Naproxen induces cell cycle arrest and apoptosis in human urinary bladder cancer cell lines and chemically induced cancers by targeting PI3-K

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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, anti-pyretic, and anti-inflammatory activities. Naproxen, as well as other NSAIDS, 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 phosphatidylinositol 3-kinase (PI3-K) is a potential target for naproxen. In vitro kinase assay data revealed that naproxen interacts with PI3-K and inhibits its kinase activity. Pull-down binding assay data confirmed that PI3-K 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 UMUC-14 urinary bladder cancer cells. Furthermore, naproxen suppressed anchorage-independent cell growth and decreased cell viability by targeting PI3-K in both cell lines. Naproxen caused an accumulation of cells at the G1 phase mediated through CDK4, cyclin D1 and p21. Moreover, naproxen induced significant apoptosis, accompanied with increased levels of cleaved caspase 3, caspase 7, and poly (ADP-ribose) polymerase (PARP) in both cell types. Naproxen-induced cell death was mainly due to apoptosis in which a prominent down-regulation of Bcl-2 and up-regulation 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 (OH-BBN).
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 to new drug development (2).

Naproxen (Fig. 1A), a potent COX-1 and COX-2 inhibitor, is one of the best known and commonly used non-steroidal anti-inflammatory drugs (NSAIDs). 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 to other NSAIDs based on a wide range of epidemiological 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 epidemiological 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 phosphatidylinositol 3-kinase (PI3-K) 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 (Ann Arbor, MI). Dulbecco’s modified Eagle’s medium and other supplements were from Life Technologies, Inc. (Carlsbad, CA). EAH-Sepharose 4B beads were purchased from GE Healthcare Biosciences (Pittsburgh, PA). The human recombinant PI3-kinase (p110α/p85α) was from Millipore Corp (Billerica, MA). The antibodies against phosphorylated Akt (Ser473), total Akt, phosphorylated mTOR, total mTOR, phosphorylated p70S6K, total p70S6K, cyclin D1, cyclin A, CDK4, CDK2, cleaved caspase 3 (Asp175), cleaved caspase 7 (Asp198), and cleaved PARP (Asp214) were purchased from Cell Signaling Biotechnology (Beverly, MA). Antibodies used to detect Bcl-2, Bax and β-actin were from Santa Cruz Biotechnology (Santa Cruz, CA). The protein assay kit was from Bio-Rad (Hercules, CA).

**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 co-crystallized
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 I).

**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 (Manassas, VA). The cells were cultured at 37°C with 5% CO₂ in DMEM supplemented with 10% FBS, 2 mM 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 two 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 ppm 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 pre-surgical approach to examining biomarkers has previously been used by us to examine the effects of gefitinib on these types of tumors (15).
**In vitro PI3-K kinase assay.** The *in vitro* PI3-K kinase assay was performed as described previously (16). Active PI3-K (100 ng) was incubated with naproxen (0, 0.5, 1, or 2 mM) or LY294002 (10 μM) for 10 min at 30°C. LY 294002, a well-known PI3-K inhibitor, was used as a positive control. The mixtures were incubated with 0.5 mg/mL phosphatidyl-inositol (MP Biomedicals, Santa Ana, CA) for 5 min at room temperature, followed by incubation with reaction buffer [10 mM Tris-HCl (pH 7.6), 60 mM MgCl2, and 0.25 mM ATP containing 10 μCi \[^{32}\text{P}\] ATP] for an additional 10 min 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, Darmstadt, Germany). The resulting \(^{32}\text{P}\)-labeled phosphatidylinositol-3-phosphate (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 PI3-K (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 (1×10^3 cells/well) in 96-well plates with 10% FBS/DMEM and incubated at 37°C in a 5% CO₂ incubator for 24 h and then treated with different doses (0, 0.5, 1 or 2 mM) of naproxen. After incubation, 20 μl of CellTiter96 Aqueous One Solution (Promega, Madison, WI) were added to each well. Cells were then incubated for 1 h at 37°C in a 5% CO₂ incubator. Absorbance was measured at 490 and 690 nm.

**Anchorage-independent cell growth.** Cells (8×10^3 cells/well) suspended in complete growth medium (DMEM or BME supplemented with 10% FBS and 1% antibiotics) were added to 0.3% agar with different doses (0, 0.5, 1 or 2 mM) of naproxen in a top layer over a base layer of 0.6% agar with different doses (0, 0.5, 1 or 2 mM) 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 (2×10^5 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 h followed by treatment for 48 h with naproxen (0, 0.5, 1, 2 mM) 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
min in the dark, and subjected to flow cytometry using the FACSCalibur flow cytometer. Data were analyzed using ModFit LT (Verity Software House, Inc., Topsham, ME).

**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, Watertown, MA) and propidium iodide according to the manufacturer’s instructions. Cells were analyzed by two-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 RIPA buffer (20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM Na2EDTA, 1 mM EGTA, 1% Triton, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 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 h 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.

**TUNEL (TdT-mediated dUTP Nick-End Labeling) assay.** Urinary bladder cancer tissues from female Fischer-344 rats, induced with the carcinogen 4-hydroxybutynitrosamin (OH-BBN) and treated with 400 mg/kg naproxen for 5 days, as well as untreated controls, were prepared for the TUNEL assay and immunofluorescence microscopy. Apoptosis was determined by using the DeadEnd Colorimetric TUNEL System (Promega, Madison, WI) 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 min at room temperature. Thereafter, slides were rinsed in PBS and incubated with TUNEL reaction mixture for 1 h at 37°C in a humidified chamber. Slides were then washed with PBS followed by a stop solution for 10 min at room temperature. The slides were counterstained with DAPI under glass coverslips. The stained tissue was examined at 200x magnification using a Nikon Eclipse TE2000-E Confocal microscope.

**Immunofluorescence microscopy.** Slides were baked at 60°C for 2 h, deparaffinized with xylene and rehydrated through a graded alcohol bath. 10 mM sodium citrate buffer (pH 6.0) was used for antigen retrieval by heating slides and buffer for 10 min in the microwave followed by 20 min of ambient cooling. Slides were washed with DI water followed by 1xPBS. For immunofluorescence, specimens were blocked in 5% donkey serum-1xPBS-0.3% Triton (PBST) buffer for ~1 h. The primary antibody was prepared according to the manufacturer’s instructions and left on overnight at 4°C. Slides were washed in 1xPBS followed by 2 h of appropriate
secondary antibody incubation prepared according to the manufacturer’s instructions. Slides were washed with 1xPBS followed by high salt PBS (23.38 g NaCl in 1 Liter 1XPBS). Fluoro-Gel II with Dapi (Electron Microscopy Sciences, #17985-50) was used to mount glass coverslips and then sealed using clear nail polish. All stained tissues were examined at 200x magnification using a Nikon Eclipse TE2000-E Confocal microscope.

**Statistical analysis.** All quantitative results are expressed as mean values ± S.D. Statistically significant differences were obtained using the Student’s *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 v9.2 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 to naproxen.

**Naproxen inhibits phosphatidylinositol 3-kinase (PI3-K) 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 (PI3-K, JNK1, Akt1, Akt2 and Src) based on their importance in cancer development and commercial availability. Naproxen only dose-dependently
suppressed the kinase activity of PI3-K (Fig. 1B, Supplementary Fig. 1). Therefore, PI3-K is the most likely target for naproxen. Next, we investigated whether naproxen interacted directly with PI3-K. The direct binding of naproxen and PI3-K was demonstrated by an *in vitro* pull-down assay (Fig. 1C). Additionally, we observed *ex vivo* binding between naproxen and PI3-K 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 (IFD) module from Schrödinger. The hierarchical docking algorithm Glide of Schrödinger-Maestro v9.2 (13) was then used to assess the possible binding orientations of naproxen and PI3-K. The simulation returned a positive result, with the theoretical naproxen/PI3-K complex shown in Fig. 1E, F. These data support PI3-K as a potential target of naproxen.

**Naproxen attenuates downstream PI3-K signaling.** EGFR is known to be involved in bladder carcinogenesis and COX-2 and Akt, among others, are all potential downstream targets of EGFR and appear to be important molecular targets of naproxen and other NSAIDs (17-19). In this study, we used two 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 PI3-K, 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 PI staining. Previous reports show that the PI3-K inhibitor, LY294002, induced G0/G1 phase arrest in many types of cancer cells (20-22). 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 h 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 p21cip, CDK4 and cyclin D1 (20). Treatment with naproxen for 48 h also increased p21cip and decreased CDK4 and cyclin D1 expression in both UM-UC-5 (Fig. 4C) and UMUC-14 (Fig. 4D) cell lines.

**Naproxen induces apoptosis in UM-UC-5 and UM-UC-14 urinary bladder cancer cells.** The PI3-K signaling pathway delivers an anti-apoptotic signal (23) and inhibition of PI3-K activity induces apoptosis (24, 25). We examined the effect of naproxen on apoptosis in UM-UC-5 and UM-UC-14 bladder cancer cells using Annexin V/PI double staining. Annexin V only stains cells representative of early apoptosis, which was increased in naproxen-treated UMUC-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 pro-apoptotic factor, Bax and pro-survival factor, Bcl-2 is important for apoptosis and both of two proteins are associated with the PI3-K 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, B). Furthermore, Akt phosphorylation (Ser473) was decreased in naproxen-treated vs. untreated urinary bladder tumors (Fig. 6 C, D).

**Discussion**

Over 70,000 new cases of urinary bladder cancer were diagnosed in the United States in 2010, with 14,680 deaths (26). Furthermore, bladder cancer treatment is the most expensive per patient compared to all other cancers. More than 90% of bladder cancers cases are urothelial carcinomas (UC). 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, 27, 28). Furthermore, the NSAID piroxicam has proven to be effective in canine bladder cancer (8-
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 (26, 29-31).

The most serious side effects associated with most NSAIDs include increased adverse cardiovascular events and gastrointestinal (GI) 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 (Lubet, Grubbs, unpublished data).

Naproxen appears to be a good agent for drug repositioning and might be a more useful drug if other applications were found. In order 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 PI3-K as an additional potential molecular target for naproxen and also elucidated its mechanism of action through this protein target. The PI3-K signaling pathway plays a key role in cancer cell growth, survival, motility, and metabolism (32) and is activated in a variety of different human cancers. PI3-K belongs to a family of lipid kinases that phosphorylate the 3'-hydroxy position of the inositol ring of phosphatidylinositides, yielding products of which the best characterized is phosphatidylinositol-3,4,5-trisphosphate (PIP3), the second messenger that recruits Akt to the cell membrane and activates Akt. Akt activation is associated with various downstream signals, which are involved in carcinogenesis (33).
Inhibitors of this pathway are under active development as anticancer agents (34). We tested the inhibitory effects of naproxen on five kinases, including PI3-K, JNK1, Akt1, Akt2 and Src in the target list of naproxen identified by our virtual screening. In this study, we first showed that naproxen binds with PI3-K and inhibits its activity resulting in suppression of Akt and p70S6K signaling downstream of PI3-K in vitro and in vivo (Fig. 7). To investigate the molecular basis of PI3-K inhibition by naproxen, we performed a docking study using a homology model structure of PI3-K. Naproxen could bind well at the ATP binding pocket of PI3-K. Several important hydrogen bonds were formed between naproxen and PI3-K, including Val882 at the backbone and Lys833 at the side chain of PI3-K. Naproxen also formed π-π stacking with Tyr867 and hydrophobic interactions with Met804, Pro810, Trp812, Ile831, Ile879, Ala885, Ile881, Met953, Phe961, and Ile963. This suggests that naproxen could be a potential inhibitor against PI3-K. These results add a new perspective to our understanding of the parameters involved in PI3-K inhibition by naproxen. Further studies using X-ray crystallography or nuclear magnetic resonance techniques are needed to confirm the exact binding mode of naproxen to PI3-K. Other targets identified by virtual screening such as urokinase, a serine protease, and PKA-PKB chimera are interesting potential targets and suggest a number of possible additional mechanism of action (35). These and other potential protein targets will be the subject of further studies.

In summary, we have shown that naproxen decreases viability and inhibits anchorage-independent growth of urinary bladder cancer cells by binding and suppressing PI3-K activity and downstream signaling. Inhibition of these signaling pathways resulted in cell cycle arrest and apoptosis. These studies demonstrate that naproxen can interact with PI3-K and that this interaction can contribute to the efficacy of naproxen and may offer some additional useful biomarkers.
References


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Figure legends

**Figure 1.** Naproxen decreases PI3-K kinase activity. (A) Chemical structure of naproxen [(2S)-2-(6-methoxynaphthalen-2-yl) propanoic acid]. (B) Naproxen inhibits PI3-K activity *in vitro*. Active PI3-K (100 ng) was mixed with naproxen (0, 0.5, 1, or 2 mM) or LY294002 (i.e., a PI3-K inhibitor, 10 µM) and then incubated with a [γ-32P] ATP mixture. The 32P-labeled PIP3 was separated by thin-layer chromatography and then visualized by autoradiography. All data are shown as means ± S.D. of values from 3 independent experiments. Band density was measured using the Image J (NIH) software program. The asterisk (*) indicates a significant difference between PI3-K kinase activity as determined by *t* test (*p* < 0.05). (C) Naproxen binds to PI3-K *in vitro*. Active PI3-K (500 ng) was incubated with naproxen-conjugated EAH Sepharose 4B beads or EAH-Sepharose 4B beads alone, and the pulled down proteins were analyzed by Western blot. (D) Naproxen binds to PI3-K *ex vivo*. Cell lysates from UM-UC-5 bladder cancer cells (500 µg) were incubated with naproxen-conjugated EAH Sepharose 4B beads or EAH-Sepharose 4B beads alone, and the pulled down proteins were analyzed by Western blot. Data are representative of 3 independent experiments that gave similar results. (E) Docking model of naproxen and the PI3-K protein structure. Naproxen is shown in sphere representation and carbons are colored white. PI3-K is shown as a cartoon model. (F) Binding site of PI3-K with naproxen. The ATP-binding site of PI3-K is shown in surface representation.

**Figure 2.** Naproxen suppresses phosphorylation of the PI3-K/Akt signaling pathway. (A) Cells were treated with naproxen (0, 0.5, 1, or 2 mM) in medium containing 10% FBS and analyzed by Western blot. Naproxen inhibits phosphorylation of Akt, mTOR and p70S6K in
UM-UC-5 (A) and UM-UC-14 (B) cells. The bands were quantified by densitometric analysis using Image J software and normalized using their nonphospho-band.

**Figure 3.** Naproxen exerts anticancer activity against urinary bladder cancer cells. (A) Naproxen inhibits bladder cancer cell growth in a dose-dependent manner. UM-UC-5 (left) or UM-UC-14 (right) cells (1 × 10³ cells/well) were seeded in 96-well plates and treated with various concentrations (0, 0.5, 1 or 2 mM) of naproxen and proliferation was measured by MTS assay. Data are shown as means ± S.D. (N = 5) and the asterisk (*) indicates a significant (p < 0.05) difference in naproxen-treated cells compared to untreated control cells. Naproxen inhibits anchorage-independent cancer cell growth. UM-UC-5 (B) or UM-UC-14 (C) cells were incubated in 0.3% agar for 10 days with various concentrations (0, 0.5, 1 or 2 mM) of naproxen. Colonies were counted using a microscope and the Image-Pro PLUS (v.6.1) computer software program. Representative photographs are shown in the left panels and data in the right panels are represented as means ± S.D. of values from triplicates and similar results were obtained from 2 independent experiments. The asterisk (*) indicates a significant (p < 0.05) decrease in colony formation induced by naproxen compared to untreated control.

**Figure 4.** Naproxen induces G1 arrest in UM-UC-5 (A) and UM-UC-14 (B) urinary bladder cancer cells. Cells were starved in serum-free medium for 24 h and then treated with naproxen (0, 0.5, 1, or 2 mM) for 48 h. Cell cycle analysis was performed using flow cytometry. Data are shown as mean percentages ± S.D. (N = 3) and the asterisk (*) indicates a significant (p < 0.05) difference in naproxen-treated cells compared to untreated control cells. Naproxen regulates the
CDK4/cyclin D1 signaling pathway in UM-UC-5 (C) and UM-UC-14 (D) cells. Cells were treated with naproxen (0, 0.5, 1, or 2 mM) in 10% FBS/DMEM. The levels of E2F, cyclin D1, cyclin A, Cdk2, Cdk4 and p21cip proteins were determined by Western blot analysis. The bands were quantified by densitometric analysis using Image J software and normalized against β-actin.

Figure 5. Naproxen induces apoptosis in UM-UC-5 (A) and UM-UC-14 (B) cells. Cells were starved in serum-free medium for 24 h and then treated with naproxen (0, 0.5, 1, or 2 mM) for 72 h. Apoptosis was measured by flow cytometry. Data are shown as means ± S.D. (N = 3) and the asterisk (*) indicates a significant (p < 0.05) difference in naproxen-treated cells compared to untreated control cells. The levels of cleaved caspase 3, caspase 7, PARP and expression levels of p53, Bcl-2, and Bax were determined in UM-UC-5 (C) and UM-UC-14 (D) cells by Western blot analysis. The bands were quantified by densitometric analysis using Image J software and normalized using β-actin.

Figure 6. Naproxen induces apoptosis and decreases phosphorylation of Akt in mouse bladder cancer tissues. Bladder cancer was induced in female Fischer-344 rats with the carcinogen 4-hydroxybutylnitrosamin (OH-BBN) and then rats were treated or not treated with 400 mg/kg of naproxen for 5 days. (A, B) Naproxen induces apoptosis in vivo. The TUNEL assay was conducted following the manufacturer’s protocol to detect apoptosis in mouse bladder cancer tissues. (C, D) Naproxen inhibits Akt phosphorylation in vivo. Fixed tumor samples from rats in A were subjected to immunofluorescence staining to detect phosphorylated Akt (Ser473) by incubating with specific primary antibodies and secondary antibodies conjugated to a

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fluorescence dye. Samples were observed by confocal microscopy. For A and C, representative images are shown from a minimum of 6 tumors with similar results. For B and D, data are shown as means ± S.D. and the asterisks (*) and **) indicate a significant ($p < 0.05$, $p < 0.01$, respectively) difference in naproxen-treated bladder cancer tissues compared to untreated bladder cancer tissues.

**Figure 7.** A scheme for illustrating the effect of Naproxen on the regulation of cell cycle and apoptosis mediated through the inhibition of the PI3-K signaling pathway.
## Table 1. Potential kinase target of Naproxen

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<td>GVN</td>
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<td>2uw5</td>
<td>PKA-PKB CHIMERA(PKB)</td>
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<td>2uvy</td>
<td>PKA-PKB CHIMERA(PKB)</td>
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<td>c-Src kinase domain</td>
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<td>0.754</td>
<td>3g0e</td>
<td>KIT kinase domain</td>
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Figure 1

A. Chemical structure of Naproxen.

B. Graph showing kinase activity as a function of Naproxen concentration.

C. Western blot showing p110 activation in different conditions.

D. Western blot showing p110 expression in cell lysates.

E. 3D model of a protein structure.

F. 3D model highlighting Lys833 and Val882 residues.
Figure 2

A

Naproxen (mM) - 0.5 1 2

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<tr>
<th>Protein</th>
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<th>0.4</th>
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<tr>
<td>pmTOR</td>
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<tr>
<td>β-actin</td>
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B

Naproxen (mM) - 0.5 1 2

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<td>p70 S6K</td>
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<tr>
<td>β-actin</td>
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</tbody>
</table>
Figure 3

A

Cell viability (% of control)

Naproxen (mM)  
-  0.5  1  2

UM-UC-5

UM-UC-14

B

Colony No./8000 cells

Naproxen (mM)  
-  0.5  1  2

a  b  c  d

C

Colony No./8000 cells

Naproxen (mM)  
-  0.5  1  2

a  b  c  d

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Figure 4

(A) and (B) Bar graphs showing the percentage of cells in different phases of the cell cycle for UM-UC-5 and UM-UC-14 cells treated with varying concentrations of naproxen. (C) and (D) Western blot analysis of cell cycle regulators and cyclins for UM-UC-5 and UM-UC-14 cells treated with different concentrations of naproxen.
Naproxen → PI3K → Akt → CASPASE-3, mTOR, p21 → CASPASE-7, p70S6K → Bcl_2, Bax → APOPTOSIS
CDK4, cyclin D1 → CELL CYCLE
Naproxen induces cell cycle arrest and apoptosis in human urinary bladder cancer cell lines and chemically induced cancers by targeting PI3-K

Mi-Sung Kim, Jong-Eun Kim, Do Young Lim, et al.


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