

NSAID Inhibition of Prostate Cancer Cell Migration Is Mediated by Nag-1 Induction via the p38 MAPK-p75^{NTR} Pathway

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Abstract

The nonsteroidal anti-inflammatory drugs (NSAID) *R*-flurbiprofen and ibuprofen have been shown to induce expression of p75^{NTR} (neurotrophin receptor) in prostate cancer cell lines. p75^{NTR}, a tumor necrosis factor receptor superfamily member, is a proapoptotic protein that functions as a tumor suppressor in the human prostate. Expression of p75^{NTR} is lost as prostate cancer progresses and is minimal in several metastatic prostate cancer cell lines. NSAIDs induce p75^{NTR} through activation of the p38 mitogen-activated protein kinase (MAPK) pathway, with a concomitant decrease in cell survival. Here, we show that treatment with *R*-flurbiprofen and ibuprofen induces expression of the NSAID-activated gene-1 (Nag-1) protein, a divergent member of the TGF beta (TGF- β) family, in PC-3 cells. Using the selective pharmacologic inhibitor of p38 MAPK, SB202190, and p38 MAPK-specific siRNA (small interfering RNA), we show that Nag-1 induction following NSAID treatment is mediated by the p38 MAPK pathway. p75^{NTR}-specific siRNA pretreatment shows that Nag-1 induction by NSAIDs is downstream of p75^{NTR} induction. Decreased survival of NSAID-treated cells is rescued by p75^{NTR}-specific siRNA but not by Nag-1 siRNA. Transwell chamber and *in vitro* wound healing assays demonstrate decreased cell migration upon NSAID treatment. Pretreatment of PC-3 cells with p75^{NTR} and Nag-1-specific siRNA shows that NSAID inhibition of cell migration is mediated by Nag-1 and p75^{NTR}. These results demonstrate a role for Nag-1 in NSAID inhibition of cell migration, but not survival. *Mol Cancer Res*; 8(12); 1656–64. ©2010 AACR.

Introduction

p75^{NTR} (neurotrophin receptor) is a member of the TNF receptor (TNFR) superfamily, capable of inducing apoptosis (1, 2). It differs from other TNFR superfamily members in its ability to induce apoptosis in a ligand-independent manner (3). Normal prostate epithelial cells express high levels of p75^{NTR}, with a loss of expression as prostate cancer progresses (4). The expression of this protein is very low in metastatic prostate cancer cell lines PC-3, DU-145, and LNCaP (5). Exogenous reexpression of p75^{NTR} in PC-3 cells led to a decreased tumor formation in SCID mice (6). In addition, reexpression of p75^{NTR} caused decreased proliferation and increased apoptosis in prostate cancer cells, showing that p75^{NTR} acts as a tumor suppressor in the prostate (7, 8). Recent studies demonstrated that p75^{NTR} is induced by nonsteroidal anti-inflammatory drugs (NSAID) via sustained activation of the p38 MAPK pathway, leading

to a p75^{NTR}-mediated increase in apoptosis (7, 9, 10). NSAID-activated gene-1 (Nag-1) is a novel divergent member of the human TGF- β (TGF- β) superfamily (11). It was reported by several groups and given the names placental transforming growth factor beta (PTGF- β), placental bone morphogenetic protein (PLAB), growth differentiating factor 15 (GDF-15), prostate-derived factor (PDF), and macrophage inhibitory cytokine 1 (MIC-1; refs. 12–16). Nag-1 mRNA is highly expressed in human prostate epithelium suggesting a role for Nag-1 in prostate homeostasis (17). Nag-1 has been reported to exhibit both antitumorigenic and proapoptotic functions in several cancer cells including prostate cancer cells (17, 18).

NSAIDs inhibit COX activity and thereby provide relief from inflammation (19). Many of these drugs have been shown to possess anticancer activity, some independent of their COX inhibitory activity (20). Flurbiprofen and ibuprofen, the 2 NSAIDs shown to be potent inducers of p75^{NTR}, have exhibited anticancer activity in the prostate. Significantly, treatment with the enantiomer *R*-flurbiprofen, which lacks COX inhibitory activity, slowed the progression of prostate cancer in the TRAMP (TRansgenic Adenocarcinoma Mouse Prostate) model (21). In the PC-3 prostate cancer cell line, the housekeeping isoform COX-1, was expressed at negligible levels and was not induced by *R*-flurbiprofen or ibuprofen (7). Furthermore, ibuprofen treatment was shown not to decrease COX-2 levels (7). Hence, it was concluded that the anticancer activity of these

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drugs in prostate cancer cell lines was COX independent. Significantly, long-term ibuprofen use has been associated with decreased prostate cancer risk, and treatment of prostate cancer cells with ibuprofen resulted in decreased cell survival (22). Because both p75^{NTR} and Nag-1 are induced by NSAIDs in prostate cancer cells; in the present study, we explored the possibility of a common signaling pathway in the NSAID induction of both these proteins. We describe, for the first time, that the p38 MAPK pathway mediates induction of Nag-1 downstream of the p75^{NTR} protein. We also describe divergent effects of NSAIDs on the role of p75^{NTR} and Nag-1 in decreased prostate cancer cell survival versus migration.

Materials and Methods

Cell culture, treatment, and drug preparation

PC-3 cell line was obtained from the tissue culture core facility of Georgetown University Lombardi Comprehensive Cancer Center and maintained in DMEM (Mediatech Inc.) containing 4.5 g/L of glucose and L-glutamine supplemented with antibiotic/antimycotic [100 units/mL of penicillin G, 100 µg/mL of streptomycin, and 0.25 µg/mL of amphotericin B (Mediatech Inc.)] and 10% heat-inactivated FBS (Omega Scientific). The PC-3 Nag-1 cell line was a gift from Dr. James Lambert, University of Colorado Denver, and was maintained in RPMI (Mediatech Inc.) containing 4.5 g/L of glucose and L-glutamine supplemented with antibiotic/antimycotic [100 units/mL of penicillin G, 100 µg/mL of streptomycin, and 0.25 µg/mL of amphotericin B (Mediatech Inc.)], 10% heat-inactivated FBS and 100 µg/mL of G418 (Sigma Chemicals Co.). Cells were incubated in the presence of 5% CO₂ at 37°C. Ibuprofen (Sigma) and R-flurbiprofen (Sigma) stock solutions were prepared by dissolving in DMSO (Sigma) at a concentration of 200 mmol/L. Cells were seeded overnight at 80% to 85% confluency and were treated with drugs at concentrations of 0.25, 0.5, 1, and 2 mmol/L for up to 48 hours. Relative cell survival was measured by MTT assay (Roche Applied Science).

Immunoblot analysis

Cells were treated with 1 mmol/L of R-flurbiprofen or 2 mmol/L of ibuprofen for 48 hours. Lysates were prepared using Nonidet lysis buffer (50 mmol/L of Tris-Cl, 10 mmol/L of NaCl, 3 mmol/L of MgCl₂, and 0.5% Nonidet P-40) for p75^{NTR} and RIPA (radioimmunoprecipitation assay) lysis buffer (50 mmol/L of Tris-HCl, 150 mmol/L of NaCl, 1% Triton X-100, 0.5% Deoxycholic Acid, 0.1% Sodium dodecyl sulfate) for Nag-1 protein detection. Cells were scraped in lysis buffer containing 1 µL/mL of cocktail protease inhibitor (P8340; Sigma). The supernatant was retained, and protein concentration was determined by the BCA (bicinchoninic acid) method according to the manufacturer's protocol (Bio-rad Laboratories). Fifty micrograms of protein were loaded onto 10% SDS-polyacrylamide gels for electrophoresis, followed by transfer to a nitrocellulose membrane (Amersham Pharmacia Bio-

tech). Membranes were blocked in 5% nonfat milk/TBST (Tris-buffered saline with Tween; Bio-Rad Laboratories) and then incubated in primary antibody: murine monoclonal anti-p75^{NTR} (1:2,000; Upstate Cell Signaling Solutions), rabbit polyclonal anti-Nag-1 (1:500; Upstate Cell Signaling Solutions), rabbit polyclonal anti-p38α (1:1,000; Cell Signaling Technology), mouse monoclonal anti-p38β (1:1,000; Zymed Laboratories), overnight at 4°C. Membranes were then washed and incubated in the appropriate horseradish peroxidase-conjugated secondary antibody (1:3,000; Bio-Rad Laboratories). Immunoreactivity was detected using the chemiluminescence detection reagent (Amersham Pharmacia Biotech). Membranes were stripped and reprobed with mouse monoclonal anti-β-actin (1:5,000; Sigma) to ensure equal loading.

siRNA transfection

PC-3 cells were transfected for 72 hours with non-targeting small interfering RNA (siRNA) or siRNA specific for p38α (J-003512-20), p38β (J-003972-11), p75^{NTR} (D-009340-03) or Nag-1 (M-019875-01; Dharmacon RNA Technologies) at a final concentration of 100 nmol/L according to the manufacturer's protocol using Dharmacon 2 transfection reagent (Dharmacon).

In vitro scratch test and Transwell chamber migration assay

Cells were allowed to grow to confluence in 6-well tissue culture plates (untreated or after pretreatment with siRNA for 72 hours). A 200-µL tip was used to introduce a scratch in the monolayer. The wells were washed with PBS followed by addition of 1 mmol/L of ibuprofen or R-flurbiprofen. The wells were imaged at 40× magnification with an Olympus 1 × 70 microscope (Olympus) at 0 and 48 hours postscratch and post-drug introduction. Scratch healing was determined by measuring the shortest distance between scratch edges in each field of view. At least 3 different fields were measured per scratch and 20 different measurements taken per field. For the Transwell chamber migration assay, chambers were rehydrated overnight and a 0.1 mL suspension of cells (70,000 live cells; untreated or after 48 hours of 1 mmol/L of drug treatment or after pretreatment with siRNA for 72 hours followed by 48 hours of 1 mmol/L of drug treatment) in serum-free media were added to 6.5-mm Transwell 8-µm Polycarbonate Membrane Inserts (3422; Corning Life Sciences) placed in 24 wells containing 0.6 mL of 10% FBS-containing media. The plates were incubated for 6 hours at 37°C. At the end of the incubation period, nonmigrating cells on the inside of the filter were removed with a cotton swab, and the filters were fixed with methanol and stained with Toliudine Blue. Following staining, filters were removed from inserts and mounted on slides for imaging and quantification. The number of migrating cells on the underside of the filter was determined by counting cells in 5 random fields from 3 filters for each treatment through 200× magnification with an Olympus 1 × 70 microscope (Olympus).

Results

Nag-1 induction by NSAIDs correlates with induction of p75^{NTR} and is dependent on the p38 MAPK pathway

NSAID (*R*-flurbiprofen and ibuprofen) treatment showed a similar time course of p75^{NTR} and Nag-1 protein expression. Both *R*-flurbiprofen (Fig. 1A) and ibuprofen (Fig. 1B) induced Nag-1 and p75^{NTR} proteins by 24 hours posttreatment. Because NSAID induction of p75^{NTR} is mediated by the p38 MAPK pathway (10), we examined whether this pathway might also be implicated in the induction of Nag-1. Pretreatment of PC-3 cells with the selective p38 MAPK inhibitor SB202190 followed by treatment with *R*-flurbiprofen or ibuprofen prevented induction of Nag-1, showing that the p38 MAPK pathway mediates NSAID induction of Nag-1 (Fig. 2A). To further

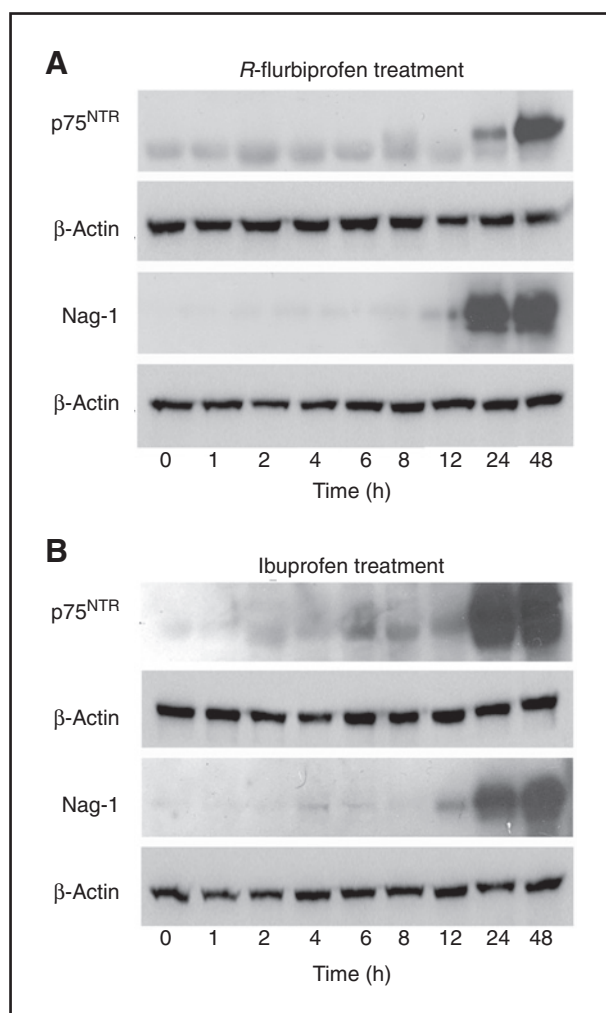


Figure 1. Immunoblot of time course of p75^{NTR} and Nag-1 protein expression following treatment with *R*-flurbiprofen (A) and ibuprofen (B). SDS-PAGE was performed using 50 μg of protein followed by immunoblot analysis with an antibody to p75^{NTR}, Nag-1 or β-actin for the loading control.

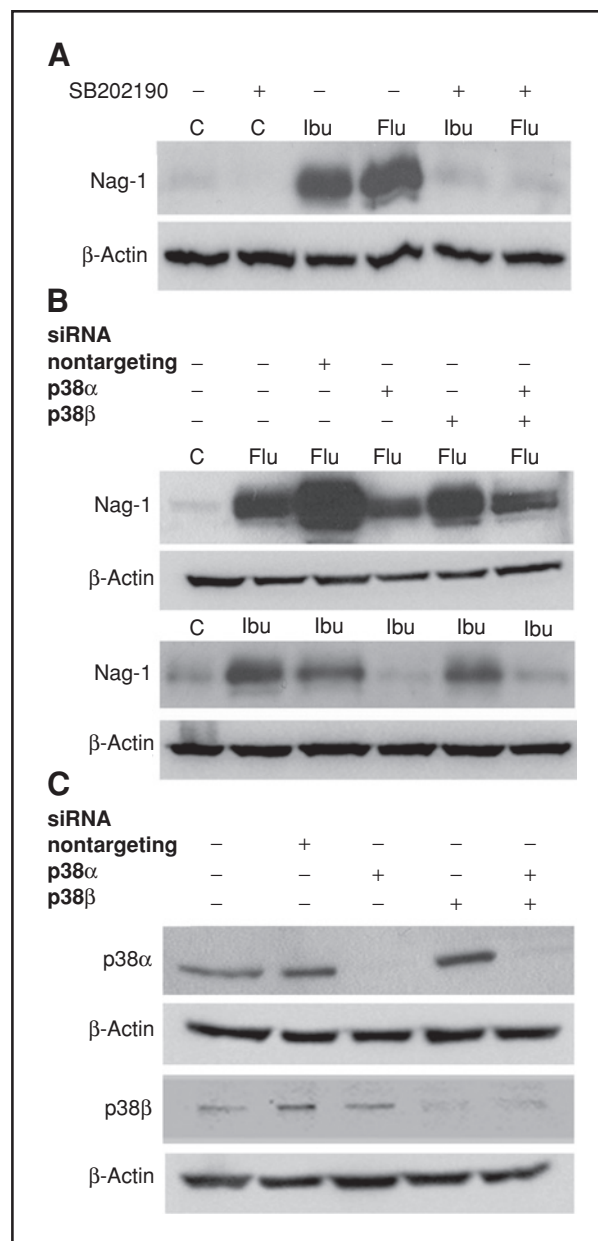


Figure 2. A, immunoblot of Nag-1 expression following inhibition of p38 MAPK pathway using a p38 MAPK-specific pharmacologic inhibitor. PC-3 cells were pretreated with 20 μmol/L of SB202190 for 1 hour, followed by 24 hour treatment with ibuprofen (Ibu) and *R*-flurbiprofen (Flu) or vehicle control (C). B, immunoblot of Nag-1 expression following inhibition of p38 MAPK pathway using siRNA specific to 2 isoforms (α and β) of p38 MAPK. PC-3 cells were pretreated with 100 nmol/L of siRNA for 72 hours, followed by 48 hours treatment with *R*-Flurbiprofen (Flu, top) and ibuprofen (Ibu, bottom) or vehicle control (C). C, validation assay for siRNA knockdown of p38 MAPK isoforms. SDS-PAGE was performed using 50 μg of protein followed by immunoblot analysis with an antibody to Nag-1 or p38 MAPK α and β. β-actin antibody was used for the loading control.

confirm the role of this pathway, we used p38 MAPK-specific siRNA to knock down the 2 ubiquitously expressed isoforms of p38 MAPK, p38 α and p38 β . The specificity of the siRNAs and the knockdown efficiency were confirmed by Western blot (Fig. 2C). Cells transfected with p38 α or a combination of p38 α and p38 β siRNAs prior to NSAID treatment prevented induction of Nag-1 in comparison to untransfected or nontargeting siRNA-transfected cells, confirming the role of p38 MAPK pathway in NSAID induction of Nag-1 (Fig. 2B). Transfection with p38 β siRNA alone was not effective in preventing induction of Nag-1, consistent with previous studies on p75^{NTR} induction (10).

NSAID induction of Nag-1 is mediated by p75^{NTR}

Because NSAIDs induced Nag-1 and p75^{NTR} in a p38 MAPK-dependent manner (Fig. 2), we further investigated any relationship between the 2 proteins. We used siRNA specific to p75^{NTR} to knock down its expression. In comparison to nontargeting siRNA, transfection with p75^{NTR} siRNA prior to *R*-flurbiprofen (Fig. 3A, top) or ibuprofen treatment (Fig. 3A, bottom) prevented induction of Nag-1, suggesting Nag-1 induction is downstream of

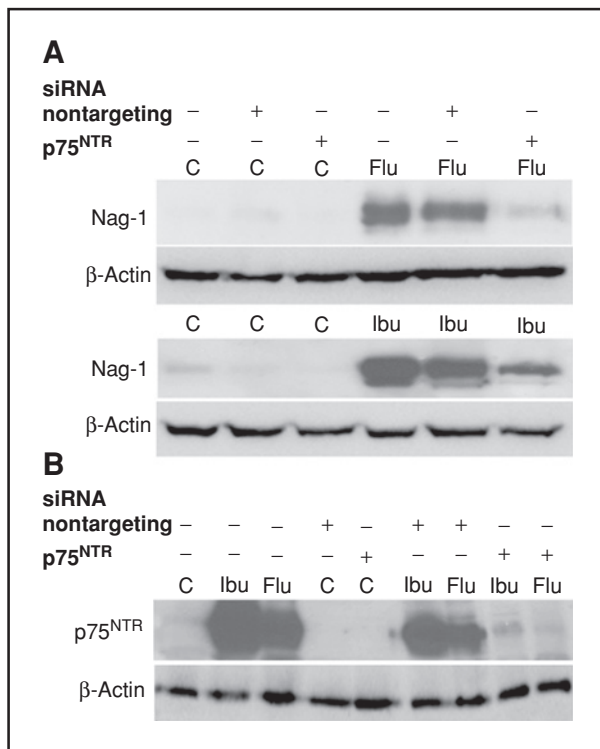


Figure 3. A, immunoblot of Nag-1 expression following p75^{NTR} inhibition using specific siRNA. PC-3 cells were pretreated with 100 nmol/L of siRNA for 72 hours, followed by 48 hour treatment with *R*-flurbiprofen (Flu, top) and ibuprofen (Ibu, bottom) or vehicle control (C). B, similarly treated cells were subjected to immunoblot for p75^{NTR} expression validating siRNA knockdown. SDS-PAGE was performed using 50 μ g of protein followed by immunoblot analysis with an antibody to Nag-1 or p75^{NTR}. β -actin antibody was used for the loading control.

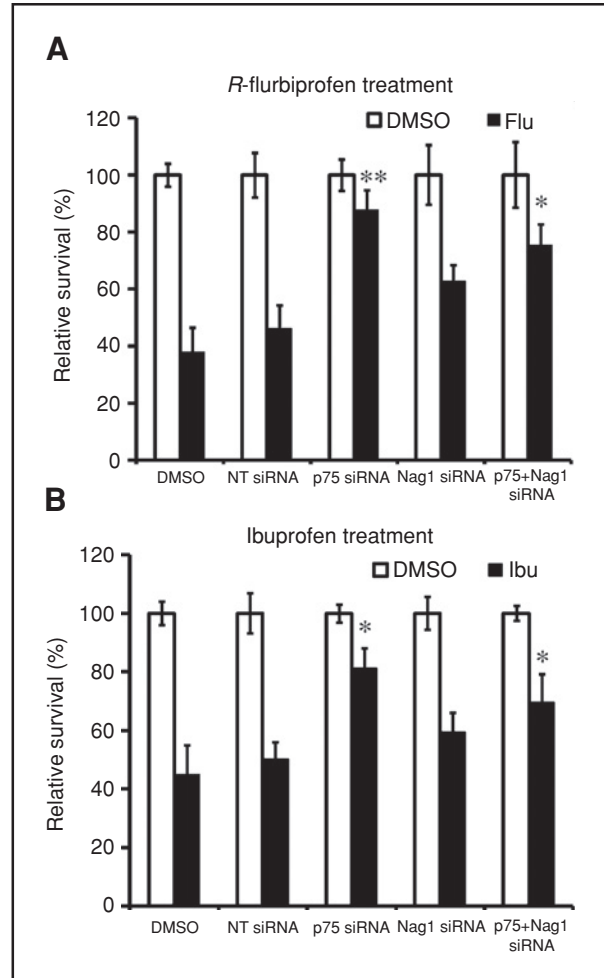


Figure 4. Cell survival analysis by MTT assay following 48 hours treatment with (A) *R*-flurbiprofen or (B) ibuprofen. Before treatment, cells were transfected with 100 nmol/L of nontargeting (NT) siRNA or Nag-1 siRNA or p75^{NTR} siRNA for 24 hours. Columns, mean relative to the vehicle control (DMSO); bars, SE of 3 independent experiments. *, $P < 0.05$; **, $P < 0.01$ relative to drug treatment.

p75^{NTR}. siRNA specificity and knockdown efficiency was determined by Western blot (Fig. 3B). Minor differences in knockdown efficiency of p75^{NTR} were observed between ibuprofen- and *R*-flurbiprofen-treated cells.

NSAID suppression of cell migration is mediated by Nag-1 downstream of p75^{NTR}

Pretreatment of PC-3 cells with p75^{NTR} siRNA rescued NSAID-mediated decreased cell survival as previously described (7). However, pretreatment of PC-3 cells with Nag-1-specific siRNA did not significantly rescue NSAID-mediated decreased cell survival, in comparison to nontargeting siRNA or siRNA specific to p75^{NTR}, as shown by the MTT cell survival assay (Fig. 4A and B). *In vitro* scratch test of PC-3 cells showed a dose-dependent decrease in cell migration in response to both ibuprofen and *R*-flurbiprofen

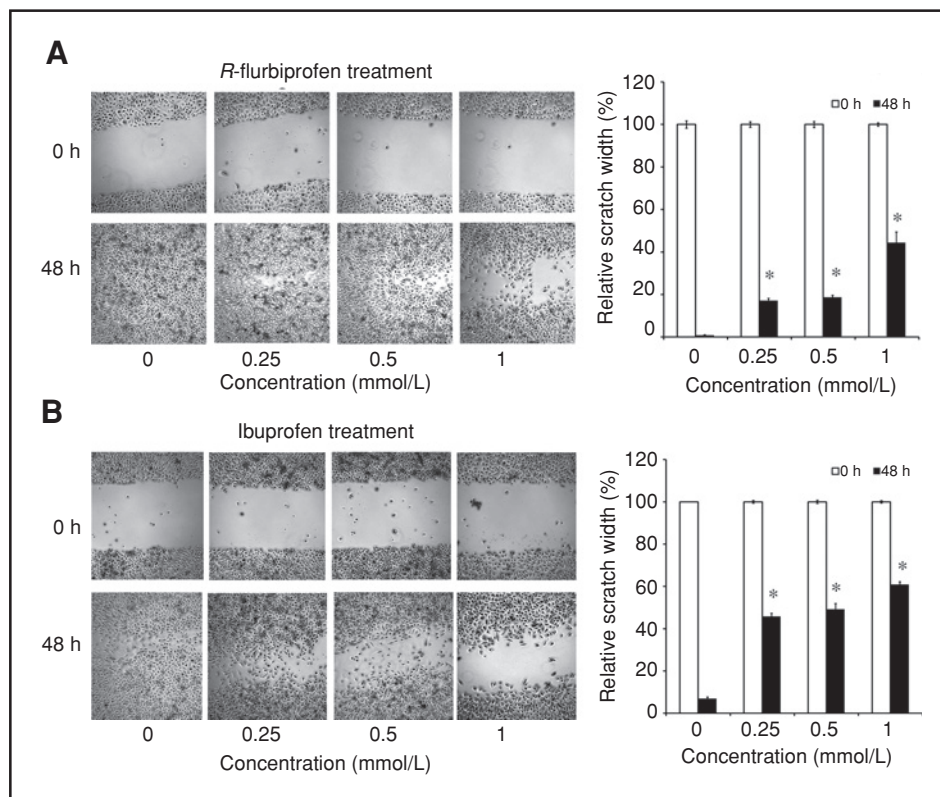


Figure 5. Cell migration measurement via the *in vitro* wound healing assay. PC-3 cells grown to confluent monolayers were wounded using a sterile tip. The wounded cells were treated with 0, 0.25, 0.5, and 1 mmol/L (A) *R*-flurbiprofen, or (B) ibuprofen. Wound healing was quantified by measuring the shortest distance between scratch edges at 0 and 48 hours postintroduction of scratch in 3 different fields per scratch. Columns, mean relative to the initial wound width (0 hour); bars, SE of 3 independent experiments. *, $P < 0.05$ relative to vehicle (DMSO).

treatment, as shown by up to 40% to 60% delay in wound closure at 48 hours posttreatment with *R*-flurbiprofen (Fig. 5A) and ibuprofen (Fig. 5B). Transfection with p75^{NTR} or Nag-1 siRNA before treatment with *R*-flurbiprofen (Fig. 6A) or ibuprofen (Fig. 6B) significantly rescued ($P < 0.01$) NSAID inhibition of PC-3 cell migration, as shown by 20% to 30% wound closure for these treatments in comparison to 50% to 60% for nontargeting siRNA-transfected cells. Hence, induction of p75^{NTR} and/or Nag-1 mediates, at least in part, the decreased cell migration observed upon NSAID treatment. To reconfirm these results, migration of cells undergoing the afore-mentioned treatments were measured through the Transwell chamber assay. Decreased cell migration upon NSAID treatment was dependent on the induction of Nag-1 downstream of p75^{NTR}, as shown by statistically significant partial rescue of relative cell migration across 8- μ m polycarbonate membrane pores toward 10% FBS-containing media, when cells were pretreated with Nag-1 or p75^{NTR} siRNA but not nontargeting siRNA ($P < 0.001$). Approximately 50% to 60% of plated cells were shown to migrate when treated with Nag-1 siRNA or p75^{NTR} siRNA in comparison to 30% for nontargeting siRNA transfected cells (Fig. 7A and B). siRNA specificity and knockdown efficiency of these same cells was

determined by Western blot (Fig. 7C). To further confirm that Nag-1 plays a significant role in suppression of cell migration, the relative migration of PC-3 cells stably expressing Nag-1 was measured in comparison to the parental cell line. Both *in vitro* scratch test and Transwell chamber assay showed that Nag-1 overexpression decreased the migratory potential of cells (Fig. 8A–C). A 40% delay in wound closure was observed for Nag-1 overexpressing cells in the *in vitro* scratch test ($P < 0.01$) and a 60% decrease in migratory potential ($P < 0.01$) in the Transwell chamber migration assay, in comparison to the parental cell line. Expression of Nag-1 in the cells was confirmed by Western blot (Fig. 8C).

Discussion

Nag-1, a TGF- β superfamily member, is induced by several chemopreventive agents, most notably by resveratrol, indole-3-carbinol, genistein, green tea, cruciferous vegetables, and NSAIDs (12, 18, 23). Several of these agents also induce p75^{NTR}, a surface receptor glycoprotein known to be a tumor suppressor, whose expression is lost in poorly differentiated prostate cancer tissue and cell lines (4, 7, 24). In this study, we explored the possibility of a common signaling mechanism between induction of

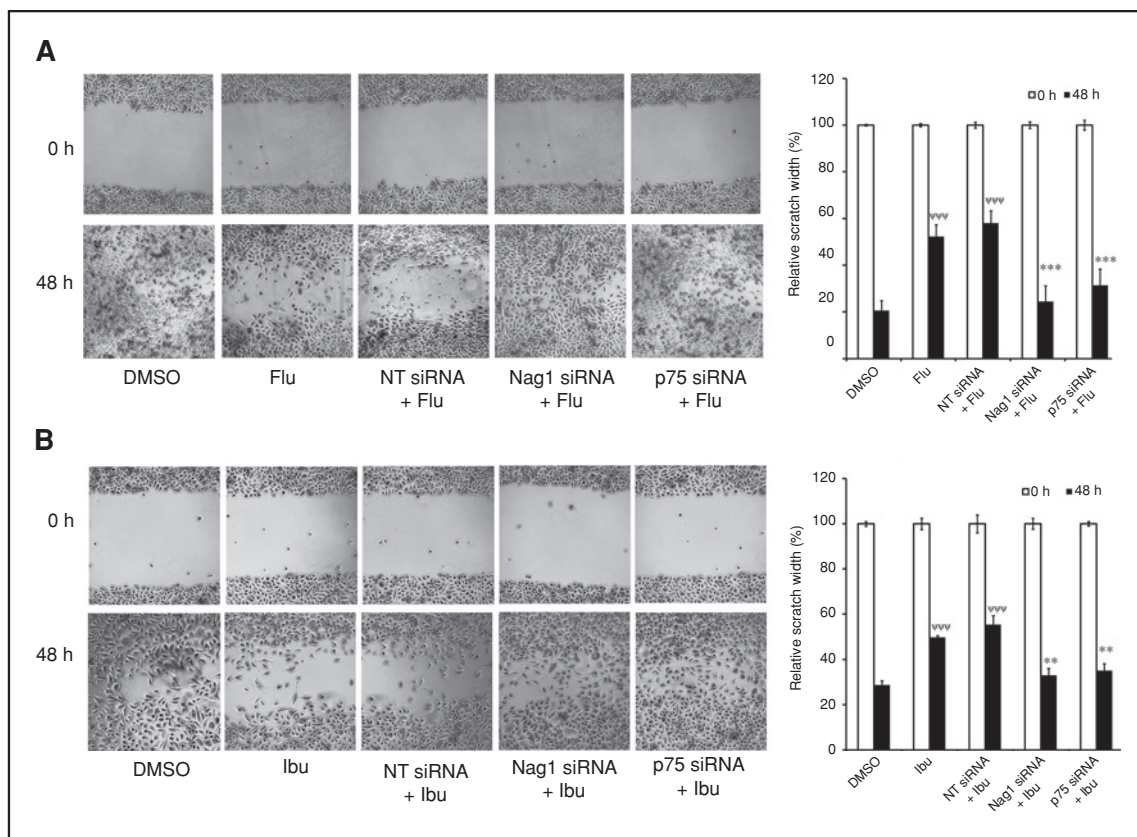


Figure 6. Cell migration measurement via the *in vitro* wound healing assay. Cells were treated with nontargeting (NT) siRNA or siRNA specific to Nag-1 or p75^{NTR} for 72 hours prior to introduction of a scratch with a sterile tip and addition of *R*-flurbiprofen (A) or ibuprofen (B). Wound healing was quantified by measuring the shortest distance between scratch edges at 0 and 48 hours postintroduction of scratch in 3 different fields per scratch. Columns, mean relative to the initial wound width (0 hours); bars, SE of 3 independent experiments. , $P < 0.001$ relative to vehicle; **, $P < 0.01$ relative to drug treatment; ***, $P < 0.001$ relative to drug treatment.

p75^{NTR} and Nag-1 by NSAIDs in prostate cancer cells. The choice of drugs in this study was based on the efficacy of p75^{NTR} induction in prostate cancer cell lines shown in previous work from this laboratory (7). *R*-flurbiprofen, an enantiomer which lacks the COX inhibitory activity, was shown to be the most effective NSAID in inducing p75^{NTR}, followed by ibuprofen (7). Significantly, induction of the p75^{NTR} protein correlated with a similar temporal induction of the Nag-1 protein. NSAID induction of Nag-1 was prevented when cells were pretreated with either pharmacologic inhibitors or siRNA specific to the p38 MAPK. This was similar to previous studies on induction of p75^{NTR} (10), establishing that both these proteins share, in part, a common pathway for protein expression. Experiments using siRNA specific to p75^{NTR} showed that NSAID induction of Nag-1 is downstream of p75^{NTR}.

To date, the functional role of Nag-1 is not fully characterized; however, both p75^{NTR} and Nag-1 have been reported to play an antisurvival role in prostate and other cancer cells (8, 12, 25, 26). Pretreatment with p75^{NTR}

siRNA followed by treatment with NSAIDs, resulted in a statistically significant rescue of cell survival. Conversely, Nag-1 siRNA pretreatment did not significantly rescue NSAID-mediated decreased cell survival. Therefore, unlike p75^{NTR}, our study did not demonstrate a role for Nag-1 in the NSAID-mediated decreased cell survival of prostate cancer cells. This is consistent with an *in vivo* study, in which orthotopic implantation of PC-3 cells overexpressing Nag-1 showed no difference in tumor weight as compared with the parental cells (27). In the absence of an effect on cell survival, we next examined whether Nag-1 may affect cell migration. Interestingly, NSAIDs have been linked to metastasis suppression in a variety of cancers including prostate cancer (28–32). p75^{NTR} has been shown to contribute to prostate cancer metastasis suppression (6, 33); therefore, a potential mechanism of NSAID-mediated metastasis suppression may be through the induction of p75^{NTR} and Nag-1. Cell migration experiments revealed a role for both p75^{NTR} and Nag-1 in the NSAID-mediated decreased cell migration. A statistically

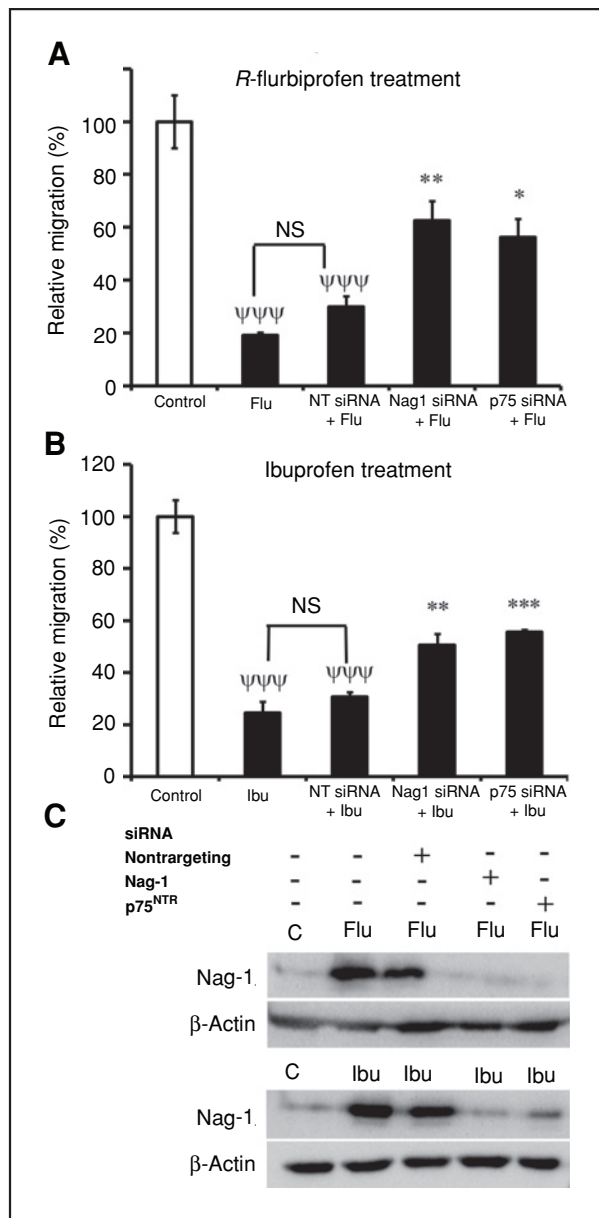


Figure 7. Cell migration measurement via the Transwell chamber assay. Cells were treated with nontargeting (NT) siRNA or siRNA specific to Nag-1 or p75^{NTR} for 72 hours prior to treatment with *R*-flurbiprofen (A) or ibuprofen (B) for 48 hours. Equal number of live cells were plated into polycarbonate inserts and allowed to migrate for 5 hours. Wound healing was quantified by measuring the shortest distance between scratch edges at 0 and 48 hours postintroduction of scratch in 3 different fields per scratch. Columns, mean relative to the initial wound width (0 hours); bars, SE of 3 independent experiments. , $P < 0.001$ relative to vehicle; *, $P < 0.05$ relative to drug treatment; **, $P < 0.01$ relative to drug treatment; ***, $P < 0.001$ relative to drug treatment. C, validation assay of siRNA knockdown of Nag-1. SDS-PAGE was performed using 50 μ g of protein followed by immunoblot analysis with an antibody to Nag-1. β -actin antibody was used for the loading control. NS, not significant.

significant rescue of NSAID-inhibited cell migration was observed upon pretreatment with siRNA specific to both p75^{NTR} and Nag-1, confirming a cause-and-effect relationship between NSAID induction of p75^{NTR} and Nag-1 proteins and p75^{NTR} and Nag-1-mediated decreased cell migration. In addition, PC-3 cells stably expressing Nag-1 exhibited decreased migration relative to the parental cell line, thereby independently confirming a role for this protein in reduced prostate cancer cell migration. These results collectively show that NSAID-mediated decreased cell migration is dependent on p75^{NTR} induction and that Nag-1 is necessary for this effect. The mechanism by which Nag-1 inhibits cell migration has not been elucidated, but it could potentially include inhibition of urokinase plasminogen activator (uPA) and the matrix metalloproteases (MMP)-2 and MMP-9, similar to that which occurs for p75^{NTR} (33).

While a number of tumor suppressor proteins have been reported in the literature, only a few of these proteins have been classified purely as metastasis suppressors that do not affect the growth of the primary tumor, such as Kangai 1 (KAI-1), mitogen-activated protein kinase kinase 4 (MKK-4), nonmetastatic gene (NM-23), breast cancer metastasis-suppressor 1 (BRMS-1), Rho-GDP Dissociation Inhibitor 2 (RHOGDI-2), vitamin D3 upregulated protein 1 (VDUP-1), cofactor required for SP1 activation (CRSP-3), and Metastin (KISS-1). Of these, MKK-4 and KISS-1 act by activation of the p38 MAPK pathway, directly or indirectly (34, 35). Because cell migration is an important step during metastasis, we propose that Nag-1, a cell migration inhibitor, may also potentially contribute to metastasis suppression of prostate cancer cells. Conversely, a recent report showed that overexpression of Nag-1 induced metastasis of human prostate cancer cells (27). However, this model was significantly different from ours, in that it compared cell lines already expressing high levels of Nag-1 to cell lines that do not, whereas our study focused on the NSAID-mediated induction of Nag-1 by p75^{NTR} through the p38 MAPK pathway in cells lacking Nag-1 expression. In addition, the afore-mentioned study showed induction of metastasis by Nag-1 was dependent upon activation of the focal adhesion kinase-RhoA signaling pathway. However, several studies have shown that NSAIDs inhibit Fak and RhoA signaling (36–38), so in an NSAID induction model of Nag-1, the effect on cell migration would differ. In summary, we have presented evidence demonstrating that the TNFR superfamily member p75^{NTR} and the TGF- β superfamily member Nag-1 are induced by NSAIDs through a common pathway by activation of the p38 MAPK. In addition, whereas p75^{NTR} exhibits both tumor suppression (5, 8) and metastasis suppression (6, 33) activity, Nag-1 expression downstream of p75^{NTR} only inhibits cell migration.

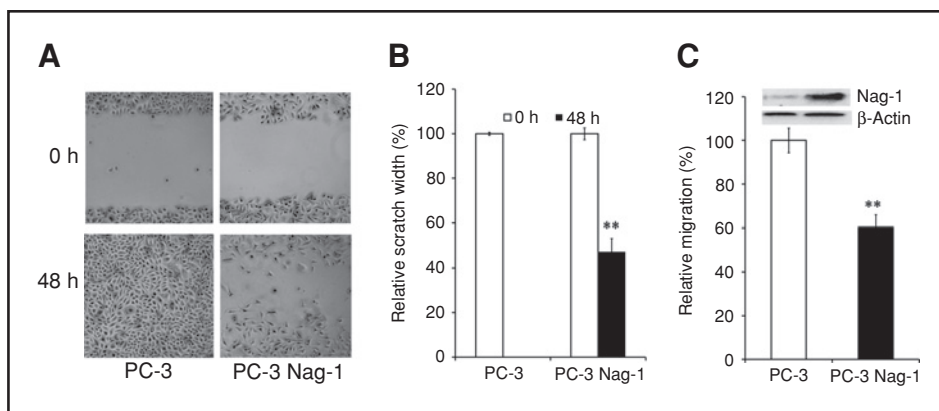


Figure 8. Measurement of cell migration of PC-3 cells stably expressing Nag-1. A and B, cell migration measurement via the *in vitro* wound healing assay. Cells were grown to confluent monolayer and wounded using a sterile tip. Wound healing was quantified by measuring the shortest distance between scratchedges at 0 and 48 hours postintroduction of scratch in 3 different fields per scratch. Columns, mean relative to the initial wound width; bars, SE of 3 independent experiments. **, $P < 0.01$ relative to parent cell line. C, measurement of cell migration via the Transwell chamber assay. Equal number of live cells were plated into polycarbonate inserts and allowed to migrate for 5 hours. Columns, mean number of migrating cells relative to the parental cell line; bars, SE of 3 independent experiments. **, $P < 0.01$ relative to parental cell line. Nag-1 expression was determined by immunoblot analysis with an antibody to Nag-1. β -actin antibody was used for the loading control.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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