Naproxen Induces Cell-Cycle Arrest and Apoptosis in Human Urinary Bladder Cancer Cell Lines and Chemically Induced Cancers by Targeting PI3K

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Abstract
Naproxen [(S)-6-methoxy-α-methyl-2-naphthaleneacetic acid] is a potent nonsteroidal anti-inflammatory drug that inhibits both COX-1 and COX-2 and is widely used as an over-the-counter medication. Naproxen exhibits analgesic, antipyretic, and anti-inflammatory activities. Naproxen, as well as other nonsteroidal anti-inflammatory drugs, has been reported to be effective in the prevention of urinary bladder cancer in rodents. However, potential targets other than the COX isozymes have not been reported. We examined potential additional targets in urinary bladder cancer cells and in rat bladder cancers. Computer kinase profiling results suggested that phosphoinositide 3-kinase (PI3K) is a potential target for naproxen. In vitro kinase assay data revealed that naproxen interacts with PI3K and inhibits its kinase activity. Pull-down binding assay data confirmed that PI3K directly binds with naproxen in vitro and ex vivo. Western blot data showed that naproxen decreased phosphorylation of Akt, and subsequently decreased Akt signaling in UM-UC-5 and UM-UC-14 urinary bladder cancer cells. Furthermore, naproxen suppressed anchorage-independent cell growth and decreased cell viability by targeting PI3K in both cell lines. Naproxen caused an accumulation of cells at the G1 phase mediated through cyclin-dependent kinase 4, cyclin D1, and p21. Moreover, naproxen induced significant apoptosis, accompanied with increased levels of cleaved caspase-3, caspase-7, and PARP in both cell types. Naproxen-induced cell death was mainly because of apoptosis in which a prominent down-regulation of Bcl-2 and upregulation of Bax were involved. Naproxen also caused apoptosis and inhibited Akt phosphorylation in rat urinary bladder cancers induced by N-butyl-N-(4-hydroxybutyl)-nitrosamine. Cancer Prev Res; 7(2): 236–45. ©2013 AACR.

Introduction
Because of the great cost and time involved in the development of new drugs, the use of drug repositioning is particularly appealing. Drug repositioning is defined as the development of existing drugs for new uses (1). Existing drugs on the market have already been tested for toxicity and safety and have favorable or validated pharmacokinetic properties for licensing from the Food and Drug Administration. Furthermore, development of a new drug usually requires 10 to 17 years, whereas drug repositioning can decrease the time needed to bring a drug to the patient. Therefore, drug repositioning could obviously save substantial money and time compared with new drug development (2).

Naproxen (Fig. 1A), a potent COX-1 and COX-2 inhibitor, is one of the best known and commonly used nonsteroidal anti-inflammatory drugs (NSAID). It was introduced in prescription form as "naprosyn" in 1976. Naproxen has been used to treat inflammation associated with conditions such as arthritis, ankylosing spondylitis, tendinitis, bursitis, or gout (3). COX-2 selective and nonselective NSAIDs have been linked to increases in the risk and number of serious cardiovascular events. However, naproxen reportedly has the least risk of adverse cardiovascular events compared with other NSAIDs based on a wide range of epidemiologic studies (4, 5). Although NSAIDs are regarded as cancer preventive agents, naproxen might have additional target molecules beyond its obvious COX inhibition, which might make it a more effective cancer preventive agent.

Previous studies have shown that naproxen could inhibit development of urinary bladder cancer in rodents (6, 7).
Furthermore, the NSAID, piroxicam, has been proven effective in reducing bladder cancer in dogs (8–10) and at least some epidemiologic studies in humans have shown significant preventive efficacy with NSAIDs (11). However, the precise mechanism and variety of potential direct target(s) of naproxen in urinary bladder cancer prevention have not been elucidated.

In this study, we used virtual screening to identify potential novel protein targets of naproxen and identified phosphoinositide 3-kinase (PI3K) as a novel target for naproxen. Naproxen showed anticancer effects against bladder cancer cells by inducing cell-cycle arrest and apoptosis. Naproxen also caused apoptosis and inhibited Akt phosphorylation in rat urinary bladder cancers induced by N-butyl-N-(4-hydroxybutyl)-nitrosamine (OH-BBN), a urinary bladder carcinogen.

**Materials and Methods**

**Reagents**

Naproxen was purchased from Cayman Chemical. Dulbecco’s Modified Eagle Medium and other supplements
were from Life Technologies, Inc. EAH Sepharose 4B beads were purchased from GE Healthcare Biosciences. The human recombinant PI3K (p110α/p85α) was from Millipore Corp. The antibodies against phosphorylated Akt (Ser473), total Akt, phosphorylated mTOR, total mTOR, phosphorylated p70S6K, total p70S6K, cyclin D1, cyclin A, cyclin-dependent kinase (CDK)4, CDK2, cleaved caspase-3 (Asp175), cleaved caspase-7 (Asp198), and cleaved PARP (Asp214) were purchased from Cell Signaling Biotechnology. Antibodies used to detect Bcl-2, Bax, and β-actin were from Santa Cruz Biotechnology. The protein assay kit was from Bio-Rad.

Virtual screening

The coordinates of naproxen were obtained from the crystal structure of 2VDB (12), which was downloaded from the Protein Data Bank (PDB). The cocrystallized ligand was selected as the query molecule structure for shape screening. This database is directly used for shape-similarity screening without any further modification. Shape similarity screening was performed using the phase module (13) in the Schrödinger Suite by comparing the volumetric similarity between naproxen and each compound from the PDB ligand database. During the screening process, polar hydrogens were included and only one alignment per ligand was recorded. In addition, the output structures were sorted by decreasing ShapeSim score and then a ranked list was created. Only the kinases with a ShapeSim score of at least 0.75 were selected (Table 1).

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Cell culture

UM-UC-5 (gefitinib sensitive) and UM-UC-14 (gefitinib resistant) human urinary bladder cancer cell lines were obtained from American Type Culture Collection. The cells were cultured at 37 °C with 5% CO2 in Dulbecco’s Modified Eagle Medium (DMEM) supplemented with 10% FBS, 2 mmol/L l-glutamine, and 100 units/ml penicillin. Cells were cytogenetically tested and authenticated before being frozen. Each vial of frozen cells were thawed and maintained for about 2 months (16 passages).

In vivo treatment with naproxen

OH-BBN was used to induce urinary bladder tumors as previously described (14). When a control OH-BBN-treated rat developed a palpable bladder tumor, the rat was treated with naproxen (400 mg/kg in diet) for 5 days. At the end of this time, animals were sacrificed and the bladder tumors were quickly removed and divided so that roughly half of each tumor was snap frozen in liquid nitrogen and the other half was fixed for immunohistochemistry or immunofluorescence. This presurgical approach to examining biomarkers has previously been used by us to examine the effects of gefitinib on these types of tumors (15).

In vitro PI3K kinase assay

The in vitro PI3K kinase assay was performed as described previously (16). Active PI3K (100 ng) was incubated with naproxen (0, 0.5, 1, or 2 mmol/L) or LY294002 (10 μmol/L) for 10 minutes at 30 °C. LY294002, a well-known PI3K inhibitor, was used as a positive control. The mixtures were incubated with 0.5 mg/mL phosphatidylinositol (MP Biomedicals) for 5 minutes at room temperature, followed by incubation with reaction buffer [10 mmol/L Tris-HCl (pH 7.6), 60 mmol/L MgCl2, and 0.25 mmol/L ATP containing 10 μCi [γ-32P] ATP] for an additional 10 minutes at 30 °C. The reaction was stopped by adding 15 μL of 4 N HCl and 130 μL of chloroform:methanol = 1:1. After mixing, the lower chloroform phase was spotted onto a silica gel plate (Merck KGaA). The resulting 32P-labeled phosphatidylinositol-3,4,5-trisphosphate (PIP3) was separated by thin layer chromatography with developing solvent (chloroform: methanol:NH4OH:H2O = 60:47:2:11.3) and then visualized by autoradiography.
**In vitro and ex vivo pull-down assays**

Naproxen-conjugated EAH (1,6-diaminohexane) Sepharose 4B beads were prepared following the instructions provided by GE Healthcare Biosciences. For in vitro or ex vivo pull-down assays, active PI3K (500 ng) or lysates from UM-UC-5 cells (500 μg) were mixed with 1 mL of naproxen-conjugated EAH Sepharose 4B beads in reaction buffer [50 mM Tris-HCl (pH 7.5), 5 mM EDTA, 150 mM NaCl, 1 mM dithiothreitol (DTT), 0.01% NP-40, 2 mg/mL bovine serum albumin, 0.02 mM phenylmethylsulfonylfluoride (PMSF), and protease inhibitor cocktail]. After incubation with gentle rocking at 4°C overnight, the beads were washed 5 times with washing buffer [50 mM Tris-HCl (pH 7.5), 5 mM EDTA, 150 mM NaCl, 1 mM DTT, 0.01% NP-40, and 0.02 mM PMSF]. The proteins bound to the beads were analyzed by Western blotting.

**Cell viability assay**

Cells were seeded (1 x 10^4 cells/well) in 96-well plates with 10% FBS/DMEM and incubated at 37°C in a 5% CO2 incubator for 24 hours and then treated with different doses (0, 0.5, 1, or 2 mM/L) of naproxen. After incubation, 20 μL of CellTiter96 Aqueous One Solution (Promega) were added to each well. Cells were then incubated for 1 hour at 37°C in a 5% CO2 incubator. Absorbance was measured at 490 and 690 nm.

**Anchorage-independent cell growth**

Cells (8 x 10^3 cells/well) suspended in complete growth medium (DMEM or Basal Medium Eagle [BME] supplemented with 10% FBS and 1% antibiotics) were added to 0.3% agar with different doses (0, 0.5, 1, or 2 mM/L) of naproxen in a top layer over a base layer of 0.6% agar with different doses (0, 0.5, 1, or 2 mM/L) of naproxen. The cultures were maintained at 37°C in a 5% CO2 incubator for 2 weeks and then colonies were counted under a microscope using the Image-Pro Plus software (v.6.2 program) (Media Cybernetics). All experiments were repeated 3 times.

**Cell-cycle assay**

Cells were seeded (2 x 10^5 cells/well) in 6-well plates with 10% FBS/DMEM and incubated overnight at 37°C in a 5% CO2 incubator. Cells were then incubated in serum-free medium for 24 hours followed by treatment for 48 hours with naproxen (0, 0.5, 1, and 2 mM/L) in 10% FBS/DMEM. The cells were trypsinized, then washed twice with cold PBS, and finally fixed with ice-cold 70% ethanol at 20°C overnight. Cells were then washed twice with PBS, incubated with 20 mg/mL RNase A and 200 mg/mL propidium iodide in PBS at room temperature for 30 minutes in the dark, and subjected to flow cytometry using the FACS-Calibur flow cytometer. Data were analyzed using ModFit LT (Verity Software House, Inc.).

**Apoptosis assay**

Annexin V and propidium iodide staining were used to visualize apoptotic cells in a similar procedure as described above but without prefixing with 70% ethanol. Cells were stained using the Annexin V-FITC Apoptosis Detection Kit (MBL International Corporation) and propidium iodide according to the manufacturer's instructions. Cells were analyzed by 2-color flow cytometry. The emission fluorescence of the Annexin V conjugate was detected and recorded through a 530/30 bandpass filter in the FL1 detector. Propidium iodide was detected in the FL2 detector through a 585/42 bandpass filter. Apoptotic cells were only those located in the bottom right quadrant that stained positive for Annexin V and negative for propidium iodide.

**Western blot analysis**

Cell lysates were prepared with radioimmunoprecipitation assay buffer [20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton, 2.5 mM sodium pyrophosphate, 1 mM β-glycerocephosphate, 1 mM Na3VO4, 1 μg/mL leupeptin]. Equal amounts of protein were determined using the RC DC protein assay (Bio-Rad). Proteins were separated by SDS-PAGE and electrophoretically transferred to polyvinylidene difluoride membranes (GE Healthcare Biosciences). Membranes were blocked with 5% nonfat dry milk for 1 hour at room temperature and incubated with appropriate primary antibodies overnight at 4°C. After washing with PBS containing 0.1% Tween 20, the membrane was incubated with a horseradish peroxidase–conjugated secondary antibody at a 1:5,000 dilution and the signal was detected with a chemiluminescence reagent (GE Healthcare Biosciences). Band density was determined using the Image J (NIH) software program.

**Terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling assay**

Urinary bladder cancer tissues from female Fischer-344 rats, induced with the carcinogen 4-hydroxybutynitrrosamin (OH-BBN) and treated with 400 mg/kg naproxen for 5 days, as well as untreated controls, were prepared for the terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling (TUNEL) assay and immunofluorescence microscopy. Apoptosis was determined by using the Dead-End Colorimetric TUNEL System (Promega) according to the manufacturer's instructions. Briefly, after deparaffinization and rehydration, the tissue sections were pretreated with 20 μg/mL proteinase K solution for 10 minutes at room temperature. Thereafter, slides were rinsed in PBS and incubated with TUNEL reaction mixture for 1 hour at 37°C in a humidified chamber. Slides were then washed with PBS followed by a stop solution for 10 minutes at room temperature. The slides were counterstained with 4,6-diamidino-2-phenylindole (DAPI) under glass coverslips. The stained tissue was examined at ×200 magnification using a Nikon Eclipse TE2000-E Confocal microscope.

**Immunofluorescence microscopy**

Slides were baked at 60°C for 2 hours, deparaffinized with xylene, and rehydrated through a graded alcohol bath. Of note, 10 mM sodium citrate buffer (pH 6.0) was used...
Statistical analysis

All quantitative results are expressed as mean ± SD. Statistically significant differences were obtained using the Student t test or by one-way ANOVA. A P < 0.05 value was considered to be statistically significant.

Results

Virtual screening for novel targets of naproxen

Shape similarity screening was used to search for the potential kinase targets of naproxen. The screening was performed using the phase module of Schrödinger-Maestro 9.2 to compare the volumetric similarity between naproxen and each compound from the PDB ligand database (12). Eight different potential kinase targets (column IV) for naproxen were identified from shape screening (Table 1). The ligands (column I) from the crystal structures (column III) of these kinase targets have shape similarity scores of at least 0.75 (column II) when compared with naproxen.

Naproxen inhibits PI3K activity directly

We examined whether naproxen had an effect on the activity of several kinases that were identified by our virtual screening. We selected 5 kinases (PI3K, JNK1, Akt1, Akt2, and Src) based on their importance in cancer development and commercial availability. Naproxen only dose dependently suppressed the kinase activity of PI3K (Fig. 1B and Supplementary Fig. S1). Therefore, PI3K is the most likely target for naproxen. Next, we investigated whether naproxen interacted directly with PI3K. The direct binding of naproxen and PI3K was demonstrated by an in vitro pull-down assay (Fig. 1C). In addition, we observed ex vivo binding between naproxen and PI3K in UM-UC-5 cell lysates (Fig. 1D). To elucidate the potential binding site, we conducted an in silico docking study using the induced fit docking module from Schrödinger. The hierarchical docking algorithm glide of Schrödinger-Maestro 9.2 (13) was then used to assess the possible binding orientations of naproxen and PI3K. The simulation returned a positive result, with the theoretical naproxen/PI3K complex shown in Fig. 1E and F. These data support PI3K as a potential target of naproxen.

Naproxen attenuates downstream PI3K signaling

EGFR is known to be involved in bladder carcinogenesis and COX-2 and Akt, among others, are all potential down-stream targets of EGFR and seem to be important molecular targets of naproxen and other NSAIAs (17,18). In this study, we used 2 cell lines with differing sensitivity to the EGFR inhibitor, gefitinib. Cell lines included UM-UC-5 (gefitinib sensitive) and UM-UC-14 (gefitinib resistant). To elucidate the mechanism underlying the inhibitory effects of naproxen, we examined the effects of naproxen on the most well-known downstream molecules of PI3K, including Akt, mTOR, and p70S6K. Importantly, exposure of UM-UC-5 (Fig. 2A) and UM-UC-14 (Fig. 2B) bladder cancer cells to naproxen decreased the phosphorylation of Akt, mTOR, and p70S6K in both cell lines.

Naproxen decreases viability and inhibits anchorage-independent growth of UM-UC-5 and UM-UC-14 urinary bladder cancer cells

Next, we determined whether naproxen could affect viability and anchorage-independent growth of UM-UC-5 and UM-UC-14 bladder cancer cells. Naproxen was found to significantly decrease viability of both UM-UC-5 (Fig. 3A,
left) and UM-UC-14 (Fig. 3A, right) cells. We also measured anchorage-independent cell growth using a soft agar assay and naproxen inhibited growth of UM-UC-5 (Fig. 3B) and UM-UC-14 (Fig. 3C) cell in soft agar.

Naproxen induces G₀–G₁ cell-cycle arrest in UM-UC-5 and UM-UC-14 urinary bladder cancer cells

To elucidate the mechanism of naproxen’s inhibitory effect on cell growth, we used flow cytometry analysis after propidium iodide staining. Previous reports show that the PI3K inhibitor, LY294002, induced G₀–G₁ phase arrest in many types of cancer cells (19–21). Naproxen also increased the percentage of cells in G₀–G₁ in both UM-UC-5 (Fig. 4A) and UM-UC-14 (Fig. 4B) cells after 48 hours treatment and a corresponding decrease was shown in S phase. LY294002 treatment was reported to arrest the cell cycle at G₀–G₁ through G₁-associated proteins including p21<sup>cip</sup>, CDK4, and cyclin D1 (19). Treatment with naproxen for 48 hours also increased p21<sup>cip</sup> and decreased CDK4 and cyclin D1 expression in both UM-UC-5 (Fig. 4C) and UM-UC-14 (Fig. 4D) cell lines.

Naproxen induces apoptosis in UM-UC-5 and UM-UC-14 urinary bladder cancer cells

The PI3K signaling pathway delivers an antiapoptotic signal (22) and inhibition of PI3K activity induces apoptosis (23, 24). We examined the effect of naproxen on apoptosis in UM-UC-5 and UM-UC-14 bladder cancer cells using Annexin V/propidium iodide double staining. Annexin V only stains cells representative of early apoptosis, which was increased in naproxen-treated UM-UC-5 (Fig. 5A) and UM-UC-14 (Fig. 5B) cells. We then studied the effect of naproxen on effectors of the cell death pathways. Naproxen induced PARP, caspase-3, and caspase-7 cleavage.
The ratio of proapoptotic factor Bax and prosurvival factor Bcl-2, which is important for apoptosis, and both proteins associated with the PI3K pathway were also examined. We found that naproxen markedly increased the Bax/Bcl-2 ratio in both UM-UC-5 (3.7/0.5–5/0; Fig. 5C) and UM-UC-14 (1.8/0.7–1.5/0.2; Fig. 5D) cells.

Naproxen induced apoptosis and inhibited Akt phosphorylation in OH-BBN–induced bladder cancer

Our previous study indicated that naproxen inhibits OH-BBN–induced bladder cancer in rats (6). Naproxen was effective in decreasing the incidence of large tumors by greater than 85% at sacrifice (weight of bladder plus lesions).
greater than 200 mg) even when treatment was initiated when microcarcinomas were already present (6). Using bladder tissue from short-term treatment with naproxen (Materials and Methods), we examined apoptosis by TUNEL and DAPI staining and Akt phosphorylation. Apoptosis was significantly increased by naproxen treatment in animal tissue samples (Fig. 6A and B). Furthermore, Akt phosphorylation (Ser473) was decreased in naproxen-treated versus untreated urinary bladder tumors (Fig. 6C and D).

Discussion

More than 70,000 new cases of urinary bladder cancer were diagnosed in the United States in 2010, with 14,680 deaths (25). Furthermore, bladder cancer treatment is the most expensive per patient compared with all other cancers. More than 90% of bladder cancers cases are urothelial carcinomas. A number of groups have reported that various NSAIDs and celecoxib are striking inhibitors of chemically induced urinary bladder cancer in rodent animal models (7, 26, 26). Furthermore, the NSAID piroxicam has proven to be effective in canine bladder cancer (8–10). Finally a number of epidemiologic studies imply the efficacy of NSAIDs in reducing human bladder cancer (11). However, in much of the epidemiologic literature, this response has appeared variable (25, 28–30).

The most serious side effects associated with most NSAIDs include increased adverse cardiovascular events and gastrointestinal ulceration (4, 5). Naproxen is a well-known NSAID that is easily purchased over-the-counter and has lower cardiovascular toxicities than any of the other NSAIDs and is in fact recommended for persons with arthritis who have a higher cardiovascular risk. Similar to most other NSAIDs and higher doses of aspirin, naproxen can cause gastric toxicity and bleeding. However, this effect can apparently be ameliorated without losing efficacy either by combining it with omeprazole or altering the dosing schedule (R.A. Lubet and C.J. Grubbs; unpublished data).

Naproxen seems to be a good agent for drug repositioning and might be a more useful drug if other applications were found. To determine other applications, exploring the molecular mechanism and direct molecular target(s) of naproxen is critical. Several previous studies have shown that naproxen has anti-urinary bladder cancer effects in vivo (6, 7). In this study, we identified PI3K as an additional potential molecular target for naproxen and elucidated its mechanism of action through this protein target. The PI3K signaling pathway plays a key role in cancer cell growth, survival, motility, and metabolism (31) and is activated in a
Naproxen could bind well at the ATP binding pocket of PI3K and inhibits its activity resulting in suppression of Akt and p70S6K signaling downstream of PI3K in vitro and in vivo (Fig. 7). To investigate the molecular basis of PI3K inhibition by naproxen, we performed a docking study using a homology model structure of PI3K. Several important hydrogen bonds were formed between naproxen and PI3K, including Val882 at the backbone and Lys833 at the side chain of PI3K. Naproxen also formed π-π stacking with Tyr867 and hydrophobic interactions with Met804, Pro810, Thr812, Ile831, Ile879, Ala885, Ile881, Met953, Phe961, and Ile963. This suggests that naproxen could be a potential inhibitor against PI3K.

In summary, we have shown that naproxen decreases viability and inhibits anchorage-independent growth of urinary bladder cancer cells by binding and suppressing PI3K activity and downstream signaling. Inhibition of these signaling pathways resulted in cell-cycle arrest and apoptosis. These studies demonstrate that naproxen can interact with PI3K and that this interaction can contribute to the efficacy of naproxen and may offer some additional useful biomarkers.

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

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Analysis and interpretation of data (e.g., statistical analysis, bioinformatics, computational analysis): M.-S. Kim, D.Y. Lim, Z. Huang, H. Chen, A. Langfald, Z. Dong, A.M. Bode
Writing, review, and/or revision of the manuscript: M.-S. Kim, J.-E. Kim, Z. Huang, H. Chen, A. Langfald, R.A. Lubet, A.M. Bode
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): Z. Dong
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