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Sulindac and its Metabolites Inhibit Invasion of Glioblastoma Cells via Down-Regulation of Akt/PKB and MMP-2

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Abstract Non-steroidal anti-inflammatory drug (NSAID), sulindac has chemopreventive and anti-tumorigenic properties, however, the molecular mechanism of this inhibitory action has not been clearly defined. The Akt/protein kinase B, serine/threonine kinase is well known as an important mediator of many cell survival signaling pathways. In the present study, we demonstrate that down-regulation of Akt is a major effect of anti-invasiveness property of sulindac and its metabolites in glioblastoma cells. Myristoylated Akt (MyrAkt) transfected U87MG glioblastoma cells showed increase invasiveness, whereas DN-Akt transfected cells showed decrease invasiveness indicating that Akt potently promoted glioblastoma cell invasion. MMP-2 promoter and enzyme activity were up-regulated in Akt kinase activity dependent manner. Sulindac and its metabolites down-regulated Akt phosphorylation, inhibited MMP-2 production, and significantly inhibited invasiveness of human glioblastoma cells. In addition, sulindac and LY294002, a selective inhibitor of phosphoinositide 3-kinase (PI3K), synergistically inhibited the invasion of glioblastoma cells. Furthermore, only celecoxib showed Akt phosphorylation reduction and an anti-invasivness in glioblastoma cells, whereas aspirin, ketoprofen, ketorolac, and naproxen did not. In conclusion, our results provide evidence that down-regulation of Akt pathway and MMP-2 may be one of the mechanisms by which sulindac and its metabolites inhibit glioblastoma cell invasion. J. Cell. Biochem. 94: 597–610, 2005.

Key words: Akt; glioblastoma; invasion; migration; MMP; NSAIDs

Abbreviations used: COX, cyclooxygenase; ECM, extracellular matrix; GSK3, glycogen synthase kinase 3; MMPs, matrix metalloproteinases; MyrAkt, myristoylated Akt; NSAIDs, non-steroidal anti-inflammatory drugs; PH, plekstrin homology; PI3K, phosphoinositide 3-kinase; PIP_3, phophatidylinositol-3,4,5-triphophate; PIP_2, phasphatidylinositol-3,-4-bisphosphate; PKB, protein kinase B.

Hyung-Chahn Lee and In-Chul Park contributed equally to this study.

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Glioblastoma is the most common and malignant form of tumor in the human central nervous system: the mean expected length of survival is less than 1 year. Local invasive growth is a key feature of primary malignant brain tumors, and cause massive tissue destruction at the border between tumor and normal brain tissue [Bjerkvig et al., 1986]. Glioblastoma spreads along nerve fiber tracts and frequently penetrates beyond the glial membrane limitans externa, leading to leptomeningeal dissemination of the tumor. The diffusely infiltrative nature of glioblastoma is one of the major obstacles to its successful surgical control [Russel and Rubinstain, 1989]. The specific mechanisms facilitating the invasive behavior

of brain cancers remain largely unclear. However, the interactions between cancer and the surrounding normal cells in addition to extracellular matrix (ECM) are thought to be the key in tumor cell invasion [Liotta, 1986]. To invade and spread through surrounding normal tissue, tumor cells must degrade multiple elements of the ECM, including fibronectin, laminin, and type IV collagen [Matrisian, 1992]. The invasiveness of glioblastoma is determined in part by their proteolytic capacity, especially by two matrix metalloproteinases (MMPs), MMP-2 and MMP-9, that degrade gelatin. In general the expression and proteolytic activity of these MMPs are up-regulated in more aggressive form of brain tumors [Rao et al., 1993; Forsyth et al., 1998].

Akt/protein kinase B (PKB), a serine/threonine-protein kinase, plays a critical role in controlling the balance between cell survival and apoptosis [Franke et al., 1997a]. Phosphorylation of Akt is promoted by phophatidylinositol-3,4,5-triphophate (PIP_3) and phasphatidylinositol-3,4-bisphosphate (PIP_2) generated by phosphoinositide 3-kinase (PI3K), thus facilitating transmembrane signaling by serving as membrane-localization elements to recruit target proteins to specific sites in response to various growth/survival factors [Burgering and Coffer, 1995; Hemmings, 1997; Klippel et al., 1997]. Four phosphorylation sites have been identified on the plekstrin homology (PH) domain of Akt: Ser124, Thr308, Thr 450, and Ser473 [Alessi et al., 1996; Datta et al., 1999]. Thr308 and Ser473 are inducibly phosphorylated after treatment of cells with extracellular stimuli, whereas Ser124 and Thr450 appear to be basally phosphorylated [Datta et al., 1999]. Previous reports have shown that Akt delivers anti-apoptotic survival signals by phosphorylating Bad and activating caspase-9 [Datta et al., 1997; Cardone et al., 1998], and inhibition of Akt signaling can induce apoptosis in some human cancer cell lines [Yuan et al., 2000; Itoh et al., 2002]. Moreover, inactivation of PI3K with a specific inhibitor has led to dephosphorylation of Akt at Ser473, consequently causing translocation of Akt to the nucleus, where it is believed to regulate the transcription of various genes that are involved in cell survival or apoptosis [Hemmings, 1997]. Although, it has been reported that Akt promotes cancer cell invasion via increased motility and metalloproteinase production [Kim et al., 2001], the function of Akt in human glioblastoma cells has not been clarified.

It was observed that patients with rheumatoid arthritis had a reduced risk of colorectal cancer, supporting the epidemiological evidence that the use of non-steroidal anti-inflammatory drugs (NSAIDs), including sulindac and its metabolites, may protect the patients from the development of cancer [Gridley et al., 1993]. Evidences suggesting that NSAIDs may reduce the risk of cancer development and subsequent mortality have been accumulated numerous epidemiological studies and studies employing animal models [Williams et al., 1999]. Sulindac, a non-selective cyclooxygenase (COX) inhibitor, causes regression of precancerous adenomatous polyps in familial colon polyposis [Steinbach et al., 2000]. Recently, sulindac sulfide, a metabolite of sulindac, effectively suppressed the TGF-β-induced invasive phenotype of A549 lung adenocarcinoma cells [Shigeoka et al., 2004]. Furthermore, sulindac sulfide and sulindac sulfone caused the inhibition of ERK phosphorylation and integrin-activated signaling molecules [Rice et al., 2001]. Based on these observations, we hypothesized that some chemopreventive NSAIDs may suppress the intracellular signal that is essential for the motility of cancer cells. Here, we propose a unique mechanism of sulindac and its metabolites for cell invasion in human glioblastoma cells. Our data demonstrated that the NSAIDs, sulindac and its metabolites, sulindac sulfide, and sulindac sulfone (Exisulind) down-regulated Akt pathway and MMP-2, thereby inhibiting invasion of glioblastoma cells in vitro.

MATERIALS AND METHODS

Cell Culture and Reagents

The human glioblastoma cell lines U87MG, U251MG, and U373MG were obtained from the American Type Culture Collection (Rockville, MD). Cell lines were maintained in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum and 50 U/ml of both penicillin and streptomycin in a humidified 5% CO_2/air atmosphere at 37°C. Primary antibodies against phospho-Akt (Ser473) were purchased from Cell Signaling (Beverly, MA), anti-Akt antibody and Protein A/G plus were purchased from SantaCruz Biotechnology (Santa Cruz, CA), sulindac, sulindac sulfone, sulindac sulfide, and LY294002 were

from Calbiochem (La Jolla, CA), and aspirin, naproxen, ketoprofen, and ketorolac were from Sigma (St. Louis, MO). Celecoxib was a kind gift from Dr. Sung Hee Hong (KIRAMS, Korea). Retroviruses containing constitutive active form of Akt (MyrAkt) and kinase-dead Akt (DN-Akt) were generously provided by Dr. Hee Yong Chung (Hanyang University, Korea).

Growth Inhibition Assay

In vitro growth inhibition effects of NSAIDs on glioblastoma cells were determined by measuring MTT dye absorbance of live cells. Briefly, cells (5×10^3 cells/well) were seeded in 96-well microtiter plate (Nunc, Roskilde, Denmark). After exposure to the drug for 72 h, 50 μl of MTT (Sigma) solution (2 mg/ml in PBS) was added to each well, and the plates were incubated for additional 4 h at 37°C. To solubilize of the formazon crystal formed in viable cells, 200 μl of DMSO were added to each well and the absorbance was measured at 570 nm.

Cell Invasion Assays

The assay was performed by the modified method of procedure previously described [Yoon et al., 2001]. 2×10^5 cells/chamber were used for each invasion assay. Fifty microliters of Matrigel (1:1 vol./vol. dilution in cold serum-free medium) was applied to upper parts of a 24-well Transwell plate (8 μm pore size; Corning, Corning, NY) and allowed to be coated at 37°C. After rinsing with PBS, 2×10^5 cells in 200 μl of serum free DMEM were added to the upper chamber. Conditioned medium containing 0.1 mg/ml BSA (Sigma) was added to the lower compartment. In experiments indicated, sulindac, sulindac sulfide, and sulindac sulfone at various concentrations were added to upper chambers. The invasion was allowed to proceed for 8 h at 37°C. After incubation, the filters were fixed and stained with Diff-Quick staining kit (Fisher Scientific, Pittsburgh, PA). The cells that reached the under side of the filter were counted. For each filter, the average number of cells in five randomly chosen microscopic fields was determined. The final values for each condition were the average from the three invasion chambers.

Cell Migration (Wound) Assays

To determine the effect of sulindac, sulindac sulfide, and sulindac sulfone on cell motility, cells were seeded onto 100 μl of 1.5% gelatin-coated Transwell membrane filters as in invasion assay. Migration in the absence or presence of three NSAIDs was measured as described in the invasion assay. In addition, migration through a wound introduced in a cell monolayer was also measured, as described [Chandrasekar et al., 2003]. Briefly, 2×10^5 cells were plated onto each well of 12-well culture plates and incubated for 24 h. Then, subconfluent monolayers of cells were wounded by scraping with a plastic pipette tip, washed twice with PBS, and the medium was replaced with complete medium containing the reagents indicated. After 24 h, the distance that the advancing cells had moved into the cell-free (wound) area was measured, after staining with Diff-Quick staining kit and photographed.

Gelatin Zymography

Production of MMPs by glioblastoma cells was analyzed by gelatin zymography, as described previously [Park et al., 2002]. Briefly, cells were incubated in serum-free media for 24 h after drug treatment. The conditioned media were mixed with SDS sample buffer without heating or reduction, and the mixtures were applied to 10% polyacrylamide gels copolymerized with 1 mg/ml gelatin. After electrophoresis, gels were washed for 2 h at room temperature in buffer containing 2.5% (v/v) Triton X-100 in 50 mM Tris-HCl (pH 7.5). The gels were then incubated in 50 mM Tris-Cl (pH 7.5) with 5 mM $CaCl_2$ and 1 μM $ZnCl_2$ for 16 h at 37°C. After staining with Coomassie Blue (0.5%), zones of gelatinolytic activity were detected as clear bands against a blue background. Densitometric analysis was performed using *Scion Image* NIH Image program.

Assay of Akt Kinase Activity

The kinase activity of Akt was detected using glycogen synthase kinase 3-β (GSK3β) as a substrate. Briefly, after exposure to the indicated reagents, cells were washed twice with ice-cold PBS and lysed in a lysis buffer solution containing 20 mM Tris-HCl (pH 7.5), 1.25 mM β-glycerophosphate, 137 mM NaCl, 1 mM EGTA, 1 mM EDTA, 2 mM NaF, 1% NP-40, 1 mM sodium orthovanadate, and Complete™ protease inhibitor mix (Roche Biochemicals, Manheim, Germany). The cell lysates were centrifuged, and 250 μg of supernatants was immunoprecipitated with anti-Akt antibody and protein A/G plus-agarose. The beads were

washed three times with a solution containing 150 mM NaCl, 20 mM Tris-HCl (pH 7.5), 1 mM EGTA, 1 mM EDTA, and 0.5% NP-40, and once with a kinase assay buffer containing 50 mM Tris-HCl (pH 7.5), 137 mM NaCl, 1 mM $MgCl_2$, 1 mM sodium orthovanadate, 2.5 mM β-glycerophosphate, 2 mM EDTA, and 5 μM ATP, and then subjected to kinase assay. Akt activities were measured in a reaction mixture consisting of a kinase assay buffer, 1 μg of GSK3β, and 5 μCi of [γ-^{32}P]ATP for 20 min at 30°C. The reaction was terminated by addition of SDS sample buffer, and the samples were subjected to 10% SDS–polyacrylamide gel electrophoresis. Phosphorylated GSK3β were visualized by autoradiography.

Western Blotting Analysis

Glioblastoma cells were washed twice with ice-cold PBS and lysed in a lysis buffer [20 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1% Triton X-100, 0.5% Non-idet P-40, 1 mM EDTA, 1 mM EGTA, 2 mM sodium orthovanadate, 2 mM NaF, and Complete protease inhibitor mix (Roche Molecular Biochemicals, Germany)] for 20 min on ice. After centrifugation, protein concentration of cell lysates was determined by Bio-Rad protein assay kit (Bio-Rad, Hercules, CA). Eighty micrograms of proteins were resolved by 10% SDS–PAGE and transferred onto nitrocellulose membrane. The membranes were blocked with 5% non-fat dry milk in TBST [10 mM Tris-HCl (pH 7.5), 100 mM NaCl, and 0.05% Tween-20] for 1 h at room temperature and then incubated with appropriate primary antibody overnight at 4°C, followed by incubation with horseradish peroxidase-conjugated secondary antibody at 1:2,000 dilution for 1 h at room temperature. The immunoblots were visualized by enhanced chemiluminescence-Western blotting detection system (Amershambiosciences, Seoul, Korea).

Luciferase Reporter Assay

pGL3 and pGL2 luciferase reporter vectors containing MMP-2 promoter regions were kindly provided by Dr. Sang Oh Yoon (Korea Advanced Institute of Science and Technology, Korea). U87MG cells in 50–60% confluency were infected with retroviruses containing MyrAkt and DN-Akt, incubated for 24 h and transfected transiently with MMP-2 reporter and control plasmid using Effecten reagents (Qiagen, Valencia, CA), according to the supplier's instructions. After 24 h, cells were treated with either sulindac, sulindac sulfide, sulindac sulfone or LY294002 for 16 h, and the luminescence was measured using a luminometer.

Statistical Analysis

Quantitative data were expressed as mean ± SD. All measurements for each condition were performed in triplicate. Statistical significance of differences was assessed using a two-tailed homoscedastic Student's *t*-test. Statistical significance was inferred if $P < 0.05$.

RESULTS

Sulindac and Its Metabolites Reduce Invasion of Glioblastoma Cells

To determine whether sulindac and its metabolites, sulindac sulfide, and sulindac sulfone (Exisulind), could reduce the invasive behavior of glioblastoma cells, we measured the invasion ability of the cells treated with various doses of sulindac and its metabolites. As shown in Figure 1, sulindac and its metabolites markedly reduced U87MG glioblastoma cell invasion in a dose-dependent manner, demonstrated by Matrigel invasion, gelatin migration, and wound assays without the loss of cell viability. Sulindac reduced the invasion by 40% at 200 μM and 55% at 300 μM in Matrigel invasion assay. Sulindac sulfide and sulindac sulfone also affected the invasion: at 150 μM, the former inhibited by 60%, and the latter 50% inhibition at 400 μM. Next, we examined the effects of sulindac and its metabolites on invasion of other glioblastoma cells, U251MG and U373MG. The inhibitory effects of sulindac and its metabolites on invasion and migration of U251MG and U373MG cells also were apparent as shown in Figure 1E.

Sulindac and Its Metabolites Down-Regulate Phosphorylation of Akt Ser473

To explore the possible mechanism responsible for the inhibition of cell invasion, we examined whether sulindac and its metabolites might attenuate the activity of the serine/threonine kinase Akt, since Akt is a key signaling component in cell survival and motility by mediating the phosphorylation of its downstream effectors and Akt activation is a multistep process and requires phosphorylation of Akt Ser473. Therefore, the phosphorylation status of Akt Ser473 was studied following stimulation of U87MG cells with serum to activ-

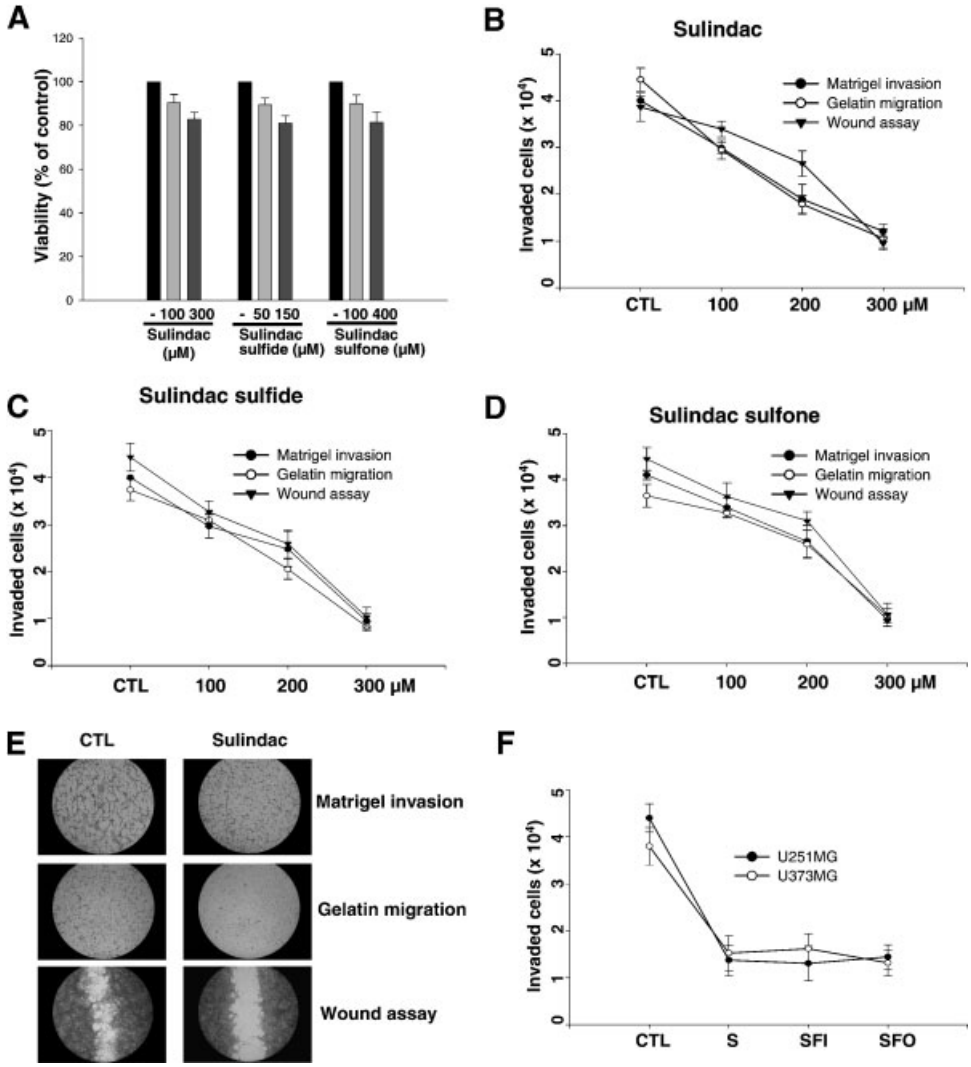

Fig. 1. Sulindac and its metabolites inhibit the invasiveness of U87MG cells. Cells not exposed to sulindac and its metabolites were used as controls (CTL). Cell viability test was performed for sulindac, sulindac sulfide, and sulindac sulfone (**A**), cell invasion assay was performed for sulindac (**B**), sulindac sulfide (**C**), and sulindac sulfone (**D**) invasion through a layer of Matrigel was determined by modified Boyden chamber method and was assessed by counting the number of invaded cells. Migration through gelatin-coated chambers was assessed as in invasion assay. Cell invasion was determined by counting the number of cells that migrated into the denuded area along the wounded edge after Diff-Quick staining. Results shown are the means of three individual experiments. **E**: Microscopic images show representative cell motility images of three independent experiment for 300 μM of sulindac. **F**: Sulindac and its metabolites inhibit the motility of glioblastoma cells. Cell lines indicated were treated with 300 μM of sulindac (S), 150 μM of sulindac sulfide (SFI) and 400 μM of sulindac sulfone (SFO) as described in ''Materials and Methods.'' Invasion through a layer of Matrigel-coated chambers was assessed as in (B). Results shown are the means of three individual experiments.

ate the growth factor-stimulated PI3K/Akt pathway, and cells were treated with sulindac or its metabolites for 5–120 min. Subsequently, whole cell extracts were probed by Western blot analysis with a Ser473 specific anti-phospho-Akt antibody. As depicted in Figure 2, sulindac or its metabolites significantly reduced the phosphorylation of Ser473. Although, the phospho-Akt level decreased marginally 15 min post-treatment, it decreased significantly at 30 min and became virtually undetectable at 60 min (Fig. 2A). These drugs also inhibited Akt phosphorylation in a dose-dependent manner when U87MG cells were treated with increasing concentrations of sulindac and its metabolites (Fig. 2B). Also, pretreatment of the cells with 10 μM LY294002, a specific inhibitor of PI3K, for 60 min markedly inhibited the Akt phosphor-

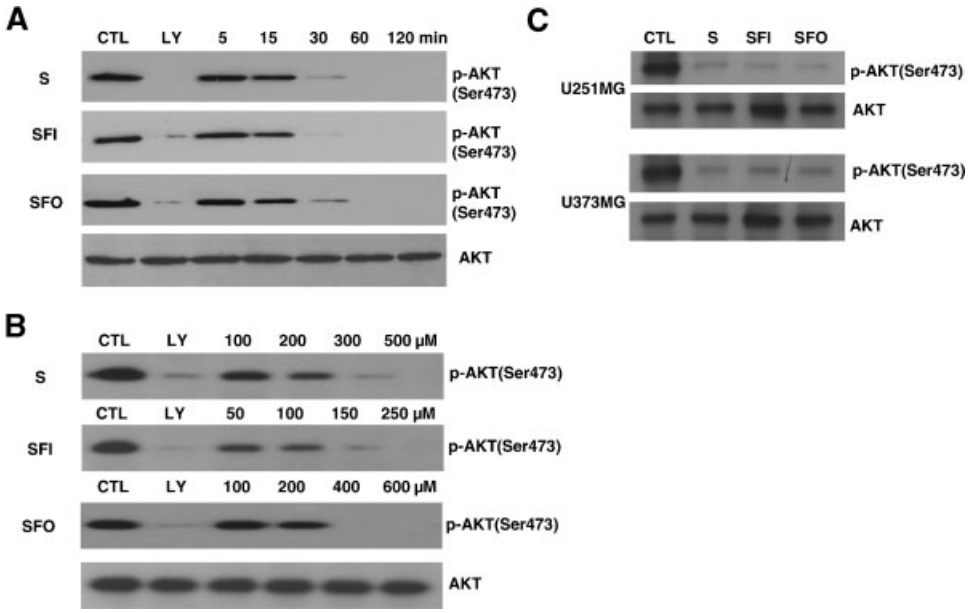

Fig. 2. Sulindac and its metabolites inhibit Akt phosphorylation. **A**: U87MG cells were treated either with 300 μM sulindac, 150 μM sulindac sulfide, or 400 μM sulindac sulfone for the indicated time period and lysed, and the cell lysates were electrophoresed and probed by Western blot with anti-P-^{473}Ser Akt antibodies. **B**: U87MG cells were exposed to sulindac or its metabolites with the indicated concentrations for 30 min and probed by Western blot as in (A). **C**: U251MG and U373MG glioblastoma cells were treated with 300 μM sulindac, 150 μM sulindac sulfide, or 400 μM sulindac sulfone for 30 min and probed by Western blot as in (A). S, sulindac; SFI, sulindac sulfide; SFO, sulindac sulfone.

ylation, indicating Akt phosphorylation is a down-stream event of PI3K activation. Furthermore, down-regulation of Akt Ser473 phosphorylation was also observed in U251MG and U373MG glioblastoma cell lines after treatment with sulindac or its metabolites (Fig. 2C).

Sulindac and Its Metabolites Inhibit Invasion Through by Akt Down-Regulation

To confirm the premise that anti-invasive effect of sulindac and its metabolites was mediated through the inhibition of Akt activation, myristoylated Akt (MyrAkt) which is anchored to the plasma membrane and has constitutively active kinase activity, and kinase-dead Akt (DN-Akt), which acts in a dominant negative manner, were transfected into U87MG cells, and the effect of sulindac on the activity of Akt in MyrAkt- and DN-Akt-transfected cells was assayed by determining the phosphorylation of GSK3β, which is an endogenous substrate of Akt, as a substrate. As seen in Figure 3A, Akt activity in the MyrAkt transfected cells was increased, whereas the cells transfected with DN-Akt had a decreased Akt activity. The invasiveness was significantly increased in MyrAkt transfected cells, whereas decreased in DN-Akt transfected cells. These findings demonstrate that Akt promotes invasion and migration of U87MG glioblastoma cells due to its membrane-translocating ability and kinase activity. This observasion was coincident with the previous report that Akt promoted the enhanced motility of squamous carcinoma cell lines [Grille et al., 2003]. We next tested the effect of sulindac and its metabolites on invasiveness of MyrAkt and DN-Akt transfected U87MG cells. Sulindac treatment significantly reduced the phosphorylation level of GSK3β in both MyrAkt and DN-Akt transfected cells (Fig. 3A, lane 2, 4, 6), and sulindac and its metabolites markedly suppressed the increase of MyrAkt-induced invasion activity and enhanced the suppression of invasion activity in DN-Akt transfected cells (Fig. 3C–E), suggesting that Akt may be at least, in part, the target of sulindac and its metabolites.

Because Akt represents one of the major downstream effectors of PI3K [Franke et al., 1997b], we also tested the effects of LY294002, a fairly specific PI3K inhibitor, on invasivness of U87MG cells. As shown in Figure 4A, 5 μM LY294002 inhibited the invasion by more than 20% (dotted bar) and co-treatment of the cells

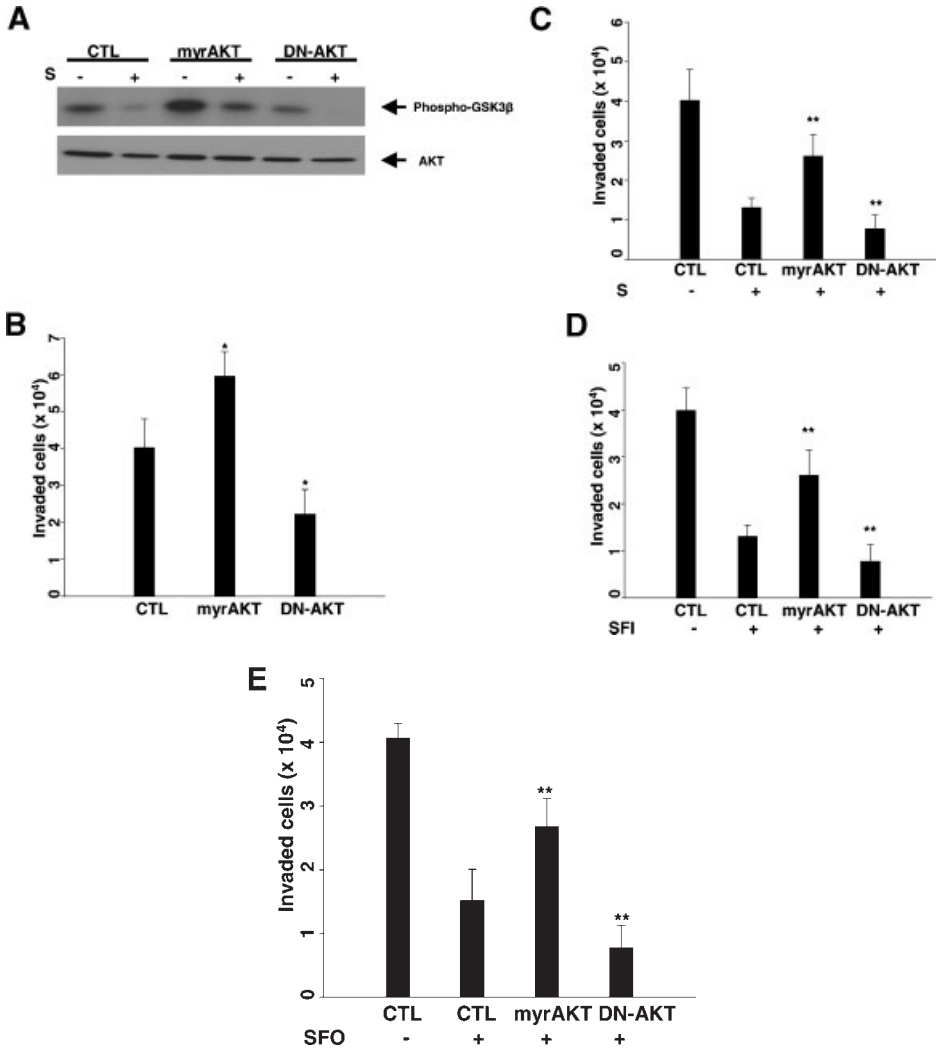

Fig. 3. Sulindac and its metabolites decrease the Akt-induced motility of glioblastoma cells. U87MG cells were infected with the recombinant retroviruses containing the constitutive active form of Akt (MyrAkt) and kinase-dead Akt (DN-Akt). **A**: U87MG cells harboring MyrAkt and DN-Akt were exposed to 300 μM sulindac for 30 min and Akt kinase assay was performed in vitro using recombinant human GSK3β as a substrate. **B**: U87MG cells harboring control retroviruses, MyrAkt and DN-Akt were used for Matrigel invasion assay as described in ''Material and Methods.'' Cells harboring control retroviruses, MyrAkt retroviruses, and DN-Akt retroviruses were exposed to 300 μM sulindac (**C**), 150 μM sulindac sulfide, (**D**), and 400 μM sulindac sulfone (**E**), and Matrigel invasion assay was performed as in (B). Results shown are the means of three individual experiments; bars, ± SD. *, $P < 0.01$. **, $P < 0.05$. S, sulindac; SFI, sulindac sulfide; SFO, sulindac sulfone.

with LY294002 and sulindac synergistically inhibited cell invasion. In addition, LY294002 also suppressed the increase of MyrAkt-induced cell invasion (Fig. 4B). These results imply that invasiveness of U87MG glioblastoma cells was involved in PI3K-induced activation of Akt pathway. Furthermore, the increased invasion activity induced by MyrAkt was not completely suppressed by LY294002. This was in agreement with the previous report that LY294002 suppressed Akt activation through blockage of the PI3K-PDK1 pathway, and Myr-Akt is the membrane-targeted version pathway. MyrAkt may be activated in part, by a LY294002-insensitive pathway [Shigeoka et al., 2004]. Therefore, Myr-Akt may partially overcome the effect of LY294002 on the motility of glioblastoma cells in our experiments.

Sulindac and Its Metabolites Inhibit MMP-2 Activity via Akt

MMPs are enzymes involved in degradation of the ECM and are also involved in the development of invasion and metastasis [Chambers

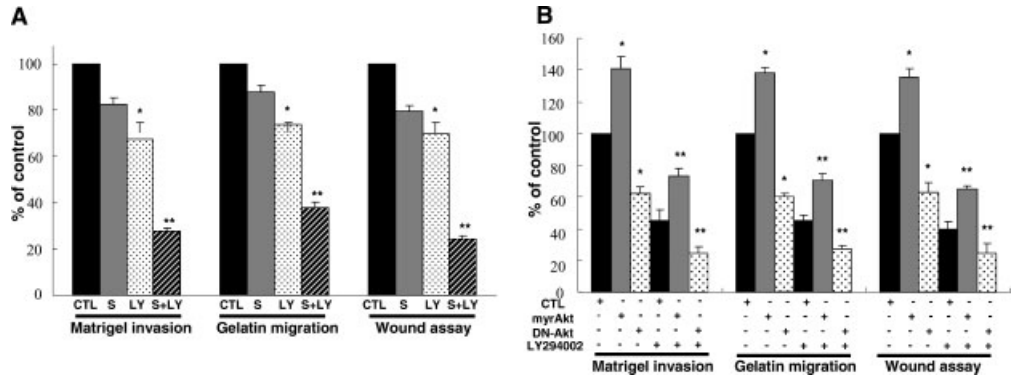

Fig. 4. PI3-kinase inhibitor, LY294002, inhibits the Akt-induced glioblastoma motility in vitro. **A**: U87MG cells were treated with 5 μM LY294002 alone or with 100 μM sulindac and Matrigel invasion, gelatin migration, and wound assay were performed as described in ''Material and Methods.'' Results shown are the means of three individual experiments. **B**: U87MG cells harboring control retroviruses, MyrAkt retroviruses, and DN-Akt retroviruses were exposed to 10 μM LY294002 and Matrigel invasion, gelatin migration, and wound assay were performed as described in ''Material and Methods.'' Results shown are the means of three individual experiments; bars ± SD. *, $P < 0.01$. **, $P < 0.05$. CTL, control; LY, LY294002; S, sulindac; SFI, sulindac sulfide; SFO, sulindac sulfone.

and Matrisian, 1997; Stetler-Stevenson and Yu, 2001]. To determine whether the activity of MMPs is associated with invasiveness, cell-conditioned medium following treatment with sulindac and its metabolites was analyzed for MMP activity by gelatin zymography. Our results showed that treatment with sulindac or its metabolites decreased the MMP-2 activity of U87MG cells in a dose-dependent manner (Fig. 5A), whereas it had only marginal effect on MMP-9 activity (data not shown). Co-treatment of the cells with LY294002 together with sulindac or its metabolites further enhanced the inhibition of MMP-2 activity at a level

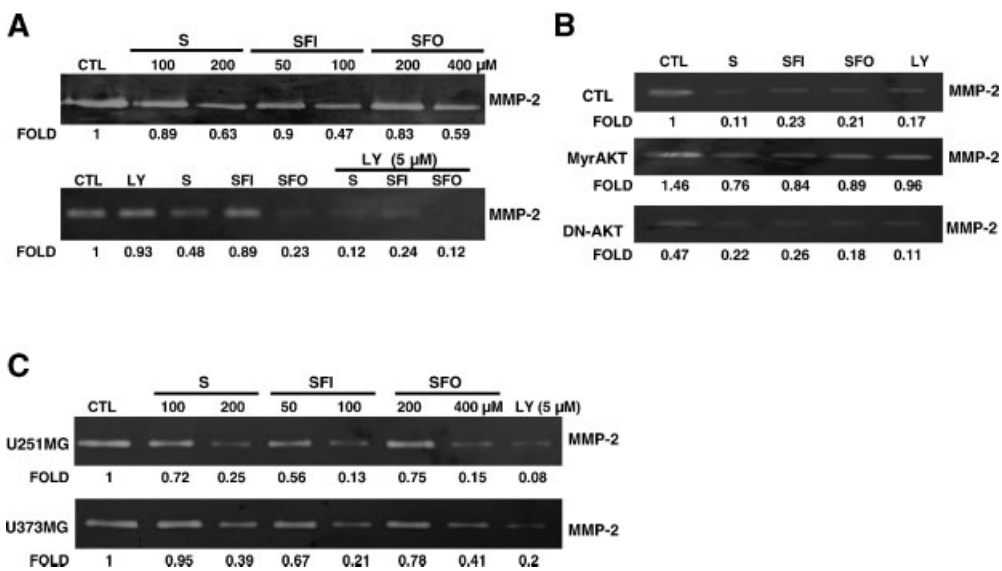

Fig. 5. Sulindac and its metabolites decrease the Akt-induced MMP-2 activity of glioblastoma cells. **A**: U87MG cells in the conditioned media were exposed to either sulindac, sulindac sulfide, sulindac sulfone, or LY294002 at the indicated concentrations for 24 h, and the activity of secreted MMP-2 was analyzed using gelatin zymography. Cells not exposed to the reagents were used as controls. **B**: U87MG cells harboring control retroviruses, MyrAkt retroviruses, and DN-Akt retroviruses were exposed to 200 μM sulindac, 100 μM sulindac sulfide, 400 μM sulindac sulfone, or 5 μM LY294002 at the indicated concentrations for 24 h, and the activity of secreted MMP-2 was analyzed as in (A). **C**: In U251MG and U373MG glioblastoma cells, the effects of sulindac, sulindac sulfide, sulindac sulfone, and LY294002 on the MMP-2 secretion were analyzed at the indicated concentrations as described in (A). CTL, control; LY, LY294002; S, sulindac; SFI, sulindac sulfide; SFO, sulindac sulfone.

higher than those treated with either reagent. In other glioblastoma cells, such as U251MG and U373MG, MMP-2 activity was also down-regulated by sulindac and its metabolites (Fig. 5B).

In order to investigate whether MMP-2 down-regulation was dependent on Akt activity, we next tested the effect of sulindac and its metabolites on MMP-2 activity in MyrAkt and DN-Akt transfected cells. As shown in Figure 5C, MyrAkt transfected cells had increased MMP-2 activity, whereas DN-Akt had significantly suppressed activity. Sulindac and its metabolites effectively suppressed the increase of MyrAkt-induced MMP-2 activity and enhanced the suppression of MMP-2 activity in DN-Akt transfected cells. These findings suggest that sulindac and its metabolites down-regulate MMP-2 activity by suppressing Akt pathway.

Sulindac and Its Metabolites Inhibit MMP-2 Promoter Activity Mediated by Akt

To examine whether the down-regulation of MMP-2 activity by sulindac and its metabolites was associated with gene expression, we investigated the effect of sulindac and its metabolites on the promoter activity of MMP-2. Therefore, U87MG cells were transiently transfected with a MMP-2 promoter-reporter construct containing 5′-flanking sequence of the promoter. The transfected cells showed about twofold decrease in the promoter activity after the cells were treated with 200, 100, and 400 μM of sulindac, sulindac sulfide, and sulindac sulfone, respectively (Fig. 6A), indicating that sulindac and its metabolites decreased the MMP-2 promoter activity in U87MG cells. To explore the importance of the Akt in the down-regulation of

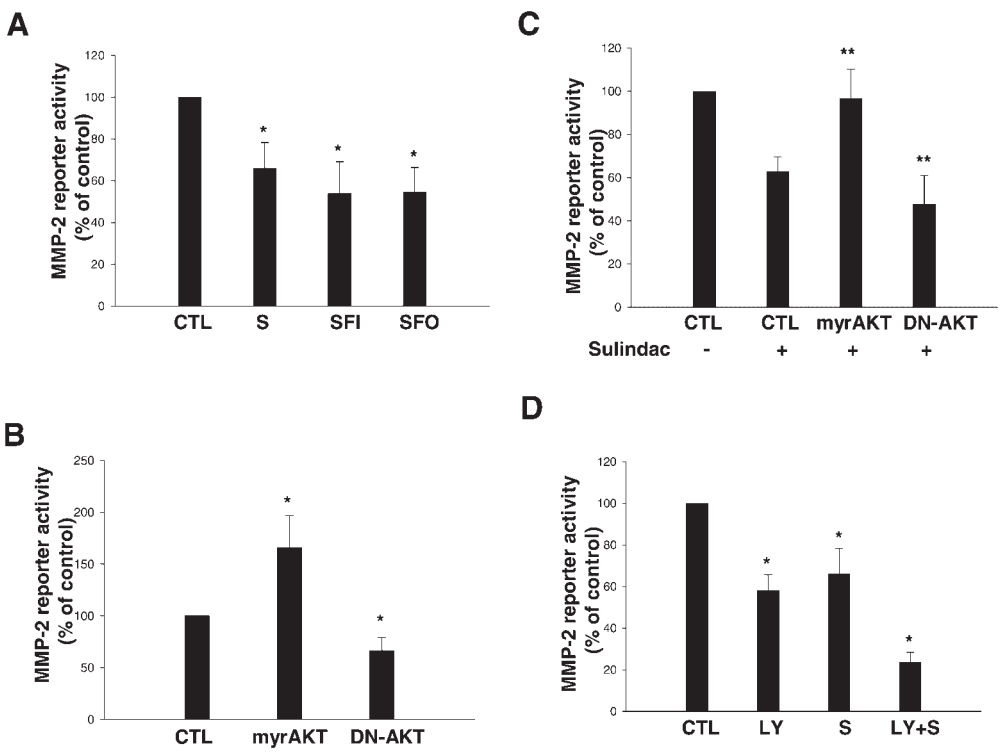

Fig. 6. Sulindac and its metabolites decrease the Akt-induced MMP-2 promoter activity of glioblastoma cells. **A**: U87MG cells were transfected with reporter vectors containing MMP-2 promoter regions and luciferase gene. After 24 h of transfection, cells were exposed to 200 μM sulindac, 100 μM sulindac sulfide, and 400 μM sulindac sulfone for 16 h. The cells were lysed, and the extracts were analyzed for luciferase activity. **B**: U87MG cells harboring control retroviruses, MyrAkt retroviruses and DN-Akt retroviruses were analyzed for luciferase activity as in (A). **C**: U87MG cells harboring control retroviruses, MyrAkt retroviruses and DN-Akt retroviruses were treated with 200 μM sulindac for 16 h. The cells were then lysed, and the extracts were analyzed for luciferase activity. **D**: U87MG cells transfected with reporter vectors containing MMP-2 promoter regions and luciferase gene, were exposed with 5 μM LY294002 alone or with 100 μM sulindac for 16 h and analyzed for luciferase activity. Results shown are the means of three individual experiments; bars ± SD. *, $P < 0.01$. **, $P < 0.05$. CTL, control; LY, LY294002; S, sulindac.

MMP-2 promoter by sulindac and its metabolites, we next tested the effect of sulindac or its metabolites on MMP-2 promoter activity in MyrAkt and DN-Akt transfected U87MG cells. As shown in Figure 6B, MyrAkt transfected cells showed increased MMP-2 promoter activity, whereas the MMP-2 promoter activity in DN-Akt transfected cells was significantly suppressed. Sulindac and its metabolites effectively suppressed the increase of MyrAkt-induced MMP-2 promoter activity, and enhanced the suppression of MMP-2 promoter activity in DN-Akt transfected cells (Fig. 6C). These results show that Akt activity is associated with MMP-2 gene expression and MMP-2 down-regulation by sulindac and its metabolites is dependent on Akt activity. Additionally treatment of the transfected cells with 5 μM LY294002 results in threefold decrease in the MMP-2 promoter activity and co-treatment of LY294002 with sulindac synergistically inhibited the MMP-2 transcriptional activity (Fig. 6D).

Not all NSAIDs can Inhibit Cell Invasion of U87MG

To test whether other NSAIDs have anti-invasive effects, the effects of various NSAIDs on the invasion of U87MG glioblastoma cells were examined after treating the cells with non-cytotoxic concentrations of various NSAIDs. As shown in Figure 7A, only celecoxib showed a statistically meaningful anti-invasion effect in U87MG cells among the NSAIDs examined, which include aspirin, celecoxib, ketoprofen, ketorolac, and naproxen, indicating that not all the NSAIDs can inhibit invasion of glioblastoma cells. At the indicated concentrations, all the NSAIDs did not induce cytotoxicity (data not shown). Since anti-invasive effect of sulidac and its metabolites is mediated via down-regulation of Akt pathway, we next measured the level of Akt phosphorylation in cells treated with NSAIDs. As shown Figure 7B, only celecoxib showed down-regulation of Akt phosphorylation implying that anti-invasion activity of celecoxib was involved in down-regulation of Akt pathway.

DISCUSSION

The invasive characteristics of glioblastoma cells depend mainly on the degradation of the ECM by proteases and the induction of cell migration, the latter being the determining step in the process of tumor invasion [Giese and Westphal, 1996]. Cell migration is a highly regulated process, which is critical for physiologic as well as pathologic tissue remodeling [Lauffenburger and Horwitz, 1996]. In the present study, we showed that treatment of glioblastoma cells with NSAIDs, especially sulindac and its metabolites, markedly reduced cell invasion in a dose dependent manner, as demonstrated by three different migration assays (Fig. 1). We also investigated the molecular mechanism by which sulindac and its metabolites inhibited cell invasion. Our results showed that suppression of glioblastoma cell invasion by sulindac and its metabolites was mediated through the Akt signaling pathway. Akt is recruited to plasma membrane by binding to phosphoinositides through its pleckstrin homology domain, and has been implicated in actin

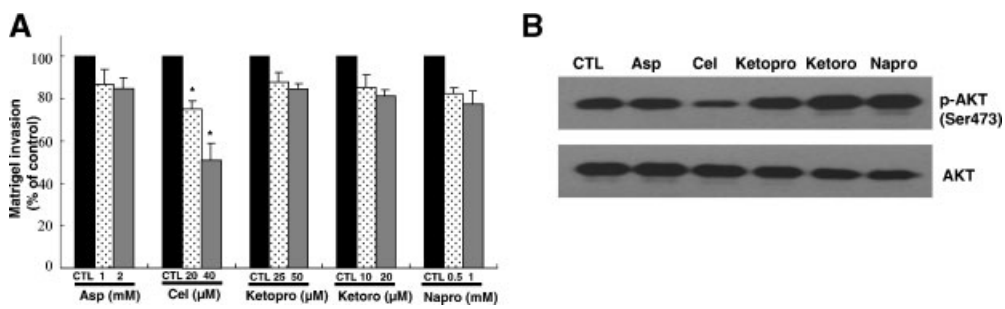

Fig. 7. Effects of NSAIDs on the invasion and Akt phosphorylation of glioblastoma cells. **A**: U87MG cells were exposed to various NSAIDs at the indicated concentrations, and invasion through a layer of Matrigel was determined by modified Boyden chamber method and was assessed by counting the number of invaded cells. Results represent mean ± SD of at least three independent experiments. **B**: U87MG cells were exposed to NSAIDs at the indicated concentrations for 1 h and lysed, and the cell lysates were electrophoresed and probed by Western blot with anti-P-^{473}Ser Akt antibodies. Results shown are the representative of three individual experiments; bars ± SD. *, $P < 0.05$. ASP, 2 mM aspirin; Cel, 40 μM celecoxib; Ketopro, 50 μM ketorprofen; Ketoro, 20 μM ketorolac; Napro, 1 mM naproxen.

reorganization and migration of microvascular endothelial cells [Morales-Ruiz et al., 2000]. A recent report suggests that activated Akt promotes cell invasion via increasing its motility [Kim et al., 2001]. Our results showed that the expression of constitutively active Akt (MyrAkt) in U87MG glioblastoma cells increased their ability to migrate through Matrigel and gelatin, the major ECM component of basement of most tissue of human organs. On the other hand, cells expressing kinase-dead Akt (DN-Akt) showed exhibited markedly decreased migration ability. Akt phosphorylation level was significantly decreased in glioblastoma cells when treated with sulindac and its metabolites. Furthermore, sulindac and its metabolites markedly suppressed the increase of MyrAkt-induced cell invasion, and enhanced the suppression of invasion in DN-Akt transfected cells. These findings demonstrated that at least, Akt promoted glioblastoma cells invasion, and that sulindac and its metabolites inhibited the glioblastoma cell invasion by down-regulating the Akt pathway. However, we cannot rule out the other possible mechanisms through which Sulindac and its metabolites suppress the glioblastoma cell motility, independent of Akt. However, in Figure 7, among other NSAIDs, only celecoxib showed down-regulation of Akt phosphorylation and suppressed the invasion. In Figure 3B, constitutive active Akt introduction enhanced the invasiveness of glioblastoma cell, and dominant-negative Akt decreased the motility. Therefore, Akt plays an important role in the invasiveness of glioblastoma cell. This result is in agreement with other previous reports that PI3K/AKT pathway is intimately involved in cancer cell motility [Park et al., 2001; Jiang et al., 2004]. And, down-regulation of Akt is a major effect of anti-invasive property of Sulindac. Although, the precise molecular targets of sulindac remain to be clarified, there are several suggestive reports with respect to the suppression of Akt activation. Celecoxib, a COX-2 inhibitor, suppresses Akt activity [Yamazaki et al., 2002] through the inhibition of 3-phosphoinositide-dependent protein kinase-1 (PDK1) [Arico et al., 2002]. And, down-regulation of Akt may be a major effect of anti-invasive property of Sulindac. Therefore, we proposed that, as a potential mechanism, sulindac and its metabolites inhibit the glioblastoma cell motility through, at least in part, the suppression of the Akt pathway.

It has recently been reported that PI3K signaling is associated with increased invasiveness and gelatinase activity of malignant glioma cells [Kubiatowski et al., 2001]. In the current study, we examined the effect of LY294002, a specific inhibitor of PI3K, on a glioblastoma cell line to discern regulation of cell invasion, and observed that LY294002 effectively inhibited invasion of U87MG glioblastoma and also suppressed the increase of MyrAkt-induced invasion. In addition, co-treatment of the glioblastoma cells with LY294002 and sulindac synergistically inhibited cell invasion (Fig. 4B). Interestingly, myrAkt-induced invasion was not completely suppressed LY294002 (Fig. 4). This phenomenon is coincident with the recent report that in human pancreatic cancer cell, the increased invasion activity induced by myr-Akt was not suppressed by LY294002 [Suzuki et al., 2004]. These results suggest that invasion of glioblastoma cells is dependent on PI3K-Akt pathway and combination therapy of PI3K inhibitor and sulindac was effective in treatment of glioblastoma.

The invasiveness of glioma cells is, in part, determined by their endogenous proteolytic capacity, and especially reflects the activity of two MMPs that degrade gelatin: MMP-2 and MMP-9. These enzymes are present in greater quantities in more aggressive gliomas [Rao et al., 1993; Giese and Westphal, 1996; Forsyth et al., 1998]. Increased MMPs levels and activity are commonly encountered in GBMs, when these tumors are assessed by gelatin zymography [Forsyth et al., 1999]. Thus, modulating the behavior of these MMPs is expected to affect tumor phenotype and favorably influence outcome. In a recent study in which a competitive inhibitor of MMPs was used in an experimental model of glioma, such an effect was indeed observed, and such a strategy is currently being clinically evaluated [Price et al., 1999]. Our results showed that sulindac and its metabolites markedly suppressed the MMP-2 expression via Akt down-regulation. In has been reported that the MMP-2 promoter contains several *cis*-acting regulatory elements, including cAMP-responsive element binding protein, Sp1, Ets-1 and AP-2 [Qin et al., 1999]. Akt is known to up-regulate Ets-2 [Smith et al., 2000] and cAMP-responsive element binding protein [Du and Montminy, 1998]. In our study, MyrAkt transfected cells had increased MMP-2 promoter activity, whereas DN-Akt cells significantly

suppressed activity, and sulindac and its metabolites effectively suppressed the MMP-2 promoter activity both in MyrAkt- and DN-Akt-transfected cells. These results indicated that Akt activity was associated with MMP-2 gene expression and MMP-2 down-regulation by sulindac and its metabolites was mediated by down-regulation of Akt activity by these reagents.

It has been shown that sulindac and other NSAIDs reduce the overall risk to develop cancer and can inhibit the growth of tumors [Rao et al., 1995; Luk, 1996; Janne and Mayer, 2000]. NSAIDs inhibit the key enzymes of the eicosanoid metabolism, the COX-1 and -2, thereby decreasing the levels of proliferation-activating eicosanoids [Vane and Botting, 1996]. Although, the COX inhibitory effect of sulindac is mediated by its main physiological metabolite sulindac sulfide, sulindac, itself and its sulfone metabolite do not affect the COX pathway [Duggan et al., 1977]. Nevertheless, sulindac sulfone, which is ineffective on COX, can induce apoptosis of tumor cells and has been shown to inhibit tumor growth [Piazza et al., 1997a]. In addition, there have been several reports demonstrating that sulindac metabolites induce apoptosis of epithelial tissue as well as some cancer cell lines independent from the eicosanoid metabolism [Pasricha et al., 1995; Hanif et al., 1996; Piazza et al., 1997b]. Indeed, it has been shown that NSAIDs, including sulindac, modulate COX-independent signaling pathways such as Ras [Herrmann et al., 1998], NF-κB [Kopp and Ghosh, 1994], AP-1[Huang et al., 1997], and p38 kinase [Schwenger et al., 1998], and others. In this respect, the anti-invasiveness effect of sulindac and its metabolites might be a signaling pathway that is independent of COX enzyme activity.

Several NSAIDs and sulindac and its metabolites, especially Exisulind, sulindac sulfone have been extensively studied for their anti-tumor effects in various types of carcinoma cells [Thompson et al., 1995; Hanif et al., 1996; Soh et al., 2000]. However, to the test of our knowledge, the data presented in this paper is the first evidence that the agents in this broad category of compounds are active as anti-invasive agents of glioblastoma. Indeed, we found that sulindac and its two metabolites, sulindac sulfide and sulindac sulfone, inhibited the invasion of three glioblastoma cell lines, U87MG, U251MG, and U373MG. Our results suggested that these compounds, when used alone or in combination with other drugs, might be useful in the treatment of patients with glioblastomas. In addition, our findings that NSAIDs such as sulindac and its metabolites inhibit cancer cell invasion by down-regulating Akt pathway and MMP-2 activation explains why some NSAIDs have anti-cancer effect, and provide new targets for drug development.

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