Carprofen induction of p75NTR-dependent apoptosis via the p38 mitogen-activated protein kinase pathway in prostate cancer cells

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Abstract
The p75 neurotrophin receptor (p75NTR) functions as a tumor suppressor in prostate epithelial cells, where its expression declines with progression to malignant cancer. Previously, we showed that treatment with R-flurbiprofen or ibuprofen induced p75NTR expression in several prostate cancer cell lines leading to p75NTR-mediated decreased survival. Using the 2-phenyl propionic acid moiety of these profens as a pharmacophore, we screened an in silico database of 30 million compounds and identified carprofen as having an order of magnitude greater activity for induction of p75NTR levels and inhibition of cell survival. Prostate (PC-3 and DU-145) and bladder (T24) cancer cells were more sensitive to carprofen induction of p75NTR, associated loss of survival than breast (MCF-7) and fibroblast (3T3) cells. Transfection of prostate cell lines with a dominant-negative form of p75NTR before carprofen treatment partially rescued cell survival, showing a cause-and-effect relationship between carprofen induction of p75NTR levels and inhibition of survival. Carprofen induced apoptotic nuclear fragmentation in prostate but not in breast cancer cells (6). Furthermore, small interfering RNA knockdown of the p38 mitogen-activated protein kinase (MAPK) protein prevented induction of p75NTR by carprofen in both prostate cell lines. Carprofen treatment induced phosphorylation of p38 MAPK as early as within 1 min.

Expression of a dominant-negative form of MK2, the kinase downstream of p38 MAPK frequently associated with signaling cascades leading to apoptosis, prevented carprofen induction of the p75NTR protein. Collectively, we identify carprofen as a highly potent profen capable of inducing p75NTR-dependent apoptosis via the p38 MAPK pathway in prostate cancer cells. [Mol Cancer Ther 2008;7(11):3539–45]

Introduction
The p75 neurotrophin receptor (p75NTR) is a 75-kDa cell surface receptor glycoprotein that shares both structural and sequence homology with the tumor necrosis factor receptor superfamily of proteins (1, 2). Some of these proteins (e.g., p75NTR, p55TNFR, Fas, DRs3-6, and EDAR) have similar sequence motifs of defined elongated structure (1) designated “death domains” based on their apoptosis-inducing function (2). In the human prostate, the p75NTR protein is progressively lost in pathologic cancer tissues (3). The proportion of epithelial cells that have retained p75NTR expression in the organ-confined pathologic prostate is inversely associated with increasing Gleason score and preoperative serum prostate-specific antigen concentrations (4). In addition, immunoblot of human prostate epithelial cell lines derived from metastases exhibit a further reduction of p75NTR expression (5). Significantly, although expression of the p75NTR protein is suppressed, the gene encoding p75NTR appears intact in these prostate cancer cells (6). The loss of p75NTR expression is a result of a loss of mRNA stability (6). Following ectopic reexpression of the p75NTR in these cancer cells, their rate of apoptosis increased (7). Additionally, the same ectopically expressing p75NTR cancer cells exhibited a retardation of cell cycle progression characterized by accumulation of cells in G1 phase with a corresponding reduction of cells in the S phase of the cell cycle (7). Consistent with these observations, the p75NTR has been characterized with both tumor suppressor and metastasis suppressor activity in prostate cancer cells (7, 8).

Several studies have shown that nonsteroidal anti-inflammatory drugs (NSAID) are effective as anticancer agents for colorectal, breast, pancreatic, squamous cell carcinoma of the head and neck, bladder, ovarian, lung, and prostate cancers (9, 10). With respect to prostate cancer, retrospective studies indicate that there is a significantly reduced risk of prostate cancer associated with regular use of NSAIDs (11–13). In vivo studies using rodents have indicated that NSAIDs can decrease the size of prostate tumors (14, 15) and suppress the metastasis of prostatic cancer (14, 16). There is no common mechanism of action underlying NSAIDs effectiveness against cancer cells. Some
NSAIDs inhibit the cyclooxygenases (COX) that convert arachidonic acid to prostaglandins (17). Prostaglandins are thought to contribute to tumor growth by inhibiting apoptosis (18) and by inducing the formation of new blood vessels needed to sustain tumor growth (19). 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 (20). In addition, NSAIDs that lack COX inhibitory activity can still have significant anticancer effects both in vivo (21) and in vitro (22). Similarly, growth of the DU-145 prostate cancer cell line that lacks expression of COX-1 and COX-2 is inhibited by NSAIDs (23). Interestingly, R-flurbiprofen and ibuprofen have been shown to induce p75NTR levels leading to apoptosis in prostate cancer cell lines (23). These profens activated the p38 mitogen-activated protein kinase (MAPK) pathway leading to stabilization of p75NTR mRNA and increased levels of p75NTR protein that subsequently induced apoptosis of the prostate cancer cells (24). In this report, we used the 2-phenyl propionic acid moiety of the profen as a pharmacophore for an in silico search of related compounds and identified carprofen as having an order of magnitude greater activity for induction of p75NTR levels and inhibition of cell survival. Carprofen activity occurred through rapid phosphorylation of p38 MAPK, which signaled through MK2 to increase levels of p75NTR protein and stimulate apoptosis in the prostate cancer cells.

Materials and Methods

Cell Lines and Culture Conditions

PC-3 and DU-145 prostate cell lines were obtained from the Tissue Culture Core Facility of the Georgetown University Lombardi Comprehensive Cancer Center. T24 bladder, MCF-7 breast, and 3T3 fibroblast cells were obtained from the American Type Culture Collection. All cell lines were maintained in DMEM (Mediatech) 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)] and 10% fetal bovine serum (Sigma). Cells were incubated in the presence of 5% CO₂ and air at 37°C.

Figure 1. Immunoblots of p75NTR levels in PC-3 and DU-145 cells following 48 h treatment with 0, 0.1, 0.25, 0.5, and 1.0 mM drug. The compound ID of each drug is given adjacent to its chemical structure. The A875 melanoma cell line was used as a positive control for p75NTR expression.
Drug Preparation, Treatment, and Cell Lysis

Using 2-phenyl propionic acid as a pharmacophore, we searched an in silico database of ~30 million compounds from which nine aryl propionic acids were selected for further analysis. Stock solutions were prepared by dissolving each aryl propionic acid in DMSO (Sigma) at a concentration of 200 mM/L. Cells were seeded overnight at 70% to 80% confluency and were then treated for 48 h at concentrations of 0, 20, 40, 60, 80, and 100 μM/L carprofen. A875 cell lysates were used as positive controls for p75NTR expression.

Immunoblot Analysis

Immunoblot analysis was done as described previously (23). Membranes were incubated in the primary antibody: murine monoclonal anti-p75NTR (1:2,000; Upstate Cell Signaling Solutions), rabbit polyclonal phosphorylated p38 MAPK (1:1,000), mouse monoclonal anti-p38 (1:1,000; Cell Signaling Technology), or murine monoclonal anti-β-actin (1:5,000; Sigma). Membranes were subsequently incubated in goat anti-mouse or goat anti-rabbit horseradish peroxidase conjugated secondary antibodies (Bio-Rad Laboratories). Immunoreactivity was visualized with a chemiluminescence detection reagent (Amersham Pharmacia Biotech). The positive control for p75NTR expression was a whole-cell lysate of A875 cells (Dr. Moses Chao, Cornell University).

Cell Survival Assay with p75NTR Dominant-Negative Transfection and Hoechst Dye Nuclear Staining

An equal number of viable cells (2 × 10³ per well) in 96-well culture plates (final volume of 100 μL/well culture medium) were incubated for 4 h at 37°C in a humidified atmosphere of 5% CO₂. Some cells were also transiently transfected with a p75NTR dominant-negative vector described previously (24–26). The ΔICD vector expresses a p75NTR gene product with the intracellular domain (ICD) deleted. The ΔICD is an edeysone-inducible p75NTR vector and therefore was cotransfected with the edeysone receptor plasmid pVgRxR. The transfection was done with Lipofect-AMINE 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 μM/L ponasterone A (Invitrogen) for 24 h to drive expression of the dominant-negative gene product. Following incubation with ponasterone A, cells were treated with carprofen (0-100 μM/L) for 48 h and relative cell survival was determined with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide labeling reagent (final concentration, 0.5 mg/mL; Roche Diagnostics). Subsequently, cells were incubated overnight with 100 μL/well solubilization solution and the samples were quantified at 570 nm using a microtiter plate reader (Bio-Rad Laboratories). Hoechst dye nuclear (DNA) staining to identify apoptotic nuclei was conducted as described previously (25). PC-3, DU-145, MCF-7, and 3T3 cells were treated for 48 h with carprofen and then fixed in 10% formalin (Electron Microscopy Sciences). Some cells were transfected with the ΔICDp75NTR plus ponasterone A before carprofen treatment.

Small Interfering RNA Transfection

Cells were transfected for 72 h with nontargeting small interfering RNA (siRNA) or siRNA specific for p38α (J-003512-20; Dharmacon RNA Technologies) at final concentrations of 100 nmol/L according to the manufacturer’s protocol. Transfection reagent DharmaFECT 1 was used for DU-145 cells, and DharmaFECT 2 was used for PC-3 cells (Dharmacon RNA Technologies). After transfection, the cells were treated with carprofen for 48 h followed by determination of p75NTR protein expression.

MK2 Dominant-Negative Transfection

PC-3 and DU-145 cells were transiently transfected with a MK2 dominant-negative vector (MK2-K76R) described previously (27). The transfection was done with LipofectAMINE reagent (Invitrogen) in serum-free medium for 6 h, after which serum-containing medium was added for 24 h to allow expression of the dominant-negative gene product. Cells were treated with carprofen (100 μM/L) for 48 h and expression of p75NTR protein was determined by immunoblot with mouse monoclonal anti-p75NTR (1:2,000; Millipore).
Statistical Analysis

The statistical differences between data sets and/or means were analyzed by ANOVA or the Mann-Whitney test using the Prizm program (GraphPad Software) and the data expressed as the mean ± SE. Data were considered statistically significant when \( P \leq 0.05 \).

Results

Carprofen Exhibits Superior Efficacy of the Aryl Propionic Acids to Induce p75NTR Levels Associated with Cell-Specific Decreased Survival

Analysis of the 2-phenyl propionic acid pharmacophore homology search identified nine aryl propionic acids that were screened for activity to induce expression of p75NTR protein in PC-3 and DU-145 human prostate cancer cells. Initially, the PC-3 and DU-145 cell lines were selected because they are the only two prostate tumor cell lines included in the NIH Developmental Therapeutics Program Anticancer Drug Discovery Program. The immunoblots showing activity of each compound to induce p75NTR were placed in rank-order (Fig. 1). In both cell lines, carprofen exhibited superior efficacy for induction of p75NTR expression at a concentration of \( \leq 100 \) \( \mu \)mol/L compared with all other aryl propionic acids examined (Fig. 1). At lower concentrations, carprofen selectively induced expression of p75NTR protein at \( \geq 40 \) \( \mu \)mol/L in PC-3 and DU-145 prostate cancer cells, as well as in the T24 bladder cancer cell line, but not in the MCF-7 breast cancer cell line or the 3T3 fibroblast cell line (Fig. 2A). The T24 bladder cancer cell line was included as a positive control because they were shown previously to be sensitive to profen (ibuprofen and R-flurbiprofen)-induced p75NTR-dependent decreased survival, whereas MCF-7 and 3T3 cells were included as negative controls because they were shown previously not to be sensitive to profen (ibuprofen and R-flurbiprofen)-induced decreased survival (25).

Carprofen treatment selectively decreased the survival of cells in rank-order with PC-3 and DU-145 prostate cancer cells exhibiting greatest sensitivity to dose-dependent decreased survival followed by the T24 bladder cancer cells and with MCF-7 and 3T3 fibroblasts the least sensitive to carprofen-induced decreased survival (Fig. 2B). Significantly, there was a strong association between the dose-dependent induction of p75NTR levels (Fig. 2A) and decreased survival of specific cell types following carprofen treatment (Fig. 2B).

Carprofen Induced Decreased Prostate Cancer Cell Survival Is Dependent on p75NTR

To establish a causal relationship between carprofen induction of p75NTR protein expression and inhibition of cell survival, we used a ponasterone A-inducible expression vector for p75NTR that exhibits a deletion of the intracellular death domain (ΔICDp75NTR) shown to function as a dominant-negative antagonist of the intact p75NTR gene product (23–26). The treatment of both PC-3 and DU-145 cells with carprofen or carprofen plus ponasterone A inhibited cell survival in a dose-dependent manner (Fig. 3). However, both PC-3 and DU-145 cell lines induced with ponasterone A to express ΔICDp75NTR exhibited a significant (\( P < 0.001 \)) partial rescue from carprofen-mediated inhibition of cell survival relative to carprofen-treated ΔICDp75NTR cells in the absence of ponasterone A (Fig. 3). Subsequently, we examined Hoechst-stained nuclear morphology to identify fragmented nuclei typical of apoptotic cells with the exception of T24 bladder cells for which we have shown previously profen-induced apoptotic nuclear fragmentation (25). Treatment of the two prostate cancer cell lines (DU-145 and PC-3) with carprofen induced a dose-dependent (0-100 \( \mu \)mol/L) fragmentation of nuclei (Fig. 4). As negative controls, the MCF-7 and 3T3 cells that were not induced by carprofen to express p75NTR (Fig. 2A) did not undergo carprofen-dependent apoptotic nuclear fragmentation (Fig. 4). Expression of the ΔICDp75NTR dominant-negative vector before carprofen treatment partially rescued nuclear fragmentation in the PC-3 and DU-145 prostate cells, whereas the MCF-7 and 3T3 negative control cells did not exhibit fragmented nuclei (Fig. 4).
Carprofen Induction of p75NTR Occurs via the p38 MAPK Pathway

An earlier study from our laboratory (24) implicated the aryl propionic acids, R-flurbiprofen and ibuprofen, in the induction of p75NTR via the p38 MAPK pathway. Because carprofen, an aryl propionic acid, exhibits an order of magnitude greater potency (Fig. 2A) than R-flurbiprofen and ibuprofen for the induction of p75NTR expression levels (23, 24), we examined the effect of siRNA knockdown of the p38α MAPK isoform on p75NTR levels following treatment with carprofen. We showed previously that p38α MAPK is the predominant isoform expressed in PC-3 and DU-145 cells (24). Whereas treatment with carprofen induced p75NTR expression levels, transfection of prostate cancer cells with p38α siRNA before carprofen treatment prevented induction of p75NTR relative to untransfected cells or cells transfected with nontargeting siRNA (Fig. 5A).

Because the MK2 kinase is downstream of p38 MAPK and was shown previously to be involved in profen induction of p75NTR (24), we used a dominant-negative expression vector for MK2 to determine involvement in carprofen induction of p75NTR. Treatment with carprofen alone induced expression of p75NTR in both PC-3 and DU-145 cells, whereas transfection of dominant-negative MK2 before carprofen treatment decreased the induction of p75NTR (Fig. 6).

Discussion

Carprofen is a propionic acid NSAID that induced p75NTR levels in prostate cancer cell lines with an order of magnitude greater efficacy than the related propionic acid NSAIDs, R-flurbiprofen and ibuprofen (23). Concomitant with the superior efficacy of carprofen to induce levels of p75NTR was its activity to inhibit cell survival via apoptosis. Our previous studies have shown a strong cause-and-effect relationship between induced levels of p75NTR and induction of apoptosis in cancer cell lines (23, 25). When expression levels are induced, p75NTR appears to be a robust marker of drug-induced apoptosis (23, 25). Because carprofen exhibited some degree of cell-specific induction of p75NTR-associated apoptosis, we focused on the PC-3 and DU-145 prostate cancer cell lines, which were most responsive to carprofen treatment, and coincidently are the...
Carprofen Induces p75NTR-Dependent Apoptosis

In prostate cancer cell lines, reexpression of p75 NTR induces modifications to several downstream signal transduction cascades leading to apoptosis. Initially, p75 NTR expression down-regulates components of the nuclear factor-κB and c-Jun NH2-terminal kinase pathways preventing nuclear translocation of both these prosurvival effectors, Smac, Bak, and Bad, and conversely a decrease in the prosurvival effector, Bcl-xL (26), leading to a reduction in X-linked inhibitor of apoptosis protein and cleavage of caspase-9 and caspase-7 followed by poly(ADP-ribose) polymerase cleavage and nuclear fragmentation in PC-3 cells (26). Hence, reexpression of p75NTR appears to promote partial redifferentiation, cell cycle arrest, and apoptosis in prostate cancer cells, thereby providing a rationale for investigation of compounds that may be used for p75NTR-dependent therapeutics.

Prostate cancer cells evade the apoptotic effects of p75NTR expression by loss of p75NTR mRNA stability with concomitant suppression of p75NTR protein levels (6). Conversely, R-flurbiprofen and ibuprofen stabilize p75NTR mRNA with concomitant expression of p75NTR protein (24) and induction of apoptosis (23) through the p38 MAPK pathway (24). Indeed, abundant evidence has been reported for the involvement of p38 MAPK in apoptosis induced by a variety of agents such as the proen NSAIDs (23, 24), Fas ligation (30), and nerve growth factor withdrawal (31). The latter is significant because nerve growth factor ligation to the p75NTR acts as a survival signal in prostate cancer cells (26). Conversely, a relative absence of nerve growth factor, either by ligand withdrawal or by

**Figure 5.** A, knockdown of p38 MAPK prevents induction of p75NTR by carprofen. PC-3 and DU-145 cells were transfected with nontargeting siRNA or siRNA for p38α for 72 h. Following transfection, cells were treated with 100 μmol/L carprofen (CAR) or DMSO vehicle control (CON) and the cell lysates were used for immunoblot analysis. A875 cell lysates were used as a positive control for p75NTR expression. p-Actin was used as the loading control. B, activation of the p38 MAPK pathway by carprofen. PC-3 and DU-145 cells were treated with 100 μmol/L carprofen for 0 min, 1 min, 5 min, 1 h, 4 h, or 8 h. Cell lysates were prepared for immunoblot analysis using antibodies to phosphorylated p38 MAPK (P-p38). Blots for phosphorylated p38 MAPK were stripped and reprobed for total p38 MAPK.

only two prostate cancer cell lines included in the NIH Developmental Therapeutics Program Anticancer Drug Discovery Program, due to their well-characterized aggressive phenotype. Hormone-responsive prostate cells were intentionally not included in these studies to maintain a focus on potential therapeutics of prostate tumor cells with phenotypes refractory to hormone ablation treatment consistent with poor prognosis. Using the prostate cancer cell lines, PC-3 and DU-145, most responsive to carprofen, we showed that a dominant-negative antagonist of p75NTR (ΔICDp75NTR) partially rescued carprofen-induced inhibition of cell survival, thereby confirming a cause-and-effect relationship between carprofen induction of p75NTR levels and p75NTR induction of apoptosis. Partial rather than complete rescue may be attributed to assay conditions or additional effects of carprofen independent of p75NTR.

In prostate cancer cell lines, reexpression of p75NTR induces modifications to several downstream signal transduction cascades leading to apoptosis. Initially, p75NTR expression down-regulates components of the nuclear factor-κB and c-Jun NH2-terminal kinase pathways preventing nuclear translocation of both these prosurvival transcriptional effectors (28). Expression of p75NTR also retards cell cycle progression through accumulation of cells in G1 at the expense of S-phase cells (7, 26). Down-regulation of cyclin/cyclin-dependent kinase holoenzyme components cyclin E, cyclin A, cyclin-dependent kinase 2, and cyclin-dependent kinase 6 contributes to hypophosphorylation of retinoblastoma along with elevated levels of p16INK4a in the p75NTR-induced cytostatic cells (26). Reexpression of p75NTR also induces elevated expression of the retinoic acid receptor β and retinoid X receptors α and β during partial redifferentiation of PC-3 cells that may also contribute to cytostasis (29). Evidence for p75NTR-dependent activation of extrinsic apoptosis in prostate cells has been limited to caspase-8 reductions in RIP, an adaptor protein that interacts with the ICD of p75NTR (28). Evidence for p75NTR-dependent activation of the intrinsic mitochondrial pathway includes an increase in proapoptotic effectors, Smac, Bak, and Bad, and conversely a decrease in the prosurvival effector, Bcl-xL (26), leading to a reduction in X-linked inhibitor of apoptosis protein and cleavage of caspase-9 and caspase-7 followed by poly(ADP-ribose) polymerase cleavage and nuclear fragmentation in PC-3 cells (26). Hence, reexpression of p75NTR appears to promote partial redifferentiation, cell cycle arrest, and apoptosis in prostate cancer cells, thereby providing a rationale for investigation of compounds that may be used for p75NTR-dependent therapeutics.

**Figure 6.** PC-3 and DU-145 cells were transfected with dominant-negative MK2 (dnMK2), after which serum-containing medium was added for 24 h to allow expression of the dominant-negative gene product. Cells were treated with 100 μmol/L carprofen for 48 h. Cell lysates were collected for immunoblot analysis of p75NTR levels. β-Actin was used as the loading control.
up-regulation of p75NTR protein to levels that initially bind residual ligand and then to higher levels that result in unbound (no ligand) p75NTR, acts as a stimulus of apoptosis in prostate cancer cells (23, 26, 28). From a potential therapeutic perspective, this mechanism of p75NTR-dependent apoptosis has the appeal that agents, such as the profens, which elevate p75NTR levels, have the same effect as ligand withdrawal leading to apoptosis of cancer cells. Activation of the p38 MAPK signal transduction pathway by carprofen was rapid, within 1 min, suggesting that carprofen is interacting with a molecule highly proximal to p38 MAPK. The observation that p38 MAPK knockdown prevented carprofen induction of p75NTR levels confirms this pathway as a mechanism responsible for p75NTR regulation. We recently reported similar observations for R-flurbiprofen and ibuprofen activation of p38 MAPK up-regulation of p75NTR-dependent apoptosis in prostate cancer cells (24). In this pathway, MK2 directly binds to the p38α isoform of MAPK during activation (32). Expression levels of MK2 are relatively robust in both PC-3 and DU-145 prostate cancer cells (24). Indeed, dominant-negative antagonism of MK2 prevented carprofen induction of p75NTR levels in prostate cancer cells. These observations suggest that carprofen initiates p75NTR-dependent apoptosis through a similar p38 MAPK signal transduction pathway to that of R-flurbiprofen and ibuprofen, albeit at an order of magnitude lower concentration of drug. Additional studies of this mechanism may lead to more potent compounds that induce p75NTR-dependent apoptosis of prostate cancer cells.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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References

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