

Ibuprofen Inhibits Survival of Bladder Cancer Cells by Induced Expression of the p75^{NTR} Tumor Suppressor Protein

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ABSTRACT

Nonsteroidal anti-inflammatory drugs (NSAIDs) are used to reduce inflammation and as analgesics by inhibition of cyclooxygenase-2. At higher concentrations, some NSAIDs inhibit proliferation and induce apoptosis of cancer cells. Although several molecular mechanisms have been postulated to explain the anticancer effects of NSAIDs, they do not involve merely the inhibition of cyclooxygenase-2, and a more proximate initiator molecule may be regulated by NSAIDs to inhibit growth. The p75 neurotrophin receptor (p75^{NTR}) is a proximate cell membrane receptor glycoprotein that has been identified as a tumor and metastasis suppressor. We observed that NSAID treatment of cell lines from bladder and other organs induced expression of the p75^{NTR} protein. Of the different types of NSAIDs examined, ibuprofen was more efficacious than aspirin and acetaminophen and comparable with (*R*)-flurbiprofen and indomethacin in induction of p75^{NTR} protein expression. This rank order NSAID induction of the p75^{NTR} protein correlated with the ability of these NSAIDs to reduce cancer cell survival. To examine a mechanistic relationship between ibuprofen induction of p75^{NTR} protein and inhibition of survival, bladder cancer cells were transfected with ponasterone A-inducible vectors that expressed a death domain-deleted (ΔDD) or intracellular domain-deleted (ΔICD) p75^{NTR} product that acts as a dominant negative antagonist of the intact p75^{NTR} protein. Expression of ΔDD and ΔICD rescued cells from ibuprofen inhibition of growth. These observations suggest that p75^{NTR} is an important upstream modulator of the anticancer effects of NSAIDs and that ibuprofen induction of the p75^{NTR} protein establishes an alternate mechanism by which ibuprofen may exert an anticancer effect.

INTRODUCTION

Nonsteroidal anti-inflammatory drugs (NSAIDs) are one of the most commonly consumed types of medicines, used primarily as analgesics for the relief of pain and to control inflammation (1). An increasing number of epidemiological, clinical, and laboratory studies have also suggested that NSAIDs may be able to inhibit the initiation and proliferation of some tumors (2–6). However, the specific mechanism(s) of NSAID action to limit neoplasia remains elusive. It is generally assumed that the analgesic and anti-inflammatory efficacy of NSAIDs arises primarily from their inhibition of cyclooxygenases (COXs) that convert arachidonic acid to prostaglandins (7). Prostaglandins are thought to contribute to tumor growth by inhibiting apoptosis (8) and by inducing the formation of new blood vessels needed to sustain tumor growth (9). Hence, COX inhibition of prostaglandin synthesis could explain part of the antitumor activity of certain NSAIDs. However, NSAIDs can also inhibit tumor formation and growth of COX-null cell lines (10). In addition, certain NSAIDs that lack COX-inhibitory activity can still have significant anticancer effects both *in vivo* (11) and *in vitro* (12). Pharmacologically, NSAIDs

generally inhibit COX activity and thus prostaglandin synthesis at concentrations 100- to 1,000-fold less than the concentrations that are associated with antitumor activity (1), suggesting the existence of additional cellular targets (9, 10, 13). Hence, COX-independent mechanisms may account for some of the anticancer effects of certain NSAIDs in which high concentrations of NSAIDs appear to be required (1). At these higher concentrations, the possible mechanisms for the antitumor activity of NSAIDs include inhibition of cell cycle progression (14) and induction of apoptosis (15). At the molecular level, the anticancer effects of NSAIDs have been associated with the down-regulation of both the antiapoptotic Bcl-2 protein (16) and the prosurvival kinase Akt (17). Other molecular mechanisms proposed to mediate the anticancer action of NSAIDs involve activation of protein kinase G (14); up-regulation of proapoptotic proteins Bax and Bcl-xl (18); inhibition of peroxisome proliferator-activated receptors β and δ (19); increasing arachidonic acid levels, leading to the production of ceramide (20); or inhibition of the transcription factor nuclear factor (NF)-κB by blocking the phosphorylation and degradation of IκBα (21, 22). For these pathways to be activated, there is a strong indication of the presence of a proximate initiator molecule that promotes transmission of a signal from the plasma membrane and the accompanying changes regulated by NSAIDs (18).

The p75^{NTR} is a *M_r* 75,000 cell surface glycoprotein that shares both structural and sequence homology with the tumor necrosis factor receptor superfamily of proteins (20, 23). Some of these proteins, including p75^{NTR}, have similar sequence motifs of defined elongated structure (20) designated “death domains” based on their apoptosis-inducing function (23). Recently, p75^{NTR} was identified as a tumor and metastasis suppressor of cancer cells derived from the urogenital system (24, 25). Interestingly, even though the gene encoding p75^{NTR} was intact in these cancer cells, expression of the p75^{NTR} protein was suppressed at the posttranscriptional level (26). Ectopic reexpression of p75^{NTR} in these cells increased their rate of apoptosis (24) via a mitochondria-mediated caspase cascade (27). Additionally, ectopic p75^{NTR} expression in these same cancer cells selectively altered expression of specific cell cycle-regulatory components to retard progression through the G₁ phase with a corresponding reduction of cells in the S phase of the cell cycle (28). These observations showed that the p75^{NTR} tumor suppressor inhibits growth by both retarding cell proliferation and promoting apoptosis. Considering that p75^{NTR} is a proximate initiator molecule that promotes inhibition of growth, comparable with that proposed as a possible target of some NSAIDs (18), we investigated whether NSAIDs could induce expression of the p75^{NTR} gene product that was causal of the inhibition of tumor cell survival.

MATERIALS AND METHODS

Cell Lines and Culture Conditions. The bladder epithelial cell line T24, previously designated TSU-pr1 (29), containing the neomycin (neo) resistance gene as well as transgenic clones expressing low, intermediate, and high levels of the p75^{NTR} have been described previously (25, 26). The RT-4 transitional cell papilloma bladder cell line and 5637 primary carcinoma bladder cell line were obtained from the American Type Culture Collection (Manassas, VA). Additional cell lines (HCT-116, MDAMB231, MCF7, HEK293, A549,

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SKOV3, and DU145) were obtained from the tissue culture core facility (Lombardi Cancer Center, Georgetown University, Washington, D.C.). All cell lines were maintained in Dulbecco's modified Eagle's medium (Mediatech Inc. Herndon, VA) containing 4.5 g/L glucose and L-glutamine supplemented with antibiotic/antimycotic (100 units/ml penicillin G, 100 μg/ml streptomycin, and 0.25 μg/ml amphotericin B; Mediatech), 5% fetal bovine serum (Sigma Chemical Co., St. Louis, MO), and McCoy's 5A medium (modified). Cell cultures were incubated in the presence of 10% CO₂ at 37°C. The A875 human melanoma cell line was used as a positive control in all p75^{NTR} Western blots as described previously (30).

Cell Treatment and Cell Lysis. Each cell line was seeded (5 × 10⁵) overnight before treatment. All NSAIDs [ibuprofen, acetaminophen, aspirin, (R)-flurbiprofen, and indomethacin (Sigma Chemical Co.)] were dissolved in dimethyl sulfoxide (Sigma Chemical Co.) at a concentration of 200 mmol/L stock solution. Each cell line was incubated with different NSAIDs of various concentrations for 48 hours. Subsequently, the cells were placed in lysis buffer [10 mmol/L Tris-Cl (pH 7.4), 10 mmol/L NaCl, 3 mmol/L MgCl₂, and 0.5% Nonidet P-40] containing protease inhibitors. The supernatant of the lysate was retained for estimation of protein concentration according to the manufacturer's instruction (Bio-Rad Laboratories, Hercules, CA).

Immunoblot Analysis. Immunoblot analyses of the cell lysates were performed as described previously (31). Briefly, 50 μg of protein were loaded onto 10% SDS-polyacrylamide gels for electrophoresis, transferred to a nitrocellulose membrane, blocked in 5% nonfat milk, and incubated in a primary murine monoclonal p75^{NTR} antibody (Upstate Cell Signaling Solutions, Lake Placid, NY) and murine monoclonal β-actin antibody (Sigma Chemical Co.).

Membranes were incubated in a horseradish peroxidase-conjugated goat anti-mouse secondary antibody (1:2,000; Bio-Rad Laboratories). Immunoreactivity was detected using the enhanced chemiluminescence detection reagent (Amersham Pharmacia Biotech, Piscataway, NJ). Densitometric analysis of protein levels was performed using a ChemImager apparatus (Alpha Innotech Corp., San Leandro, CA).

Transfection Assays. T24 cells were transiently transfected with ecdysone-inducible p75^{NTR} dominant negative vectors, as described previously (32), that expressed a death domain-deleted p75^{NTR} gene product (ΔDD) or an intracellular domain-deleted p75^{NTR} gene product (ΔICD). Transient transfection was performed with LipofectAMINE reagent (Invitrogen, Carlsbad, CA). The cells were cotransfected for 6 hours in serum-free medium with the ecdysone receptor plasmid pVgR_{xR} and either ΔDDp75^{NTR} or ΔICDp75^{NTR}, followed by the addition of serum-containing medium for 18 hours. Subsequently, incubation of cells in ponasterone A (1 μmol/L; Invitrogen) for 24 hours was used to drive expression of the dominant negative gene products. A control transforming growth factor β receptor II (TGFβRII) expression vector was transiently transfected into T24 cells as described above. The TGFβRII-transfected cells were used as a positive control for immunoblot of 48-hour ibuprofen treatment of cells probed with TGFβRII antibody (1:200 dilution, Santa Cruz Biotechnology, Santa Cruz, CA).

Cell Survival Assay. The number of cells in each well after treatment (48 hours) with NSAIDs was estimated using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. MTT labeling reagent (final concentration, 0.5 mg/ml; Roche Diagnostics Corp., Indianapolis, IN) was added to each of the NSAID-treated T24 cells, ponasterone A alone-treated cells,

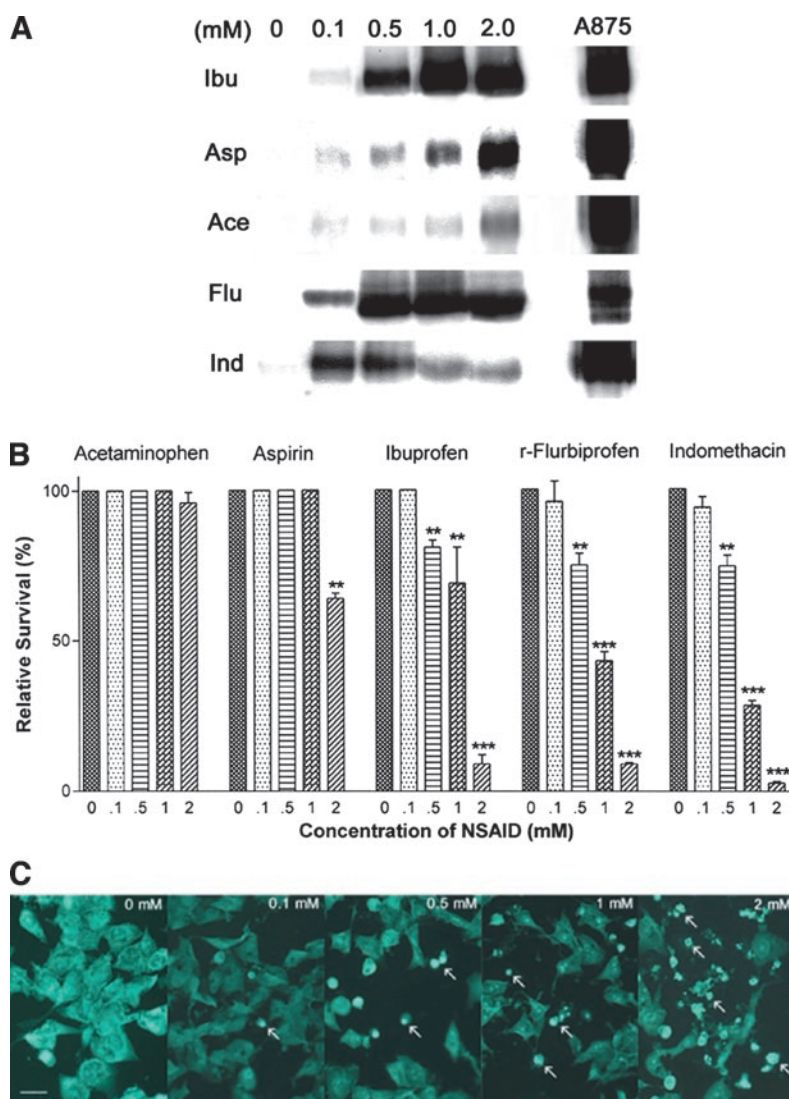


Fig. 1. A, expression of the p75^{NTR} protein in T24 bladder cells after 48-hour treatment with 0 to 2.0 mmol/L ibuprofen (*Ibu*), aspirin (*Asp*), acetaminophen (*Ace*), (R)-flurbiprofen (*Flu*), and indomethacin (*Ind*), respectively. Cell lysates containing 50 μg of T24 protein or 5 μg of protein from the A875 melanoma cell line (positive control) were subjected to SDS-PAGE and immunoblot analysis with a monoclonal antibody to the human p75^{NTR} protein. B, MTT analysis of T24 cell survival after 48-hour treatment with 0 to 2.0 mmol/L acetaminophen, aspirin, ibuprofen, (R)-flurbiprofen, and indomethacin, respectively. Results are expressed as the mean ± SE. **, *P* < 0.01; ***, *P* < 0.001, relative to the control (0 mmol/L) for each type of NSAID. C, Hoechst staining of T24 bladder cells exhibiting apoptotic nuclear fragmentation (arrows) after 48-hour treatment with 0 to 2.0 mmol/L ibuprofen. Cells were fixed in 10% formalin, rinsed, resuspended in PBS, and stained in a 1:1,000 dilution of aqueous Hoechst stain solution (Hoechst 33258). Cells were visualized under a fluorescence microscope. Scale bar = 8 μm.

Δ DDp75^{NTR}-transfected cells plus ponasterone A, and Δ ICDp75^{NTR}-transfected cells plus ponasterone A (2×10^3 cells/well) in 96-well culture plates (final volume, 100 μ l culture medium/well) and incubated for 4 hours at 37°C in a humidified atmosphere of 10% CO₂. Subsequently, cells were incubated overnight with 100 μ l of solubilization solution per well, and the samples were quantified at 570 nm using a microtiter plate reader (Bio-Rad Laboratories, Richmond, CA).

Hoechst Dye Nuclear (DNA) Staining. Hoechst staining (33) with modifications was used to distinguish between intact cell nuclei and fragmented nuclei undergoing cell death. Briefly, T24 cells treated for 48 hours with varying concentrations of ibuprofen were fixed in 10% formalin (Fischer Scientific, Pittsburgh, PA), rinsed with PBS (pH 7.4), and centrifuged, after which the cell pellet was resuspended in PBS. The cells were dried to a slide, rehydrated with PBS, covered with a 1:1,000 dilution of aqueous Hoechst stain solution (Hoechst 33258; bis-benzamide, 10 mg/ml; Molecular Probes, Eugene, OR), rinsed, dried, and mounted under a coverslip with aqueous-based Shur/Mount (Electron Microscopy Sciences, Washington, PA). The stained cells were visualized using a Axioplan 2 imaging fluorescence microscope (Zeiss, Jena, Germany).

Statistical Analysis. The statistical differences between data sets and/or means were analyzed by analysis of variance, Student's *t* test, or correlation analysis using the Prizm program (Graph Pad Software), and the data are expressed as the mean \pm SE. Data were deemed statistically significant when $P < 0.05$.

RESULTS

NSAID-Induced Expression of the p75^{NTR} Gene Product Is Associated with Inhibition of Cell Survival. Pharmacokinetic studies of ibuprofen have indicated that clinically tolerable high doses (*i.e.*, up to 3,200 mg/d) can produce levels (*i.e.*, 0.5–1 mmol/L) in the blood (34–36) that fall within the range used for these studies. In comparison, at clinically tolerable high doses of aspirin, peak serum concentrations are usually not much more than 0.5 mmol/L (37). Clinically tolerable high doses of acetaminophen can achieve peak serum concentrations of not much more than 0.1 mmol/L (38, 39), whereas blood levels in excess of 14 μ mol/L indomethacin are considered toxic (40). Hence, to provide comparisons, we examined the effect of various NSAIDs that span those concentrations achievable for ibuprofen. In this context, treatment (48 hours) of T24 bladder cancer cells with five common NSAIDs [ibuprofen, aspirin, acetaminophen, (*R*)-flurbiprofen, and indomethacin] induced expression of the p75^{NTR} gene product in a dose-dependent manner (Fig. 1A). The A875 melanoma cell line that has been characterized to constitutively express abundant levels of the p75^{NTR} gene product was included as a positive control. In rank order, ibuprofen, its analog (*R*)-flurbiprofen, and indomethacin were most efficacious in induction of the p75^{NTR} gene product, followed by aspirin; acetaminophen was the least effective compound (Fig. 1A). Similarly, in rank order, ibuprofen, (*R*)-flurbiprofen, and indomethacin were most efficacious in inhibiting the survival of T24 cells, followed by aspirin, and acetaminophen was the least effective inhibitor of survival (Fig. 1B). At high concentrations of indomethacin (1–2 mmol/L), the rapid onset of apoptosis resulted in degradation of some p75^{NTR} protein. Significantly, the rank order of NSAID-induced expression of the p75^{NTR} gene product (Fig. 1A) was similar to the rank order of NSAID-induced inhibition of cell survival (Fig. 1B). Treatment of T24 cells with NSAIDs induced nuclear involution and fragmentation indicative of apoptosis (Fig. 1C). In rank order, ibuprofen (Fig. 1C), (*R*)-flurbiprofen, and indomethacin were most effective in promoting the dose-dependent apoptosis of T24 cells, followed by aspirin, and acetaminophen was the least effective compound.

Because the ibuprofen class of NSAIDs appeared highly efficacious in the induction of the p75^{NTR} gene product (Fig. 1A) and inhibition of survival (Fig. 1B and C), we examined whether this relationship

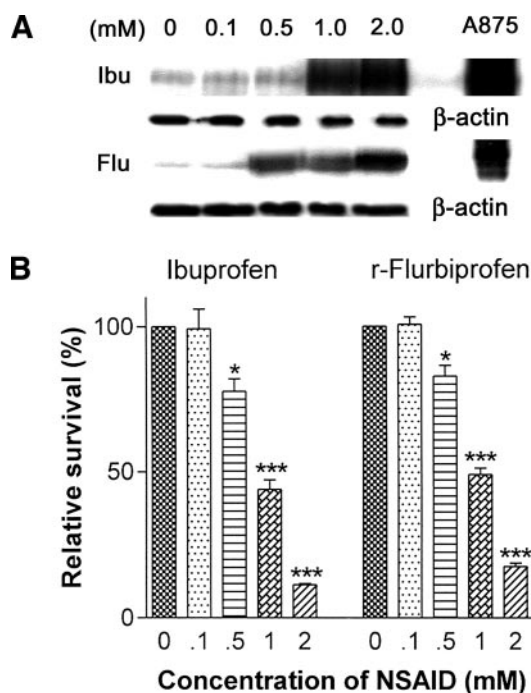


Fig. 2. *A*, expression of the p75^{NTR} protein in the COX-deficient HCT-116 colon cell line after 48-hour treatment with 0 to 2.0 mmol/L ibuprofen (*Ibu*) and its analog, (*R*)-flurbiprofen (*Flu*), which lacks COX-inhibitory activity. Cell lysates containing 50 μ g of HCT-116 protein or 5 μ g of protein from the A875 melanoma cell line (positive control) were subjected to SDS-PAGE and immunoblot analysis with a monoclonal antibody to the human p75^{NTR} protein. The same cell lysates of HCT-116 cells were similarly subjected to immunoblot analysis with a monoclonal antibody to the human β -actin protein as a loading control. *B*, MTT analysis of COX-deficient HCT-116 cell survival after 48-hour treatment with 0 to 2.0 mmol/L ibuprofen (*Ibu*) and its analog, (*R*)-flurbiprofen (*Flu*), which lacks COX-inhibitory activity. Results are expressed as the mean \pm SE. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$, relative to the control (0 mmol/L) for each type of NSAID.

may be associated with inhibition of COXs (Fig. 2A and B). In the COX-deficient HCT-116 cell line (41, 42), ibuprofen and (*R*)-flurbiprofen, the latter of which lacks COX-inhibitory activity (43), both induced expression of the p75^{NTR} gene product (Fig. 2A), which was associated with the inhibition of survival (Fig. 2B) in a dose-dependent manner. Hence, the dose-dependent induction of expression of the p75^{NTR} gene product and associated inhibition of survival by ibuprofen and (*R*)-flurbiprofen appeared independent of COX-inhibitory activity.

Specificity of Ibuprofen-Induced Expression of the p75^{NTR} Gene Product. Treatment of additional bladder cancer cell lines with ibuprofen also induced expression of the p75^{NTR} gene product in a dose-dependent manner (Fig. 3A). Ibuprofen treatment significantly induced the dose-dependent expression of the p75^{NTR} gene product of the well-differentiated (RT-4) bladder cell line (Fig. 3B; $P < 0.05$), moderately differentiated (5637) bladder cell line (Fig. 3C; $P < 0.05$), and poorly differentiated (T24) bladder cell line (Fig. 3D; $P < 0.05$). Although the T24 cell line expressed the highest levels of ibuprofen-induced p75^{NTR} gene product among the three cell lines, ibuprofen did not induce expression of another cell surface receptor protein, the TGF β RII (Fig. 3A). As a positive control, T24 cells transfected with a TGF β RII expression vector were shown to express the TGF β RII gene product (Fig. 3A).

Cell lines derived from a number of different organs were also examined for their response to ibuprofen induction of the p75^{NTR} gene product (Fig. 4A). Cell lines derived from the human bladder (RT-4, 5637, and T24), kidney (HEK293), ovary (SKOV3), and prostate (DU145) exhibited substantial induction of the p75^{NTR} gene

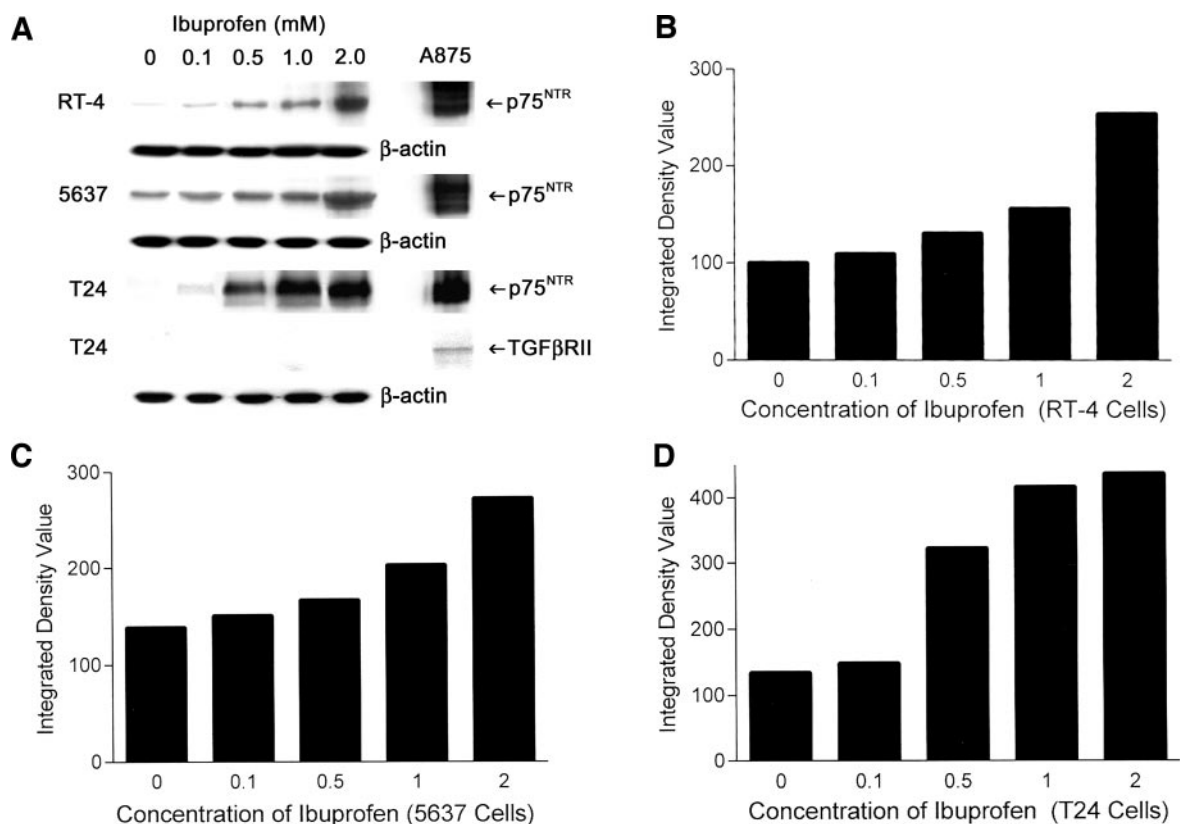


Fig. 3. Expression of the p75^{NTR} protein in three bladder cell lines (RT-4, 5637, and T24) after 48-hour treatment with 0 to 2.0 mmol/L ibuprofen. Cell lysates containing 50 μ g of protein or 5 μ g of protein from the A875 melanoma cell line (positive control) were subjected to SDS-PAGE and immunoblot analysis with a monoclonal antibody to the human p75^{NTR} protein (A). The same cell lysates were similarly subjected to immunoblot analysis with a monoclonal antibody to the human β -actin protein as a loading control. Expression of the TGF β RII protein was not observed in T24 cells treated with 0 to 2 mmol/L ibuprofen (A). Immunoblot of TGF β RII transiently transfected T24 cells was used as positive control. Densitometry of p75^{NTR} protein normalized to β -actin levels is shown for RT-4 cells (B), 5637 cells (C), and T24 cells (D).

product (Figs. 3B–D and 4B and C), with associated inhibition of survival (Table 1), in response to treatment with 2 mmol/L ibuprofen. Conversely, cell lines derived from human lung (A549), estrogen-refractory breast (MDAMB231), and estrogen-responsive breast (MCF7) exhibited either a minimal or more modest induction of the p75^{NTR} gene product (Fig. 4B and C) and inhibition of survival (Table 1) in response to treatment with 2 mmol/L ibuprofen (Fig. 4A–C). The relative survival of each cell line incubated in 2 mmol/L ibuprofen was inversely correlated with the levels of the p75^{NTR} relative to T24 cells ($r = -0.786$; $P < 0.001$).

Dominant-Negative p75^{NTR} Expression Vectors Rescue Ibuprofen Inhibition of Growth. To establish a causal relationship between ibuprofen induction of the p75^{NTR} gene product and inhibition of cell survival, we used ponasterone A-inducible expression vectors of p75^{NTR} constructs (Fig. 5A) that exhibit a deletion of the death domain (Δ DDp75^{NTR}) and a larger deletion of the intracellular domain (Δ ICDp75^{NTR}), both of which have been shown to function as dominant negative antagonists of the intact p75^{NTR} gene product (32). The treatment of T24 cells with ibuprofen or ibuprofen + ponasterone A inhibited cell survival in a dose-dependent manner (Fig. 5B). However, T24 cells that expressed the ponasterone A-induced Δ DDp75^{NTR} (Fig. 5A) and treated with ibuprofen exhibited a rescue from inhibition of cell survival relative to ibuprofen-treated Δ DDp75^{NTR} cells in the absence of ponasterone A (Fig. 5B). Similarly, T24 cells that expressed the ponasterone A-induced Δ ICDp75^{NTR} (Fig. 5A) and treated with ibuprofen exhibited a rescue from inhibition of cell survival relative to ibuprofen-treated Δ ICDp75^{NTR} cells in the absence of ponasterone A (Fig. 5B). Hence, two ponasterone A-induced truncated forms of the p75^{NTR} were able

to rescue the T24 cells from ibuprofen induction of p75^{NTR} expression and inhibition of cell survival.

DISCUSSION

A recent population-based case-control study with individuals matched by sex, age, and race showed that intake of many classes of NSAIDs was associated with reduced bladder cancer risk (44). Taking into consideration the diversity of chemical structures with the associated differences in pharmacological properties encompassed within the broad classification of NSAIDs, it was also shown that the strength of the protective effect of NSAIDs against developing bladder cancer varied by category of formulation (44). When the categories of NSAIDs were ranked according to their association with reduced bladder cancer risk, the propionic acids (*e.g.*, ibuprofen) and acetic acids (*e.g.*, indomethacin) were more effective than acetylsalicylic acids (*e.g.*, aspirin), and acetaminophen (*i.e.*, Tylenol) was the least effective agent (44). Significantly, the rank order of NSAID induction of p75^{NTR} expression and inhibition of bladder cancer cell survival observed *in vitro* was similar to the rank order of these compounds shown to have reduced bladder cancer risk in the population-based study (44).

The p75^{NTR} was recently identified as a tumor suppressor and metastasis suppressor of both bladder and prostate cancer cells (24, 25). Although the gene encoding p75^{NTR} was intact in these cells, expression of p75^{NTR} protein was suppressed at the posttranscriptional level (26). Significantly, treatment of bladder cancer cells with several common NSAIDs restored p75^{NTR} expression and concurrently inhibited cell survival in a dose-dependent manner. Ibuprofen;

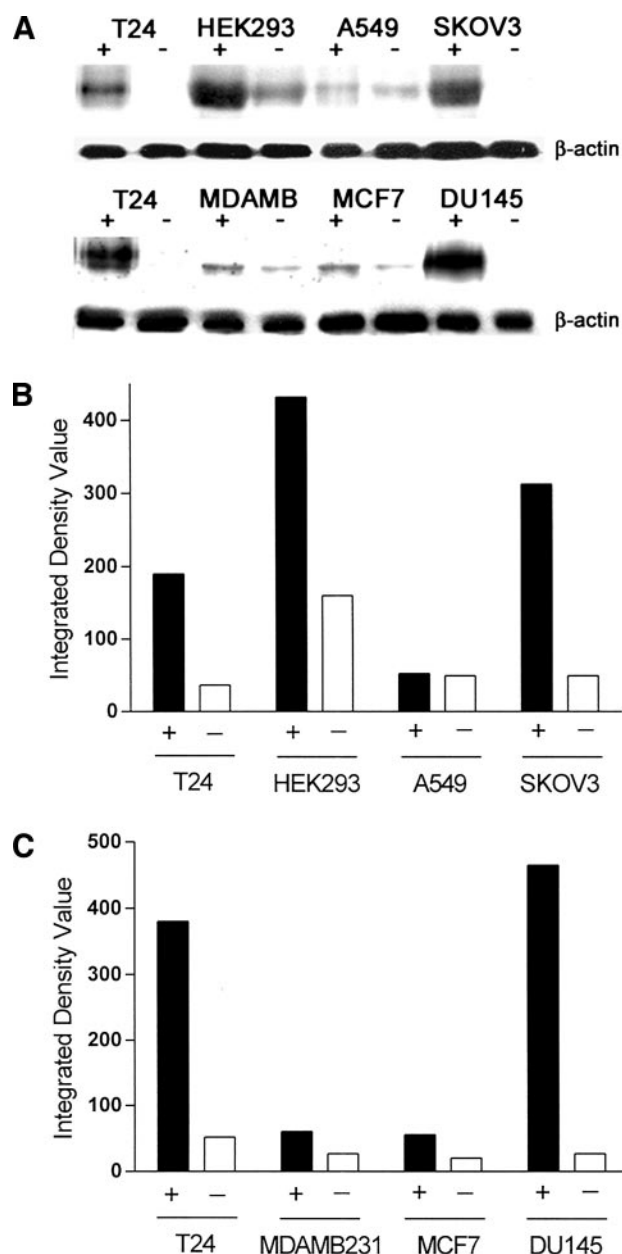


Fig. 4. Expression of the p75^{NTR} protein in tumor cells derived from the (A) bladder (T24), kidney (HEK293), lung (A549), ovary (SKOV3), estrogen-refractory breast (MDAMB), estrogen-responsive breast (MCF7), and prostate (DU145) after 48-hour treatment with 2 mmol/L ibuprofen (+) or vehicle (-). Cell lysates containing 50 µg of protein were subjected to SDS-PAGE and immunoblot analysis with a monoclonal antibody to the human p75^{NTR} protein. The same cell lysates were similarly subjected to immunoblot analysis with a monoclonal antibody to the human β-actin protein as a loading control. Densitometry of p75^{NTR} protein normalized to β-actin levels in T24 cells is shown in comparison with cell lines of the (B) kidney (HEK293), lung (A549), and ovary (SKOV3) and (C) estrogen-refractory breast (MDAMB231), estrogen-responsive breast (MCF7), and androgen-refractory prostate (DU145).

its analog, (R)-flurbiprofen, which lacks COX-inhibitory activity (43); and indomethacin were more efficacious in their induction of the p75^{NTR} protein compared with both aspirin and acetaminophen. Ibuprofen-induced expression of the p75^{NTR} protein appeared relatively receptor specific because ibuprofen did not induce expression of another cell surface tumor suppressor protein, the TGFβRII protein. Tissue specificity was variable, with tumor cells derived from the urogenital system (*i.e.*, bladder, prostate, kidney, and ovary) and colon exhibiting greater ibuprofen induction of p75^{NTR} expression than cell lines from the lung or breast. This correlation between ibuprofen

induction of p75^{NTR} expression and loss of survival of cancer cells derived from a variety of organs suggests a more general anticancer effect that may share a common mechanism mediated through induction of the p75^{NTR} tumor suppressor.

Evidence to support a mechanism of ibuprofen induction of p75^{NTR} expression causing the inhibition of bladder cancer cell growth is derived from three observations. The first observation is that the rank order of NSAID induction of p75^{NTR} protein levels correlated with the same rank order of NSAID inhibition of cell survival *in vitro* and in population-based studies of analgesic users (44). In rank order, the

Table 1 Relative survival and levels of p75^{NTR} of various cell lines after incubation in 2 mmol/L ibuprofen for 48 hours

Cell line	Organ of origin	Survival (%) after 48-h treatment with 2 mmol/L ibuprofen	Levels of p75 ^{NTR} relative to T24 cells
T24	Bladder	9 ± 3*	1.0
5637	Bladder	22 ± 10*	0.65
RT-4	Bladder	35 ± 4*	0.61
SKOV3	Ovary	58 ± 1*	1.64
HEK293	Kidney	45 ± 2*	2.27
DU145	Prostate	21 ± 4*	1.22
MDAMB231	Breast	86 ± 3	0.16
MCF-7	Breast	108 ± 4	0.15
A549	Lung	93 ± 6	0.27

NOTE. Relative survival is shown as the mean ± SE. Correlation between cell survival and levels of p75^{NTR}, *r* = -0.786; *P* < 0.001.

* *P* < 0.01.

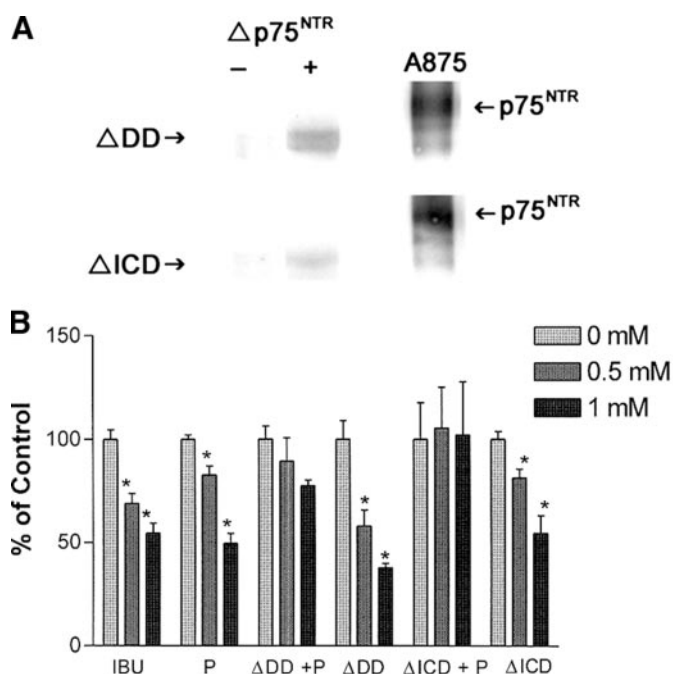


Fig. 5. A, immunoblot of the p75^{NTR} protein in either the A875 melanoma cell line (positive control) or T24 bladder cells transfected with two types of vector that can express truncated forms of the p75^{NTR} protein (Δp75^{NTR}). The cells were cotransfected for 6 hours in serum-free medium with the ecdysone receptor plasmid pVgR_XR and either ΔDDp75^{NTR} or ΔICDp75^{NTR}, followed by addition of serum containing medium for 18 hours. Subsequently, incubation of cells in 1 µmol/L ponasterone A for 24 hours was used to drive expression of the dominant negative gene products. The vectors expressed a truncated p75^{NTR} protein that lacked the death domain (ΔDD) or lacked an intracellular domain (ΔICD) after induction with ponasterone A (+), but not in the presence of vehicle alone (-). B, MTT analysis of T24 cell survival after 48-hour treatment with 0, 0.5, and 1.0 mmol/L ibuprofen (IBU), respectively. The cells were cotransfected with ponasterone A-inducible ecdysone receptor plasmid pVgR_XR and either ΔDDp75^{NTR}, which can express truncated p75^{NTR} protein that lacks the death domain (ΔDD), or ΔICDp75^{NTR}, which can express truncated p75^{NTR} protein that lacks an intracellular domain (ΔICD). Subsequently, incubation of cells in 1 µmol/L ponasterone A (P) was used to drive expression of the dominant negative gene products. Results are expressed as percentage of the vehicle-treated cells (0 mmol/L ibuprofen) showing the mean ± SE. *, *P* < 0.001 relative to the control.

propionic acids [e.g., ibuprofen and (*R*)-flurbiprofen] and acetic acids (e.g., indomethacin) induced the expression of the p75^{NTR} gene product with associated inhibition of cell survival to a greater degree than aspirin, whereas acetaminophen was the least effective compound to induce p75^{NTR} protein levels and the least effective inhibitor of bladder cancer cell survival. Hence, it appears that ibuprofen induction of the p75^{NTR} gene product may have caused, at least in part, the inhibition of bladder cancer cell survival. Because (*R*)-flurbiprofen has been shown to lack COX-inhibitory activity (43), the inhibition of survival would appear to be independent of COX. This is consistent with studies showing that specific NSAIDs can also inhibit tumor formation and growth of COX-null cell lines (45). In addition, other NSAIDs that lack COX-inhibitory activity have also been shown to have significant anticancer effects both *in vivo* (7) and *in vitro* (46). Indeed, the results from the COX-deficient HCT-116 cell line (41, 42) demonstrating that ibuprofen and (*R*)-flurbiprofen both induced expression of the p75^{NTR} gene product with associated inhibition of survival are clearly consistent with a mechanism of action independent of COX-inhibitory activity. Moreover, NSAIDs generally inhibit COX at concentrations 100- to 1,000-fold less than the concentrations that are associated with antitumor activity (47), consistent with the existence of additional cellular targets (9, 10, 13) such as the p75^{NTR} tumor suppressor. In this context, because the p75^{NTR} is a proximate receptor, it could have wide-ranging downstream effects to inhibit growth. Although the details of the p75^{NTR} signal transduction pathway have largely been extrapolated from other tumor necrosis factor receptor family members (48), inhibition of NF- κ B has been linked to p75^{NTR} (49), which is consistent with studies showing that NSAIDs can also inhibit NF- κ B (21, 22). The second observation to support a mechanism of ibuprofen induction of p75^{NTR} expression causing inhibition of bladder cancer cell growth is the dose-dependent relationship between the induction of p75^{NTR} protein levels and inhibition of survival. In addition to ibuprofen, the other two most efficacious NSAIDs examined [(*R*)-flurbiprofen and indomethacin] exhibited induction of p75^{NTR} and inhibition of cell survival at a much lower concentration when compared with either aspirin or acetaminophen. The reduction in the levels of p75^{NTR} in T24 cells treated with the higher concentrations of indomethacin appeared to result from protein degradation resulting from the rapid onset of p75^{NTR}-associated cell death. The third observation to support a mechanism of ibuprofen induction of p75^{NTR} expression causing the inhibition of bladder cancer cell growth is that dominant negative antagonism of the p75^{NTR} protein rescued ibuprofen inhibition of cell survival. The two truncated p75^{NTR} expression vectors (32) that rescued ibuprofen inhibition of survival exhibit intracellular deletions of either the death domain (Δ DDp75^{NTR}) or a larger deletion of the intracellular domain (Δ ICDp75^{NTR}) of the gene products. The intracellular moieties deleted from these two truncated p75^{NTR} expression vectors contain sequence motifs of defined elongated structure (20) designated death domains based on their apoptosis-inducing function (23). Hence, these truncated gene products (Δ DDp75^{NTR} and Δ ICDp75^{NTR}) have been shown to function as dominant negative antagonists of the full-length p75^{NTR} gene product (32). Thus, taken together, the rank order of NSAID induction of p75^{NTR} expression and inhibition of cell survival, the correlation between the dose-dependent induction of p75^{NTR} expression and inhibition of cell survival for individual NSAIDs, and the rescue of ibuprofen inhibition of cell survival by dominant negative antagonism of ibuprofen-induced p75^{NTR} protein expression all support a novel mechanism of ibuprofen inhibition of bladder cancer cell growth by induction of p75^{NTR} expression. Considering the pharmacological actions of ibuprofen, it seems possible that additional mechanisms of action may also contribute to inhibition of growth. Nevertheless, our observations provide strong support for

a novel mechanism for ibuprofen induction of p75^{NTR} expression causing inhibition of bladder cancer cell growth.

From a clinically relevant perspective, among the different types of NSAIDs, ibuprofen is one of the least damaging to the gastric mucosa (49). Patients have been maintained on high doses of ibuprofen for years without serious adverse effects (34), with advanced age having very little effect on the pharmacokinetics of ibuprofen (50). Hence, the chronic use of ibuprofen as an analgesic for many conditions, such as arthritis, could inadvertently provide a chemopreventive benefit to individuals at risk of developing bladder cancer. Because serum ibuprofen readily distributes throughout the body, a possible benefit may also extend beyond organ-confined bladder cancers to target distant locations throughout the body, thereby further facilitating the potential utility of ibuprofen as a potential agent for chemoprevention and possible treatment of NSAID-sensitive neoplasias. However, this activity of ibuprofen against bladder cancer and NSAID-sensitive neoplasias should first be confirmed in animal models for safety and efficacy. In addition, considering the broad spectrum of biological activities exhibited by NSAIDs *in vitro* and *in vivo* (44), it may be useful to screen combinatorial chemistry derivatives of the propionic and acetic acid NSAIDs for efficacy of p75^{NTR}-induced expression as it relates to chemoprevention and anticancer efficacy in similar animal models.

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