The Aryl Propionic Acid R-Flurbiprofen Selectively Induces p75NTR-Dependent Decreased Survival of Prostate Tumor Cells

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Abstract

Epidemiologic studies show that patients chronically consuming nonsteroidal anti-inflammatory drugs (NSAID) for arthritis exhibit a reduced incidence of prostate cancer. In addition, some NSAIDs show anticancer activity in vitro. NSAIDs exert their anti-inflammatory effects by inhibiting cyclooxygenase (COX) activity; however, evidence suggests that COX-independent mechanisms mediate decreased prostate cancer cell survival. Hence, we examined the effect of selected aryl propionic acid NSAIDs and structurally related compounds on the decreased survival of prostate cancer cell lines PC-3, DU-145, and LNCaP by induction of the p75NTR protein. p75NTR has been shown to function as a tumor suppressor in the prostate by virtue of its intracellular death domain that can initiate apoptosis and inhibit growth. The most efficacious compounds for induction of p75NTR and decreased survival, in rank-order, were R-flurbiprofen, ibuprofen, oxaprozin, feno-profen, naproxen, and ketoprofen. Because R-flurbiprofen and ibuprofen exhibited the greatest efficacy, we examined their dose-dependent specificity of induction for p75NTR relative to other members of the death receptor family. Whereas treatment with R-flurbiprofen or ibuprofen resulted in a massive induction of p75NTR protein levels, the expression of Fas, p55TNFR, DR3, DR4, DR5, and DR6 remained largely unchanged. Moreover, transfection of either cell line before R-flurbiprofen or ibuprofen treatment with a dominant negative form of p75NTR to antagonize p75NTR activity or p75NTR small interfering RNA to prevent p75NTR protein expression rescued both cell lines from decreased survival. Hence, R-flurbiprofen and ibuprofen selectively induce p75NTR-dependent decreased survival of prostate cancer cells independently of COX inhibition. [Cancer Res 2007;67(7):3254–62]

Introduction

Prostate cancer is the most commonly diagnosed cancer and ranks as the second leading cause of cancer-related deaths among men in the United States (1, 2). Recent epidemiologic studies found a correlation between long-term nonsteroidal anti-inflammatory drug (NSAID) use and decreased prostate cancer risk (3–6). In addition, many in vitro studies involving various human prostate cancer cell lines consistently show decreased proliferation and increased apoptosis in response to select NSAID treatment (7, 8). NSAIDs exert their anti-inflammatory activity by inhibiting cyclooxygenase (COX), the enzyme which catalyzes the conversion of arachidonic acid to prostaglandins. Two isoforms of COX exist; COX-1 is a housekeeping gene that is constitutively expressed at low levels in most cell types, whereas COX-2 is highly inducible in response to cytokines, hormones, and growth factors. COX-2 seems to play a significant role in the promotion of colon cancer with 50% of precancerous adenomatous polyps and 85% of colon carcinomas exhibiting COX-2 overexpression (9). However, the data pertaining to the role of COX-2 in prostate cancer are less conclusive. Although some studies show overexpression, others show expression is low or absent relative to normal tissue (10–14). In addition, there is not a consensus regarding the status of COX-2 expression in established prostate cancer cell lines, including LNCaP, DU-145, and PC-3 (13, 15–17). However, regardless of COX-2 expression, these cell lines all show susceptibility to select NSAID treatment (17–19). Furthermore, the anticancer activity of NSAIDs occurs at concentrations several orders of magnitude higher than those required to inhibit COX-2, and different NSAIDs show varying levels of anticancer activity (20–22). These results suggest the existence of a COX-independent mechanism by which some NSAIDs initiate apoptosis and inhibit proliferation.

The p75NTR (neurotrophin receptor) is a member of the tumor necrosis factor receptor (TNFR) superfamily capable of inducing apoptosis through a conserved intracellular death domain (23, 24). It binds neurotrophin ligands with similar affinity; however, unlike other members of the TNFR superfamily, p75NTR induces cell death and suppresses growth in the unbound state (25–29). Recently, p75NTR was identified as a tumor and metastasis suppressor in the prostate and bladder (27, 28). Although normal prostate epithelial cells express high levels of p75NTR, this expression becomes suppressed as prostate cancer progresses (30). In addition, the human prostate cancer cell lines PC-3, DU-145, and LNCaP, all derived from metastases, show little to no p75NTR expression (31). However, the p75NTR gene in these cells has remained intact, indicating that the potential for up-regulation may exist (31). Furthermore, exogenous reexpression of p75NTR in PC-3 cells suppressed growth and increased apoptosis (28, 32). This suggests potential for treatments that result in the up-regulation and re-expression of p75NTR in prostate cancer cells.

Ibuprofen and flurbiprofen belong to the aryl propionic acid class of NSAIDs. Treatment of T24 bladder cancer cells and HCT-116 colon cancer cells with ibuprofen or the enantiomer R-Flurbiprofen, which lacks COX inhibitory activity, induced a dose-dependent up-regulation of p75NTR and a corresponding decrease in survival (33). Rescue experiments involving transfection of dominant negative forms of p75NTR before ibuprofen treatment of T24 cells showed that the observed induction of p75NTR was causal of the decreased survival (33). Significantly, R-flurbiprofen treatment of TRAMP mice has been shown to lower the incidence of primary tumors and metastases of prostate cancer (34). In this context, we show that R-flurbiprofen and ibuprofen most...
effectively up-regulate p75<sub>NTR</sub> expression in both PC-3 and DU-145 cell lines relative to other aryl propionic acids. This up-regulation occurs in a dose-dependent manner and corresponds to a decrease in cell survival. The use of p75<sub>NTR</sub> dominant negatives and p75<sub>NTR</sub>-targeting small interfering RNA (siRNA) shows that the observed decreased survival is directly mediated through p75<sub>NTR</sub>, a COX-independent mechanism.

Materials and Methods

Cell lines and culture conditions. PC-3 and DU-145 cell lines were obtained from the tissue culture core facility of the Georgetown University Lombardi Comprehensive Cancer Center. LNCaP cells were obtained from the American Type Culture Collection (Manassas, VA). All cell lines were maintained in DMEM (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 Inc.)] and 5% fetal bovine calf serum (Sigma Chemical Co., St. Louis, MO). Cells were incubated in the presence of 5% CO<sub>2</sub> and air at 37°C.

Drug preparation, treatment, and cell lysis. Stock solutions were prepared by dissolving each aryl propionic acid [ibuprofen, ketoprofen, naproxen, oxaprozin, fenoprofen, and R-flurbiprofen (Myriad Pharmaceuticals Inc., Salt Lake City, UT)] in DMSO (Sigma) at a concentration of 200 mmol/L. Cells were seeded overnight at 70% to 80% confluency and were then treated for 48 h at concentrations of 0, 0.1, 0.25, 0.5, 1.0, and 2.0 mmol/L. Cell lysates of treated cells were prepared using lysis buffer [10 mmol/L Tris-Cl (pH 7.4), 10 mmol/L NaCl, 3 mmol/L MgCl<sub>2</sub>, and 0.5% Nonidet P-40] containing 1 µL/mL cocktail protease inhibitor...
The supernatant was retained, and protein concentration was determined according to the manufacturer’s protocol (Bio-Rad Laboratories, Hercules, CA). For long-term treatment of cells, ~1000 cells were seeded overnight and treated the next day with 0, 0.1, 0.2, 0.3, 0.4, and 0.5 mmol/L ibuprofen or \( R \)-flurbiprofen twice per week for 2 weeks. After 2 weeks, lysates were prepared as described above, or cells were trypsinized, resuspended, and counted using a hemocytometer.

**Immunoblot analysis.** Immunoblot analysis was done by loading 50 µg of protein onto 10% SDS-polyacrylamide gels for electrophoresis, followed by transfer to a nitrocellulose membrane (Amersham Pharmacia Biotech, Piscataway, NJ). Membranes were blocked in 5% nonfat milk (Bio-Rad Laboratories) and then incubated in the primary antibody: murine monoclonal anti-p75NTR (1:2,000, Upstate Cell Signaling Solutions, Lake Placid, NY), murine monoclonal anti-Fas (1:100, Santa Cruz Biotechnologies, Santa Cruz, CA), murine monoclonal anti-p55TNFR (1:100, Santa Cruz Biotechnologies), rat polyclonal anti-DR3 (1:200, Santa Cruz Biotechnologies), murine monoclonal anti-DR4 (1:100, Santa Cruz Biotechnologies), rat polyclonal anti-DR5 (1:500, ProSci Inc., Poway, CA), rat polyclonal anti-DR6 (2 µg/mL; Upstate Cell Signaling Solutions), mouse monoclonal anti–COX-1 (1:200, Santa Cruz Biotechnologies), goat polyclonal anti–COX-2 (1:200, Santa Cruz Biotechnologies), or murine monoclonal anti–β-actin (1:5000, Sigma). Following incubation in the primary antibody, membranes were incubated in the appropriate horseradish peroxidase–conjugated secondary antibody (1:3000, Bio-Rad Laboratories). Immunoreactivity was detected using the chemiluminescence detection reagent (Amersham Pharmacia Biotech). Several positive controls were used: A875 whole cell lysate (WCL; Tissue Culture Core Facility, Georgetown University) for p75NTR, A431 WCL (Santa Cruz Biotechnologies) for Fas, MCF-7 WCL (Santa Cruz Biotechnologies) for p55TNFR, Jurkat WCL (Upstate Cell Signaling Solutions) for DR3 and DR6, HeLa WCL (ProSci Inc.) for DR4 and DR5, U-937 WCL (Santa Cruz Biotechnologies) for COX-1, and RAW 264.7 WCL (Santa Cruz Biotechnologies) for COX-2.

**Reverse transcription-PCR.** PC-3 and DU145 cells were treated with 2.0 mmol/L \( R \)-flurbiprofen or ibuprofen. RNA was isolated following 0, 2, 4, 8, 12, 18, and 24 h of treatment using TRIzol Reagent (Invitrogen, Carlsbad, CA) according to the manufacturer’s protocol. Reverse transcription-PCR (RT-PCR) was done using the SuperScript III One-Step RT-PCR System with Platinum Taq DNA Polymerase (Invitrogen) using 250 ng RNA for p75NTR and 125 ng RNA for glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Primers were designed using Primer Quest, and their sequences are as follows: p75NTR forward 5'-AGG TGA CCT TCT GGG AAA TGG CTT-3', p75NTR reverse 5'-ATT TCC TCC GAT GCT TCT CTG GCA-3', p55TNFR forward 5'-CCA CCC ATG GCA AAT TCC ATG GCA-3', GAPDH forward 5'-CCA CCC ATG GCA AAT TCC ATG GCA-3', and GAPDH reverse 5'-TCT AGA CGG CAG GTG TCC ACC-3' (Integrated DNA Technologies, Coralville, IA). cDNA synthesis was done at 47°C for 30 min followed by denaturation at 94°C for 2 min and then 30 cycles of PCR at 94°C for 1 min, 60°C for 1 min, and 72°C for 1 min, with final extension at 72°C for 5 min. PCR products were separated on 1.5% agarose gels.

**Hoechst dye nuclear (DNA) staining.** Hoechst staining to identify apoptotic nuclei was conducted as described previously (33). PC-3, DU-145, and LNCaP cells were treated for 48 h with aryl propionic acids and then fixed in 10% formalin (Electron Microscopy Sciences, Hatfield, PA). Cells were collected, washed in PBS, centrifuged, resuspended in PBS, and dried on slides. Slides were rehydrated with PBS, washed with distilled water, and covered with a 1:1,000 aqueous dilution of Hoechst 33258 stain (Molecular Probes, Eugene, OR) for a final concentration of 10 µg/mL. After 10 min, slides were washed with distilled water, mounted, and viewed using a fluorescence microscope (Zeiss Axioplan 2 Imaging, Jena, Germany).

![Figure 2](cancerres.aacrjournals.org)
p75NTR dominant negative transfection. PC-3 and DU-145 cells were transiently transfected with one of two p75NTR dominant negative vectors described previously (35). The ∆DD vector expresses p75NTR with the death domain deleted, and the ∆ICD vector expresses p75NTR with the slightly larger intracellular domain deleted. Both are ecdysone-inducible p75NTR vectors and, therefore, were each cotransfected with the ecdysone receptor plasmid pVgRxR. The transfection was done with LipofectAMINE reagent (Invitrogen) in serum-free medium for 6 h, after which serum-containing medium was added. After 18 subsequent hours, cells were incubated in 1 μmol/L ponasterone A (Invitrogen) for 24 h to drive the expression of the dominant negative gene products. Following incubation with ponasterone A, cells were treated with aryl propionic acids for 48 h, and relative cell survival was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (Roche Applied Science, Indianopolis, IN).

siRNA transfection. PC-3 and DU-145 cells were transfected for 24 h with nontargeting or p75NTR-specific siRNA [Dharmacon RNA Technologies (Dulplex D-009340-03), Lafayette, CO] at a final concentration of 100 nmol/L according to the manufacturer's protocol using transfection reagents DharmaFECT 1 for DU-145 cells and DharmaFECT 2 (Dharmacon RNA Technologies) for PC-3 cells. After transfection, the cells were treated with aryl propionic acids for 48 h, followed by determination of p75NTR protein expression or relative cell survival by MTT assay (Roche Applied Science).

Results

Aryl propionic acids selectively induce p75NTR expression and decrease cell survival. We examined the ability of R-flurbiprofen and ibuprofen, as well as four other members of the aryl propionic acid family, including oxaprozin, fenoprofen, naproxen, and ketoprofen to induce the expression of p75NTR protein in PC-3 and DU-145 human prostate cancer cells. In both cell lines, R-flurbiprofen was the most efficacious for the induction of p75NTR expression followed by ibuprofen, oxaprozin, fenoprofen, naproxen, and finally, ketoprofen, which was the least effective compound (Fig. 1A). For subsequent experiments, only R-flurbiprofen and ibuprofen were used because they were the most effective of the six aryl propionic acids tested. Treatment with R-flurbiprofen or ibuprofen also resulted in an induction of p75NTR in LNCaP cells, which are androgen responsive (Fig. 1B). To elucidate the mechanism by which these aryl propionic acids may be inducing p75NTR protein expression, we determined relative p75NTR mRNA levels at various time points following 2.0 mmol/L R-flurbiprofen or ibuprofen treatment (Fig. 1C). In both cell lines, R-flurbiprofen and ibuprofen significantly induced p75NTR message level. This induction is first noticeable within 4 h of treatment in both cell lines with both drugs.

The aryl propionic acids are commonly used as NSAIDs that act through COX inhibition. Therefore, we examined the status of COX expression in both cell lines and determined whether the expression level of either isoform changed in response to treatment with R-flurbiprofen and ibuprofen (Fig. 1D). Both PC-3 and DU-145 cells expressed COX-1 at low levels, and expression remained unchanged following R-flurbiprofen or ibuprofen treatment. PC-3 cells lacked COX-2 expression; however, expression was induced upon treatment with R-flurbiprofen or ibuprofen. DU-145 cells also lacked COX-2 expression, and no induction was observed following treatment with either compound.

Examination of the effect of R-flurbiprofen and ibuprofen on survival of PC-3, DU-145, and LNCaP cells after 48-h treatment resulted in a dose-dependent decrease in survival that corresponded with the observed induction of p75NTR (Fig. 2A and B). Again, R-flurbiprofen was more efficacious than ibuprofen, resulting in a greater dose-dependent decrease in survival. Because p75NTR contains an intracellular death domain capable of initiating apoptosis, we used Hoechst staining to identify fragmented nuclei typical of apoptotic cells. Treatment with 2.0 mmol/L R-flurbiprofen or ibuprofen resulted in an induction of apoptotic cells, indicating that apoptosis was at least partially responsible for the observed decrease in survival (Fig. 2C and D).

p75NTR is a member of the TNFR superfamily whose members contain an intracellular death domain capable of inducing apoptosis (23, 24). Although 48-h treatment with R-flurbiprofen resulted in a substantial dose-dependent induction of p75NTR, the other death receptors, including Fas, p55TNFR, DR3, DR4, DR5, and DR6, showed little, if any, induction in PC-3 cells (Fig. 3A). Similar results were observed following treatment with ibuprofen (Fig. 3B). There seemed to be a slight induction of DR4 with ibuprofen; however, the magnitude of the response was small relative to the induction observed for p75NTR expression (Fig. 3B). The addition of the ligand corresponding to each death receptor had no effect on the induction of death receptors by R-flurbiprofen or ibuprofen (data not shown). Therefore, R-flurbiprofen and ibuprofen selectively induced p75NTR, whereas protein expression of the other TNFR family members remained largely unchanged.
Long-term R-flurbiprofen and ibuprofen treatment results in p75<sup>NTR</sup> induction and decreased survival at lower drug concentrations. Epidemiologic studies have shown a correlation between long-term continuous NSAID use and a decreased risk of prostate cancer (3, 4). Therefore, we examined whether chronic treatment with R-flurbiprofen or ibuprofen for 2 weeks at lower concentrations of 0, 0.1, 0.2, 0.3, 0.4, and 0.5 mmol/L may be equally effective or more effective than the 48-h acute treatment at higher concentrations in PC-3 and DU-145 cells. Long-term treatment with R-flurbiprofen resulted in a dose-dependent decrease in cell growth with severe growth arrest even at 0.1 mmol/L and almost a complete loss of growth at 0.3 mmol/L for PC-3 cells and 0.5 mmol/L for DU-145 cells (Fig. 4A). In addition, there was a dose-dependent increase in p75<sup>NTR</sup> levels beginning at 0.1 mmol/L, which corresponds to the concentration at which decreased growth first occurred (Fig. 4B). Similarly, long-term treatment with ibuprofen resulted in a dose-dependent decrease in survival and induction of p75<sup>NTR</sup> at lower concentrations than observed with 48-h ibuprofen treatment, but was not as efficacious as long-term induction of p75<sup>NTR</sup> at lower concentrations than observed with ibuprofen (Fig. 4C and D).

Decreased survival caused by R-flurbiprofen and ibuprofen is mediated through p75<sup>NTR</sup>. The results presented thus far show that the induction of p75<sup>NTR</sup> is associated with decreased survival of PC-3 and DU-145 cells in response to R-flurbiprofen or ibuprofen treatment. To determine if the observed decreased survival is causally mediated through R-flurbiprofen- and ibuprofen-dependent induction of p75<sup>NTR</sup>, ponasterone A-inducible expression vectors for one of two different dominant negative forms of p75<sup>NTR</sup> were transfected into PC-3 and DU-145 cells before R-flurbiprofen or ibuprofen treatment. The dominant negative vectors both express truncated forms of p75<sup>NTR</sup>. ΔDDp75<sup>NTR</sup> has a deletion of the death domain, and ΔICDp75<sup>NTR</sup> has a deletion of the larger intracellular domain that includes the death domain. PC-3 and DU-145 cells treated with 0, 0.5, or 1.0 mmol/L R-flurbiprofen alone or with ponasterone A resulted in a similar dose-dependent decrease in survival (Fig. 5A and B). R-flurbiprofen treatment of cells transfected with either ΔDDp75<sup>NTR</sup> or ΔICDp75<sup>NTR</sup> but not treated with ponasterone A yielded the same result (Fig. 5A and B). However, cells transfected with either of the two dominant negative expression vectors followed by ponasterone A treatment to induce expression of the gene product exhibited increased survival following R-flurbiprofen treatment compared with cells not expressing dominant negative p75<sup>NTR</sup> (Fig. 5A and B). Similar results were observed with ibuprofen in PC-3 and DU-145 cells, where expression of the dominant negative forms of p75<sup>NTR</sup> partially prevented decreased survival due to ibuprofen (Fig. 5C and D).

We did similar rescue experiments using siRNA targeted against p75<sup>NTR</sup>. Transfection of PC-3 cells with p75<sup>NTR</sup> siRNA before R-flurbiprofen treatment almost completely prevented the induction of p75<sup>NTR</sup> protein, whereas transfection with nontargeting siRNA did not prevent the induction of p75<sup>NTR</sup> protein (Fig. 6A). Treatment with R-flurbiprofen at 0, 0.5, and 1.0 mmol/L in untransfected PC-3 cells or in PC-3 cells transfected with nontargeting siRNA resulted in a similar dose-dependent decrease in cell viability (Fig. 6A). However, treatment of PC-3 cells transfected with p75<sup>NTR</sup> siRNA partially prevented R-flurbiprofen-dependent decreased survival at 0.5 and 1.0 mmol/L concentrations (Fig. 6A). Similarly, transfection of PC-3 or DU-145 cells with p75<sup>NTR</sup>-targeting siRNA before ibuprofen treatment also almost completely blocked the induction of p75<sup>NTR</sup>, whereas transfection with nontargeting siRNA did not block induction (Fig. 6C and D). Treatment with 0, 0.5, and 1.0 mmol/L ibuprofen

![Figure 4](image-url)
led to a dose-dependent decrease of cell survival in untransfected cells and in cells transfected with nontargeting siRNA, whereas transfection with p75NTR siRNA before ibuprofen treatment prevented a decrease in survival at the 1.0 mmol/L concentration (Fig. 6C and D). Transfection of DU-145 cells with p75NTR-targeting siRNA only partially prevented p75NTR induction upon R-flurbiprofen treatment (Fig. 6B). Consistently, only a partial rescue from R-flurbiprofen–dependent decreased survival was observed in these cells.

Discussion

The profens, also referred to as 2-aryl propionic acid derivatives, are a class of NSAIDs that share several characteristics. They can be reversible inhibitors of COX, are highly bound to plasma albumin, and are weak acids (36). They exist as enantiomer pairs, and it is generally the S-enantiomer, but not the R-enantiomer, that possesses potent COX inhibitory activity (37). Some profens have been in use for roughly 30 years, most commonly as treatment for inflammation due to rheumatoid arthritis (38–43). However, based on emerging studies associating NSAIDs with anticancer activity, various profens have been examined for their efficacy as chemopreventive and chemotherapeutic agents in a variety of cancer types (44–46). In this study, we examined the ability of six profens, including R-flurbiprofen, ibuprofen, oxaprozin, fenoprofen, naproxen, and ketoprofen, to induce the expression of the p75NTR tumor suppressor in prostate cancer cells. We observed a dose-dependent induction of p75NTR by these drugs; however, the level of efficacy varied with R-flurbiprofen followed by ibuprofen as the most effective. Consistently, previous reports identify these two drugs as promising agents in prostate cancer treatment. R-flurbiprofen was shown to inhibit the progression of prostate cancer in the TRAMP mouse, whereas ibuprofen was shown to reduce survival of LNCaP and DU-145 human prostate cancer cells (8, 21, 34). In addition, treatment of the human colon cancer COX-null cell line HCT-116 with either of these drugs resulted in reduced cell survival, indicating that these drugs possess the ability to inhibit growth through a COX-independent mechanism (33). The remaining four profens induced p75NTR to a lesser degree with oxaprozin as the next most effective followed by fenoprofen, naproxen, and ketoprofen as the least effective. Of these four drugs, naproxen has also been shown to suppress growth of prostate cancer cells; however, it was less effective when compared with ibuprofen (21).

The results showed that an induction of p75NTR was associated with significantly decreased survival of PC-3, DU-145, and LNCaP cells. Although these cell lines exhibited low levels of p75NTR expression at lower concentrations of R-flurbiprofen and ibuprofen, the levels of p75NTR were below the threshold for decreased survival. As the concentration of R-flurbiprofen or ibuprofen increased, some variability between levels of p75NTR and decreased survival was observed.
survival may have resulted from discordance around inflection points. At higher concentrations of \( R \)-flurbiprofen and ibuprofen, levels of p75\( ^{NTR} \) were concordant with decreased survival. This cause and effect relationship between higher levels of p75\( ^{NTR} \) and decreased survival was subsequently shown in the rescue experiments with dominant negatives and siRNA to p75\( ^{NTR} \).

The exact mechanism by which \( R \)-flurbiprofen and ibuprofen induce p75\( ^{NTR} \) protein expression remains under investigation. However, an examination of p75\( ^{NTR} \) mRNA levels by RT-PCR at various time points showed that treatment of PC-3 and DU-145 cells with 2.0 mmol/L \( R \)-flurbiprofen or ibuprofen resulted in a relatively strong induction of p75\( ^{NTR} \) mRNA in all cases within 4 h and continuing until between 8 and 12 h, after which p75\( ^{NTR} \) mRNA remained constant. These results suggest that the observed induction of p75\( ^{NTR} \) protein expression is a result of an increase in p75\( ^{NTR} \) mRNA. This may occur by an increase in mRNA stability because previous work showed that p75\( ^{NTR} \) protein expression is lost in prostate cancer cell lines due to increased mRNA instability that is mediated through the 3' untranslated region (31). Therefore, it is possible that treatment with \( R \)-flurbiprofen or ibuprofen alleviates this instability.

COX-2 catalyzes the conversion of arachidonic acid to prostaglandins, which are associated with increased survival, decreased apoptosis, and promotion of angiogenesis (13). Hence, inhibition of COX-2 has been suggested as a mechanism by which NSAIDs decrease cancer cell survival. However, an increasing body of literature suggests that many NSAIDs act through a COX-independent mechanism to achieve anticancer activity in the prostate (13). To elucidate whether COX plays a role in aryl propionic acid–dependent decreased prostate cancer cell survival, we examined the status of COX-1 and COX-2 expression. As expected, the housekeeping isoform COX-1 was expressed at low levels in PC-3 and DU-145 cells, and neither \( R \)-flurbiprofen nor ibuprofen induced its expression. Because COX-1 is not overexpressed in prostate cancer, is not generally associated with tumor development, and was minimally expressed in both cell lines at a

![Figure 6](cancerres.aacrjournals.org)
constant level, it seems that R-flurbiprofen and ibuprofen inhibited survival through a mechanism other than COX-1 inhibition. DU-145 cells lacked any COX-2 expression, whereas PC-3 cells only exhibited expression upon R-flurbiprofen or ibuprofen treatment. An induction of COX-2 following NSAID treatment has been observed previously in PC-3 cells, as well as several other cell types (47–49). Although ibuprofen treatment led to an up-regulation of COX-2, the effect would be negated because ibuprofen would inhibit COX-2 activity. Because R-flurbiprofen lacks COX inhibitory activity, COX-2 could potentially remain active upon induction by R-flurbiprofen. However, 48-h R-flurbiprofen treatment of PC-3 cells was more potent for decreased survival than R-flurbiprofen in DU-145 cells, as well as more potent than ibuprofen treatment in both PC-3 and DU-145 cells. Therefore, there is no evidence that an induction of COX-2 in PC-3 cells promotes survival or reduces the effect of R-flurbiprofen treatment. Indeed, induction of COX-2 in PC-3 cells was associated with the greatest decrease in cell survival. This further supports the hypothesis that these drugs act as anticancer agents independently of COX-2.

p75NTR belongs to the TNFR superfamily, which includes Fas, p55TNFR, DR3, DR4, DR5, and DR6 (35). These receptors initiate instructive apoptosis through a homologous intracellular death domain (50). With the exception of p75NTR, they induce apoptosis upon ligand binding (51). In contrast, p75NTR initiates apoptosis and suppresses growth in a ligand-independent manner in prostate and bladder cancer cells (26–29). Previous studies showed that some NSAIDs up-regulate expression of various death receptors (52, 53). To eliminate the possibility that the observed decrease in survival was due to apoptosis initiated by another member of the TNFR family, we examined expression of the remaining six family members in PC-3 cells following R-flurbiprofen or ibuprofen treatment. Neither p55TNFR nor DR6 were detected with or without treatment. Fas and DR5 were weakly expressed and remained constant upon treatment. DR3 expression was stronger than the other family members, but weak relative to p75NTR, and remained constant following treatment. DR4 seems slightly up-regulated by ibuprofen; however, its expression levels were minimal relative to the robust induction of p75NTR. Similar results were observed in the presence of the appropriate ligand for each receptor. Therefore, other than p75NTR, the death receptors capable of initiating apoptosis do not seem to play a significant role in the decreased survival caused by R-flurbiprofen and ibuprofen treatment of PC-3 and DU-145 prostate cancer cells.

It seems that R-flurbiprofen and ibuprofen decrease survival of PC-3 and DU-145 cells by a COX-independent mechanism. p75NTR is the only TNFR superfamily member significantly up-regulated by treatment with aryl propionic acids, and its up-regulation is associated with decreased survival in both cell lines. This suggests p75NTR induction as a COX-independent mechanism initiating the observed decrease in survival. Hence, three different approaches including p75NTR-targeting siRNA and two different p75NTR dominant negatives were used to examine a causal relationship between p75NTR levels and decreased cell survival. Each method increased survival of PC-3 and DU-145 cells treated with R-flurbiprofen or ibuprofen relative to untransfected cells. Collectively, these results provide strong evidence that p75NTR is at least partially causal of the decreased survival due to treatment with R-flurbiprofen or ibuprofen.

Because chemopreventive and chemotherapeutic drugs are often administered chronically, we examined whether long-term treatment enabled R-flurbiprofen and ibuprofen to maintain their anticancer activity at lower concentrations than observed with acute treatments (48 h). Both R-flurbiprofen and ibuprofen were highly effective in inducing p75NTR and reducing growth at lower concentrations after chronic treatment. In particular, long-term R-flurbiprofen treatment at the lowest concentration of 0.1 mmol/L exhibited an 85% reduction of growth relative to the control and essentially total loss of growth at concentrations of 0.3 mmol/L or greater in PC-3 cells. Likewise, in DU-145 cells, 0.1 mmol/L long-term R-flurbiprofen treatment resulted in 66% reduction of growth and almost a total loss of growth at 0.5 mmol/L treatment. This dramatic loss of growth also correlates with the induction of p75NTR, which occurred in a dose-dependent manner starting at 0.1 mmol/L. Similarly, ibuprofen exhibited a substantial loss of growth due to chronic treatment at lower concentrations relative to acute 48-h treatment at higher concentrations in both PC-3 and DU-145 cells. The results of the chronic treatment experiments are especially significant because they indicate that these drugs, especially R-flurbiprofen, could be highly effective in inhibiting prostate cancer growth at clinically achievable concentrations with long-term use. In clinical trials, R-flurbiprofen and ibuprofen have been given at doses that resulted in plasma concentrations of 0.14 and 0.48 mmol/L, respectively (54). These concentrations are within the range of concentrations used in our chronic study. Hence, the data collectively demonstrate the activity of R-flurbiprofen and ibuprofen as anticancer agents in the prostate and convincingly implicate p75NTR induction as a COX-independent mechanism by which this anticancer activity is achieved.

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