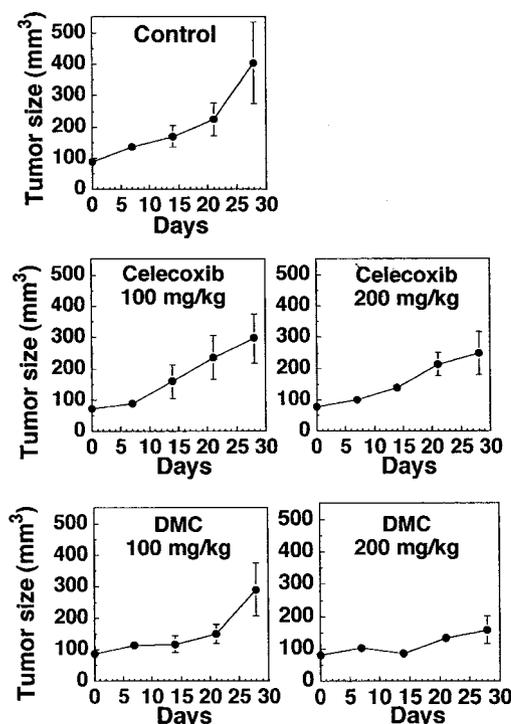


Fig. 4. Effect of celecoxib and DMC {4-[5-(2,5-dimethylphenyl)-3(trifluoromethyl)-1H-pyrazol-1-yl]-benzene-sulfonamide} on the growth of established PC-3 tumors in nude mice. Each mouse was inoculated s.c. in the dorsal flank with 5 × 10^{5} PC-3 cells suspended in 0.1 ml of serum-free medium containing 50% Matrigel. When the tumor reached a volume of approximately 80 mm[3], mice received single daily oral treatments of celecoxib or DMC at 100 and 200 mg/kg body weight/day for the duration of the study. Controls received vehicle consisting of 0.5% methylcellulose and 0.1% polysorbate 80 in sterile water. Values are means ± SE ($n = 6$).
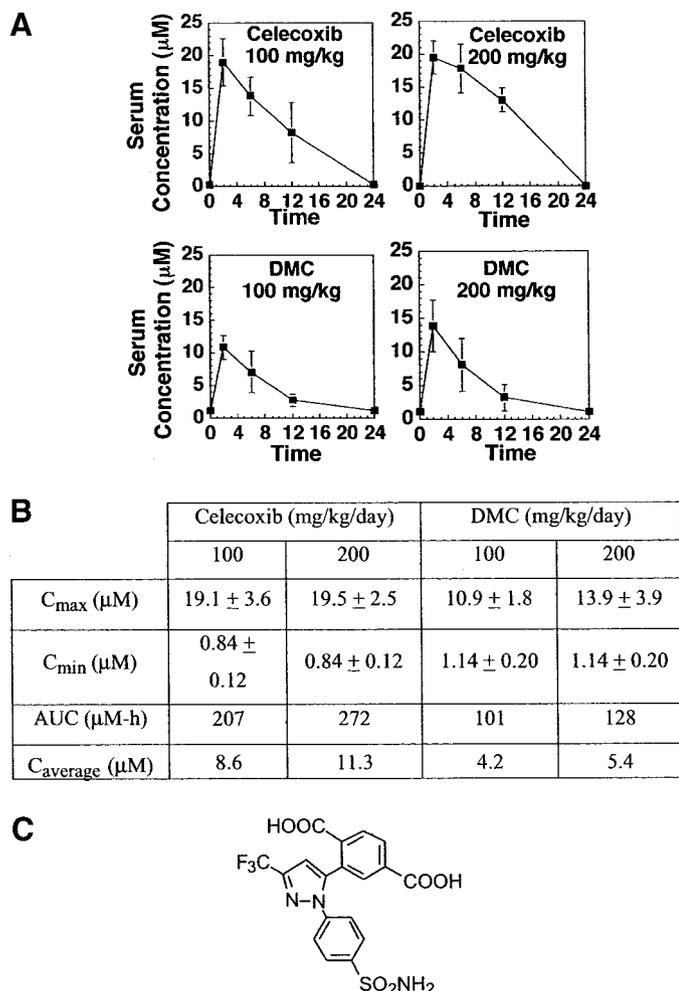
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**A**



**B**

| | Celecoxib (mg/kg/day) | | DMC (mg/kg/day) | |
|---|---|---|---|---|
| | 100 | 200 | 100 | 200 |
| $C_{max}$ ($\mu$M) | $19.1 \pm 3.6$ | $19.5 \pm 2.5$ | $10.9 \pm 1.8$ | $13.9 \pm 3.9$ |
| $C_{min}$ ($\mu$M) | $0.84 \pm 0.12$ | $0.84 \pm 0.12$ | $1.14 \pm 0.20$ | $1.14 \pm 0.20$ |
| AUC ($\mu$M-h) | 207 | 272 | 101 | 128 |
| $C_{average}$ ($\mu$M) | 8.6 | 11.3 | 4.2 | 5.4 |

**C**



Fig. 5. Pharmacokinetic profiles of celecoxib and DMC {4-[5-(2,5-dimethylphenyl)-3(trifluoromethyl)-1*H*-pyrazol-1-yl]-benzene-sulfonamide}. *A,* serum concentrations of celecoxib and DMC at different time points during the 24-h period after the final administration of test agents. Values are means $\pm$ SD ($n = 3$). *B,* pharmacokinetic parameters calculated from the serum concentration data in *A. $C_{max}$,* maximum serum concentration; $C_{min}$, minimum serum concentration; *AUC,* area under curve; $C_{average}$, average serum concentration, is defined as AUC/time. *C,* putative structure of the major metabolite of DMC extracted from serum, as determined by high-resolution mass spectrometry (HRMS). The metabolite, after high-performance liquid chromatography purification, was subject to HRMS analysis. The molecular formula of the putative metabolite is $C_{18}H_{12}O_6N_3F_3S$, of which the molecular ion (M) has a theoretical mass of 455.0398. The actual mass of the (M + H) species determined by HRMS was 456.0489.

nearly 20 $\mu$M, neither dose led to significant inhibition of tumor growth in nude mice. This finding is in line with the *in vitro* data that celecoxib at 20 $\mu$M could not effectively suppress PC-3 cell growth. It may also dampen the possibility that inhibition of COX-2 in either the tumor or the host tissues plays a major role in the *in vivo* antitumor effect of celecoxib in this model of prostate cancer. In contrast, treatment with 200 mg/kg/day DMC, which gave rise to a $C_{max}$ of 14 $\mu$M, showed significant inhibition of the PC-3 tumor growth, which parallels the results in cultured PC-3 cells in which significant growth inhibition was seen at a concentration around 15 $\mu$M. Accordingly, there exists a correlation between the effectiveness of DMC in inhibiting *in vitro* PC-3 cell proliferation and its ability to inhibit *in vivo* PC-3 tumor growth through the inhibition of PDK-1/Akt signaling.

These data suggest that PDK-1/Akt signaling represents a major non-COX-2 target underlying the *in vitro* and *in vivo* antiproliferative effects of celecoxib and DMC in prostate cancer cells. It has been demonstrated that there was no consistent overexpression of COX-2 in established prostate cancer or high-grade prostatic intraepithelial neoplasia, *vis-à-vis* adjacent normal prostate tissue (47). This finding suggests that COX-2 might not play as critical a role in prostatic carcinogenesis as in other types of cancer.

Nevertheless, this COX-2-independent mode of action might be a cancer type-specific phenomenon. Several studies in the literature have elegantly demonstrated the importance of the inhibition of COX-2-derived prostaglandins, particularly prostaglandin $E_2$, as a major mechanism underlying the *in vivo* antitumor action of celecoxib in different xenograft tumors, including those of HCA-7 colon cancer cells (22) and HNSCC 1483 head and neck cancer cells (48). Presumably, in these xenograft models, stromal or host-derived COX-2-dependent processes may play a prominent role in tumorigenesis and/or angiogenesis (49–51), rendering these tumors more susceptible to the COX-2-inhibitory effect of celecoxib. Consequently, growth of
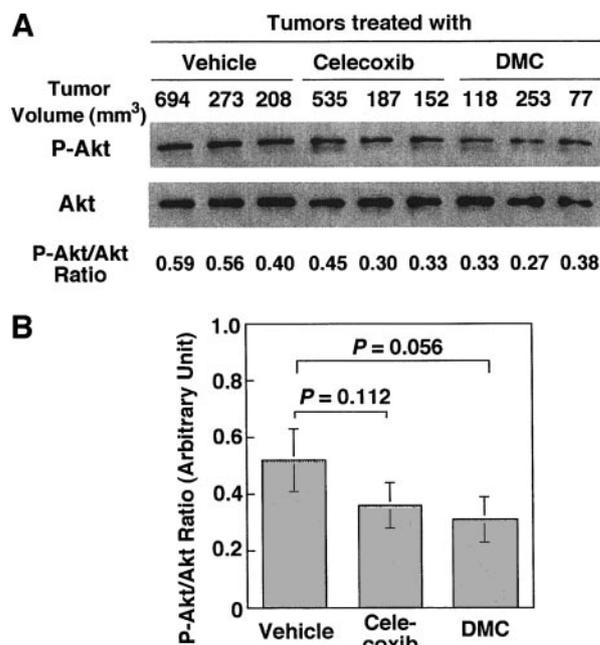
higher concentrations, induced apoptosis with relative potency consistent with the respective activities in blocking Akt activation. In addition, we reported that celecoxib and DMC facilitated the dephosphorylation of extracellular signal-regulated kinase 2 (23, 25). However, our data suggest that extracellular signal-regulated kinase 2 down-regulation might not be as important as PDK-1 inhibition in celecoxib-induced cell death, because treatment of PC-3 cells with the inhibitor of mitogen-activated protein kinase kinase, PD98059, did not lead to apoptotic death.[1] Moreover, at concentrations as low as 10 $\mu$M, celecoxib was able to perturb intracellular calcium by inhibiting endoplasmic reticulum $Ca^{2+}$-ATPases (46). Together, these data underscore the pharmacological complexity in COX-2-independent signaling mechanisms that celecoxib uses to mediate antiproliferative effects *in vitro*.

Our study with PC-3 xenograft tumors indicates that although peak serum concentrations for celecoxib at 100 and 200 mg/kg/day were

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[1] H-P. Lin, C-C. Yang, and C-S. Chen, unpublished observations.

**A**



**B**



Fig. 6. *In vivo* effect of celecoxib and DMC {4-[5-(2,5-dimethylphenyl)-3(trifluoromethyl)-1*H*-pyrazol-1-yl]-benzene-sulfonamide} on the phospho (P)-Akt status in PC-3 tumors. *A,* Western blot analysis of Akt and P-Akt levels in the homogenates of three representative PC-3 tumors with different volumes from tumor-bearing mice treated with vehicle, celecoxib, or DMC for 35 days. These immunoblots were scanned by a Photodyne image system and quantitated using a FOTO/Analyst PC image program (version 3.0) to determine the ratios of the levels of P-Akt to Akt, *B,* P-Akt/Akt ratios (in arbitrary units) in different drug-treated groups. Values are means $\pm$ SD ($n = 3$).

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these xenografts could be attenuated by low serum concentrations of celecoxib via a COX-2-dependent mechanism.

In summary, the impetus of the present study is at least 3-fold. First, to the best of our knowledge, this is the first study using a celecoxib analog to discern the relative contribution of COX-2-dependent *versus* COX-2-independent signaling mechanisms in the *in vivo* antitumor effects of celecoxib. Second, our present data demonstrate that PDK-1/Akt signaling represents a unique non-COX-2 target for celecoxib, whereas rofecoxib (Vioxx) and other COX-2 inhibitors have no appreciable-inhibitory effect on PDK-1 (data not shown). This discrepancy explains why celecoxib is substantially more potent than other COX-2 inhibitors in inducing apoptosis and cell cycle arrest in cancer cells. Third, separation of the effect on PDK-1/Akt signaling from the COX-2-inhibitory activity allows us to use celecoxib as a starting point to design a novel class of antitumor agents, of which the proof of principle has been demonstrated in our previous paper (26). Use of this strategy to develop more potent PDK-1/Akt signaling inhibitors is currently underway in this laboratory.

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# 3-Phosphoinositide-Dependent Protein Kinase-1/Akt Signaling Represents a Major Cyclooxygenase-2-Independent Target for Celecoxib in Prostate Cancer Cells

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