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3,3′-Diindolylmethane Induction of p75^{NTR}-Dependent Cell Death via the p38 Mitogen-Activated Protein Kinase Pathway in Prostate Cancer Cells

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Abstract The p75^{NTR} functions as a tumor suppressor in prostate epithelial cells, where its expression declines with progression to malignant cancer. Previously, we showed that treatment with the nonsteroidal anti-inflammatory drug, indomethacin, induced p75^{NTR} expression in the T24 cancer cell line leading to p75^{NTR}-mediated decreased survival. Utilizing the indole moiety of indomethacin as a pharmacophore, we identified in rank-order with least efficacy, ketorolac, etodolac, indomethacin, 5-methylindole-3-acetic acid, indole-3-carbinol, and 3,3′-diindolylmethane (DIM) exhibiting greatest activity for induction of p75^{NTR} levels and inhibition of cell survival. Prostate (PC-3, DU-145) and bladder (T24) cancer cells were more sensitive to DIM induction of p75^{NTR}-associated loss of survival than breast (MCF7) and fibroblast (3T3) cells. Transfection of the PC-3 prostate cell line with a dominant-negative form of p75^{NTR} before DIM treatment significantly rescued cell survival demonstrating a cause and effect relationship between DIM induction of p75^{NTR} levels and inhibition of survival. Furthermore, siRNA knockdown of the p38 mitogen-activated protein kinase (MAPK) protein prevented induction of p75^{NTR} by DIM in the PC-3 prostate cell line. DIM treatment induced phosphorylation of p38 MAPK as early as within 1 minute. Collectively, we identify DIM as an indole capable of inducing p75^{NTR}-dependent apoptosis via the p38 MAPK pathway in prostate cancer cells.

Indole derivatives represent a unique class of compounds that exhibit a broad range of activities including chemotherapeutic and chemopreventive efficacy against cancer cells. The indoles, indomethacin and etodolac, are nonsteroidal anti-inflammatory drugs that inhibit cyclooxygenase activity and also induce apoptosis of cancer cells via cyclooxygenase-independent mechanisms (1, 2). The nonsteroidal anti-inflammatory drug ketorolac, containing an incomplete indole moiety, does not seem to exhibit significant apoptotic activity (3). Conversely, dietary indoles derived from cruciferous vegetables, such as indole-3-carbinol (I3C), and 3,3′diindolylmethane (DIM), exhibit significant anticancer efficacy *in vivo* (4) and *in vitro* (5, 6). The dietary indoles (I3C, DIM) exert anticancer activity by inducing apoptosis (4–6), exert antimetastatic properties (4, 10), inhibit angiogenesis gene products (11), and induce cell cycle arrest (7–9) in G_1 (5, 9) through modulation of cyclin-cdk holoenzyme components. These indoles down-regulate cyclin D1 (4, 7, 12) and its associated cdk4 (7, 12) and cdk6 (5, 7, 9, 12) holoenzyme components in mid-G_1. They also down-regulate cdk2 (9, 12) at the G_1-S checkpoint. These effects, in turn, prevent hyperphosphorylation of the retinoblastoma protein (5), thereby preventing cell cycle progression into S phase. With regard to apoptosis, dietary indoles have been shown to down-regulate mitochondrial Bcl-2 (5, 10, 12, 13), Bcl-xL, and IAPs (10, 12), and up-regulate Bax (5) leading to PARP cleavage (5), DNA ladder formation (5, 6), and nuclear fragmentation (6). Also, dietary indoles exhibit antimetastatic properties by down-regulation of matrix metalloproteinase (MMP)-2 (10, 11) and MMP-9 (11) associated with tumor cell invasion. Lastly, dietary indoles have been shown to down-regulate the angiogenic factor vascular endothelial growth factor (11), as well as MMP-2 (10, 11) and MMP-9 (13) that can liberate matrix associated vascular endothelial growth factor. Hence, these aggregate anticancer activities provide a rational basis upon which dietary indoles from cruciferous vegetables can be associated with epidemiologic evidence for reduced risk of prostate cancer (14, 15).

The multiplicity of dietary indole anticancer activities is also reflected in a number of signal transduction pathways associated with inhibition of growth. Several studies have shown dietary indole inhibition of the prosurvival PI3K/Akt signal transduction pathway. Indoles inhibit levels of PI3K (4, 11) and PI3K phosphorylation (4), as well as inhibit levels of Akt (4, 11, 12) Akt phosphorylation (11) consistent with inhibition of survival (4, 11) and levels of mammalian target

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of rapamycin (16). Similarly, dietary indoles inhibit the prosurvival nuclear factor-κB signal transduction pathway. Indoles inhibit phosphorylation of IκBα (7) and reduce levels of nuclear factor-κB (7, 12) preventing nuclear translocation (7), consistent with inhibition of survival (14). Indoles also downregulate mitogen-activated protein kinases (MAPK), MAPK2s (12), and levels of the androgen receptor (11). Because the pleiotropic effects of dietary indoles have been associated with several cognate signal transduction pathways (12), it seems plausible that additional pathways exist that may provide a mechanistic insight into their inhibition of tumor cell growth.

Previous work from our laboratory has shown that the p75^{NTR} death receptor exhibits tumor suppressor activity in prostate and bladder cancer cells. In this article, a comparison among indoles showed superior efficacy of DIM to inhibit survival of prostate cancer cells and induce increased levels of the p75^{NTR} death receptor in a dose-dependent manner. A dominant-negative antagonist of wild-type p75^{NTR} partially rescued DIM-induced cell death, thereby demonstrating a cause and effect relationship between DIM induction of p75^{NTR} and DIM-induced cell death. DIM induction of p75^{NTR} occurred through the p38 MAPK signal transduction pathway within 1 minute of DIM treatment of prostate cancer cells. Hence, we identify a novel mechanism of action of DIM inhibition of prostate cancer cell growth via DIM induction of the p75^{NTR} death receptor levels initiated through the p38 MAPK signal transduction pathway.

Materials and Methods

Cell lines, culture conditions, drug preparation, treatment, and cell lysis

The acquisition of PC-3, DU-145, T24, MCF7, and 3T3 cell lines, as well as their standard culture conditions were previously described (17, 18). Stock solutions were prepared by dissolving ketorolac, etodolac, indomethacin, I3C (Sigma Co.), 5-methylindole-3-acetic acid (Aldrich Co), and 3,'3-diindolylmethane (Bioresponse Nutrients) in DMSO (Sigma Co.) at a concentration of 200 mmol/L. Cells were seeded overnight at 70% to 80% confluency and were then treated with drug for 48 h at concentrations of 0, 0.05, 0.1, 0.2, 0.3, 0.4, and 0.5 mmol/L. Cell lysates of treated cells were prepared as previously described (17). The supernatant was retained and protein concentration was determined according to the manufacturer's protocol (Bio-Rad Laboratories).

Immunoblot analysis, cell survival assay, and p75^{NTR} dominant-negative transfection

Immunoblot analysis was done as previously described (17). Membranes were blocked in 5% nonfat milk (Bio-Rad Laboratories) and then incubated in the primary antibody: murine monoclonal anti-p75^{NTR} (1:2,000; Upstate Cell Signaling Solutions), rabbit polyclonal phosphorylated p38 MAPK (1:1,000), mouse monoclonal anti-p38α (1:1,000; Cell Signaling Technology), or murine monoclonal anti–β-actin (1:5,000; Sigma Co.). After incubation in the primary antibody, membranes were incubated in goat-anti-mouse and goat-anti-rabbit horseradish peroxidase–conjugated secondary antibodies (Bio-Rad Laboratories) at a dilution of 1:2,000. Immunoreactivity was detected using the chemiluminescence detection reagent (Amersham Pharmacia Biotech). As a positive control for p75^{NTR} expression, a whole cell lysate of A875 cells was used (Dr. Moses Chao, Cornell University, New York, NY).

The number of viable cells in each well after treatment (48 h) with drug was estimated using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay as previously described (17). MTT labeling reagent (final concentration, 0.5 mg/mL; Roche Diagnostics Corporation) was added to the drug-treated cells, ponasterone A alone–treated cells, and ΔICDp75^{NTR}-transfected cells plus ponasterone A (2×10^3 cells per well) in 96-well culture plates (final volume of 100 μL culture medium per well) and incubated for 4 h at 37°C in a humidified atmosphere of 5% CO_2. Subsequently, cells were incubated overnight with 100 μL of solubilization solution per well and the samples quantified at 570 nm using a microtiter plate reader (Bio-Rad Laboratories).

PC-3 cells were transiently transfected with a p75^{NTR} dominant-negative vector as previously described (17–19). The ΔICD is an ecdysone-inducible p75^{NTR} vector, and therefore was cotransfected with the ecdysone receptor plasmid pVgRxR. Transfections were done as previously described (17). After incubation with 1 μmol/L ponasterone A, cells were treated with DIM for 48 h and relative cell survival was determined by MTT assay.

siRNA transfection

Cells were transfected for 72 h with nontargeting 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 2 was used for PC-3 cells (Dharmacon RNA Technologies). After transfection, the cells were treated with DIM for 48 h, followed by determination of p75^{NTR} protein expression.

Results

DIM exhibits efficacy to induce p75^{NTR} levels associated with cell-specific decreased survival

The immunoblots demonstrating activity of each compound to induce p75^{NTR} levels were placed in rank-order (Fig. 1). In the PC-3 cell line, DIM exhibited superior efficacy for induction of p75^{NTR} expression at a concentration of 100 μmol/L, followed by I3C, 5-methylindole-3-acetic acid, indomethacin, etodolac, and ketorolac the least efficacious compound (Fig. 1). In an MTT assay, each of these compounds exhibited a comparable rank-order of efficacy to inhibit the survival of PC-3 cells (Fig. 2) to their induction of p75^{NTR} levels (Fig. 1). DIM exhibited superior efficacy for the dose-dependent inhibition of survival, followed by I3C, with 5-methylindole-3-acetic acid, indomethacin, and etodolac exhibiting comparable activity to each other, whereas ketorolac was the least efficacious compound to inhibit cell survival (Fig. 2).

At lower concentrations, DIM selectively induced expression of p75^{NTR} protein at ~50 μmol/L and above in PC-3 and DU-145 prostate cancer cells (Fig. 3). 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. DIM induced expression of p75^{NTR} levels in the T24 bladder cancer cell line at ~200 μmol/L, whereas DIM induced little if any p75^{NTR} protein in the MCF-7 breast cancer cell line or the 3T3 fibroblast cell line (Fig. 3). The T24 bladder cancer cell line was included as a positive control because they were previously shown to be sensitive to ibuprofen-induced p75^{NTR}-dependent decreased survival, whereas MCF-7 and 3T3 cells were included as negative controls because they were previously shown not to be sensitive to ibuprofen-induced p75^{NTR}-dependent decreased survival (17).

DIM 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

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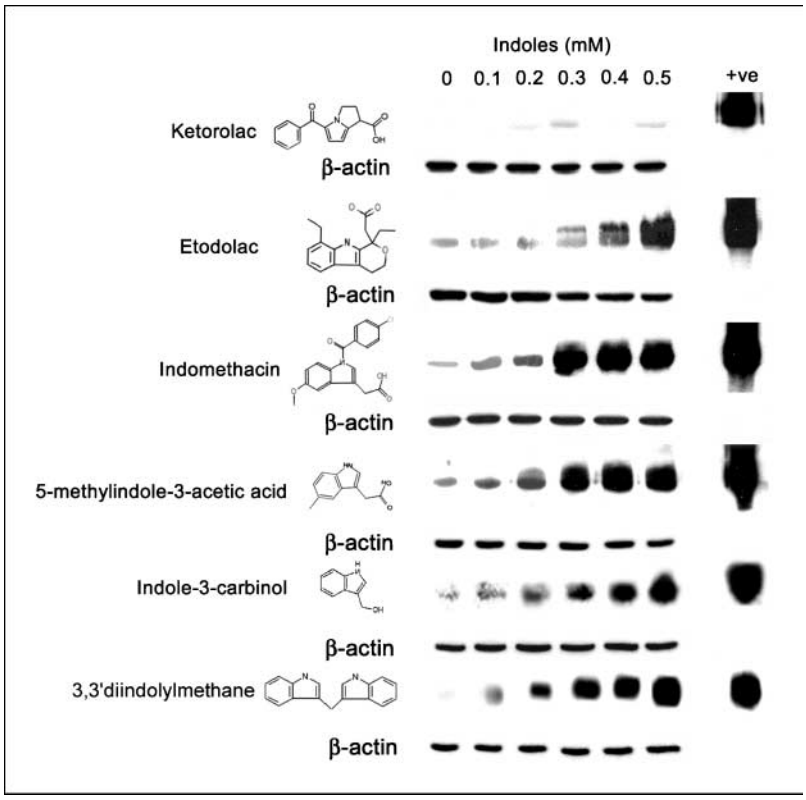

Fig. 1. Immunoblots of $p75^{NTR}$ levels with corresponding β-actin loading controls in PC-3 cells after 48 h of treatment with 0, 0.1, 0.2, 0.3, 0.4, and 0.5 mmol/L of ketorolac, etodolac, indomethacin, 5-methylindole-3-acetic acid, I3C, and 3,3′-diindolylmethane. The A875 melanoma cell line was used as a positive control (*+ve*) for $p75^{NTR}$ expression.

MCF-7 and 3T3 fibroblasts the least sensitive to DIM-induced decreased survival (Fig. 4). Significantly, there was a strong association between the dose-dependent induction of $p75^{NTR}$ levels (Fig. 3) and decreased survival of specific cell types after DIM treatment (Fig. 4).

DIM-induced decreased prostate cancer cell survival is dependent on $p75^{NTR}$

To establish a causal relationship between DIM induction of $p75^{NTR}$ protein expression and inhibition of cell survival, we used a ponasterone A inducible expression vector for $p75^{NTR}$ that exhibits a deletion of the intracellular death domain (ΔICD$p75^{NTR}$) shown to function as a dominant-negative antagonist of the intact $p75^{NTR}$ gene product (17–19). The treatment of PC-3 cells with DIM or DIM plus ponasterone A inhibited cell survival in a dose-dependent manner (Fig. 5). However, the PC-3 cell line induced with ponasterone A to express ΔICD$p75^{NTR}$ exhibited a significant ($P < 0.001$) partial rescue from DIM-mediated inhibition of cell survival relative to DIM-treated ΔICD$p75^{NTR}$ cells in the absence of ponasterone A (Fig. 5). Within each treatment group (DIM only, DIM + P, DIM + ICD, DIM + ICD + P) the data were expressed as a percentage relative to the control (100%) treatment (0 μmol/L DIM).

DIM induction of $p75^{NTR}$ occurs via the p38 MAPK pathway

An earlier study from our laboratory (20) implicated drug induction of $p75^{NTR}$ via the p38 MAPK pathway. Hence, we examined the effect of siRNA knockdown of the p38α MAPK isoform on $p75^{NTR}$ levels after treatment with DIM. We previously showed that p38α MAPK is the predominant isoform expressed in PC-3 cells (20). Whereas treatment with DIM induced $p75^{NTR}$ expression levels, transfection of prostate cancer cells with p38α siRNA before DIM treatment prevented induction of $p75^{NTR}$ relative to untransfected cells or cells transfected with nontargeting siRNA (Fig. 6A).

Because the p38 MAPK is activated by phosphorylation, we determined the phosphorylation status of p38 MAPK at several time points in PC-3 cells after treatment with DIM.

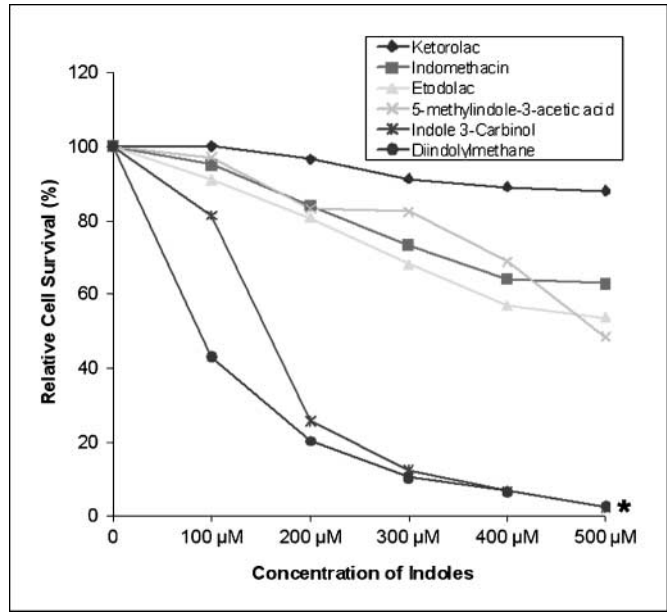

Fig. 2. A MTT cell survival assay of PC-3 prostate cancer cells, after 48 h of treatment with 0, 0.1, 0.2, 0.3, 0.4, and 0.5 mmol/L of ketorolac, etodolac, indomethacin, 5-methylindole-3-acetic acid, I3C, and 3,3′-diindolylmethane.

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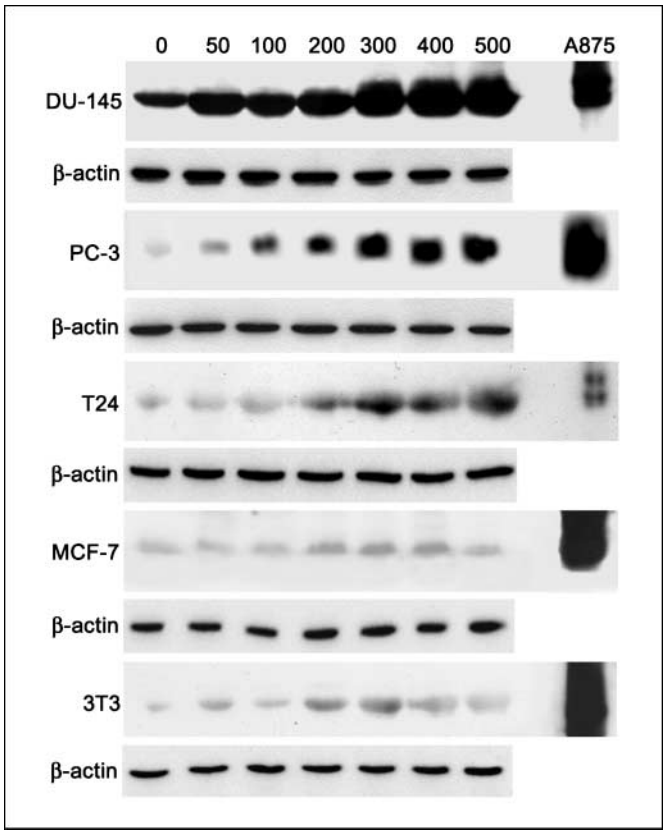

Fig. 3. Immunoblots of p75^{NTR} levels with corresponding β-actin loading controls in DU-145 and PC-3 prostate cancer cells, T24 bladder cancer cells, MCF-7 breast cancer cells, and 3T3 fibroblasts after 48 h of treatment with 0, 50, 100, 200, 300, 400, and 500 μmol/L DIM. A875 cell lysates were used as positive controls for p75^{NTR} expression.

DIM treatment stimulated rapid phosphorylation of p38 MAPK as early as within 1 minute of treatment, and subsequently led to the sustained activation of the p38 MAPK pathway that could be observed even 8 hours after treatment of each cell line (Fig. 6B).

Discussion

Cancer chemoprevention strategies encompass pharmacologic intervention with naturally occurring compounds or synthetic agents that prevent, inhibit, or suppress the development of tumor cells (21). The rationale implementation of chemoprevention strategies includes a mechanistic understanding of carcinogenesis at the molecular level that can identify specific targets against which agents can exert modulatory activity. Strategies for chemoprevention of prostate cancer currently include examination of the effects of endocrine agents, vitamins in combination with micronutrients, and dietary products such as soy, green tea, and lycopene among many others (22, 23). Significantly, a diet rich in fat has been linked to increased risk of prostate cancer (24). Mechanisms by which fat may increase the risk of prostate cancer include uptake of fat soluble pesticides, changes in androgen levels, altered role of fatty acids such as linolenic acid, and the role of fat as a pro-oxidant during oxidative stress (23). As a corollary, a high-fat diet may be conversely associated with a lower intake of fruits and vegetables. A recent epidemiologic study provided the best evidence to date that a high intake of cruciferous vegetables is associated with reduced risk of aggressive prostate cancer, particularly, extraprostatic disease (25). An active component of cruciferous vegetables is I3C, which, in the acidic environment of the stomach, is converted to DIM and related oligomers (26). Both I3C and DIM have been widely shown to exert anticancer effects *in vivo* (4) and *in vitro* (5, 6). Use of an absorption enhanced formulation of DIM (27) in mice has shown a significant inhibition of C4-2B prostate tumors thereby validating efficacy in a tumor model of prostate cancer growth (28).

Ectopic reexpression of the p75^{NTR} in human prostate cancer cells has shown both tumor suppressor and metastasis suppressor activity in severe combined immunodeficient mice (29, 30). In addition, gene therapy with p75^{NTR} expression vectors injected into s.c. PC-3 tumors grown in severe combined immunodeficient mice reduced tumor volume by induction of apoptosis, confirming tumor suppressor activity (31). Although the gene encoding p75^{NTR} has remained intact in prostate cancer cells, expression of the p75^{NTR} protein is suppressed (32). This loss of p75^{NTR} expression was shown to be caused by a loss of mRNA stability in the cancer cells similar to that which has been shown to occur for several other tumor suppressor proteins (32). After stable reexpression of the p75^{NTR} in these cancer cells, their rate of apoptosis increased (29). Additionally, the same p75^{NTR} stably expressing cancer cell lines exhibited a retardation of cell cycle progression characterized by accumulation of cells in G_1 phase with a corresponding reduction of cells in the S phase of the cell cycle (29). Hence, increased p75^{NTR} expression in prostate cancer cells by stable reexpression (29, 30) restored tumor suppressor activity. Significantly, we recently discovered that expression of p75^{NTR} in cancer cells can be selectively induced with drugs in a dose-dependent manner (17, 33), and that drug-induced up-regulation of p75^{NTR} levels was causal of apoptosis in a ligand-independent manner (18, 20, 33). The selective up-regulation of p75^{NTR} is highly significant because it suggests the existence of a specific molecular target leading to p75^{NTR} expression. It is significant that many of the effects of DIM on prostate cancer cells are similar to the effects of genetic (transient or stable plasmid transfection)

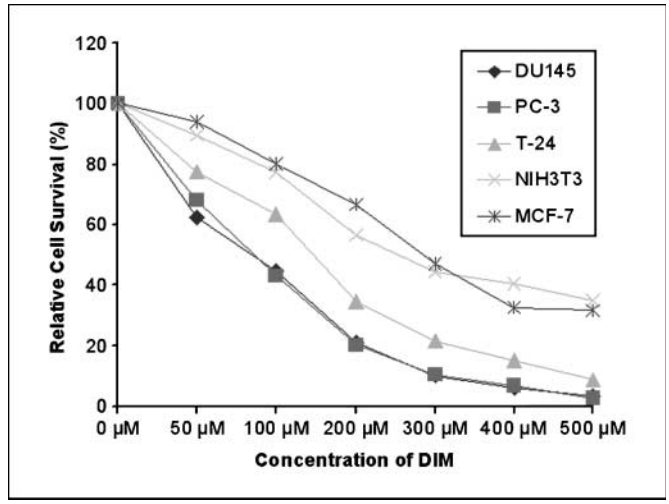

Fig. 4. A MTT cell survival assay of DU-145 and PC-3 prostate cancer cells, T24 bladder cancer cells, MCF-7 breast cancer cells, and 3T3 fibroblasts after 48 h of treatment with 0, 50, 100, 200, 300, 400, and 500 μmol/L DIM.

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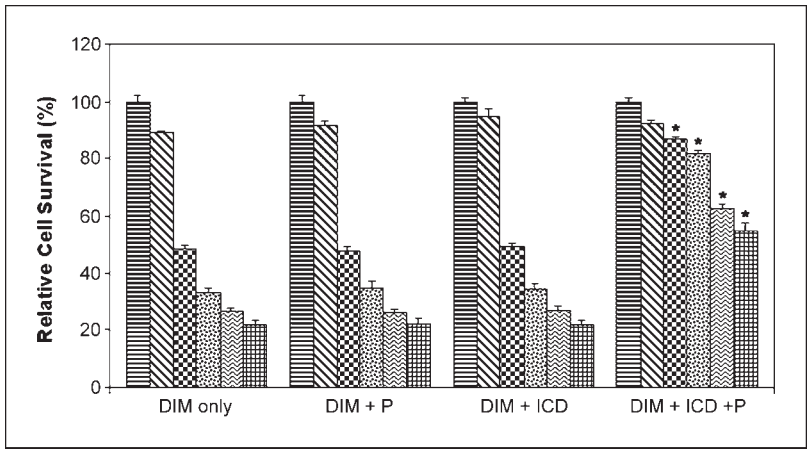

Fig. 5. PC-3 cell survival analysis by MTT assay after 48 h of treatment with 0 μmol/L (*cross-hatched*), 100 μmol/L (*diagonal*), 200 μmol/L (*large checkered*), 300 μmol/L (*stippled*), 400 μmol/L (*wavy*), and 500 μmol/L (*small checkered*) DIM. Before treatment, cells were cotransfected with a ponasterone A–inducible ecdysone receptor plasmid pVgRxR and ΔICDp75^{NTR} (*ICD*). After transfection, cells were incubated in serum containing medium for 18 h, and then incubated in 1 μmol/L ponasterone A (*P*) for 24 h to drive expression of the dominant-negative gene products (*ICD*). Results are expressed relative to the control (0 μmol/L). *, $P < 0.001$

reexpression of p75^{NTR} in prostate cancer cells, presumably because dietary indoles induce p75^{NTR} reexpression, which then mediates growth inhibitory effects. In this context, cell cycle arrest in G_1 is induced by treatment with DIM (6, 7) and/or genetic p75^{NTR} reexpression (29, 19), associated with down-regulation of cyclin D1, cdk4, and cdk6 with DIM (7, 34), and/or genetic reexpression of p75^{NTR} (19). Apoptosis is induced by treatment with DIM (7, 35) and/or genetic p75^{NTR} reexpression (19). DIM-mediated cell death occurs by DNA ladder formation (6) leading to nuclear fragmentation and apoptosis (6), in a manner similar to that induced by reexpression of p75^{NTR} (17–19). Lastly, MMP-9 and urokinase-type plasminogen activator associated with invasion and angiogenesis are inhibited by DIM (36) and also by genetic reexpression of p75^{NTR} (37). Hence, the similarities in the activities of DIM and genetic reexpression of p75^{NTR} to inhibit prostate cancer cells suggest the modulation of signal transduction pathways common to both mechanisms of action. Interestingly, DIM has been shown to up-regulate expression of another death receptor family member DR5 (38) in cancer cells. In this context, we have shown up-regulation of the p75^{NTR} death receptor by genetic reexpression (19, 29, 30), and by drug-induced reexpression (17, 18, 20, 33), and now from a dietary component, DIM, that mediates p75^{NTR} reexpression, which causes apoptosis in a ligand-independent manner. Hence, it seems plausible that an additional mechanism of action of DIM to inhibit the growth of prostate cancer cells occurs via DIM induction of p75^{NTR} dependent apoptosis.

The mechanism of action by which DIM induces p75^{NTR}-dependent apoptosis occurs, in part, through the rapid phosphorylation of p38 MAPK. A similar mechanism of action was recently shown for the nonsteroidal anti-inflammatory drugs r-flurbiprofen, ibuprofen, and carprofen, which also induced rapid phosphorylation of p38 MAPK leading to increased levels of the p75^{NTR} protein (20, 33) and apoptosis (18, 33). This mechanism of action is further supported by the observation that DIM can also stimulate IFN γ expression via the specific activation of p38 MAPK (39). Indeed, phosphorylation of p38 MAPK has been implicated in cellular responses to inflammation, control of the cell cycle, apoptosis, development, differentiation, senescence, and tumorigenesis (40). Evidence for activation of p38 MAPK in apoptosis induced by nerve growth factor withdrawal (41) is consistent with ligand-independent activation of p75^{NTR} in prostate cancer cells (18, 19, 30, 42), whereby small molecules such as DIM, and the profens (20, 33), may interact with moieties proximal to the p38 MAPK to initiate up-regulation of p75^{NTR}-dependent apoptosis. Hence, these results provide a rational basis upon which the consumption of cruciferous vegetables containing I3C converted to DIM in the acid environment of the stomach can exert an anticancer effect via p38 MAPK-dependent up-regulation of the p75^{NTR} tumor suppressor.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

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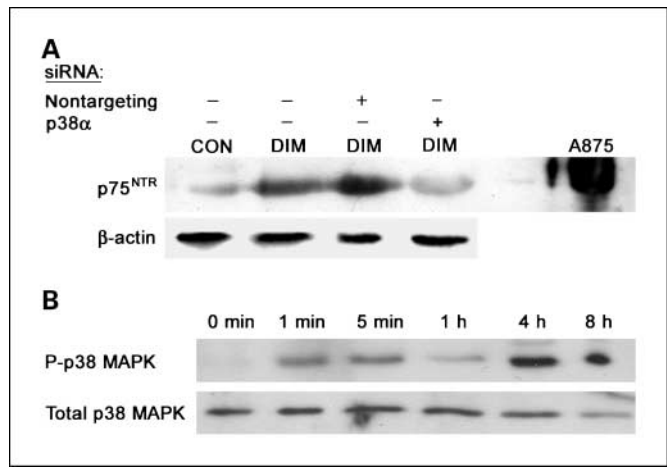

Fig. 6. *A*, knockdown of p38 MAPK prevents induction of p75^{NTR} by DIM. PC-3 cells were transfected with nontargeting siRNA or siRNA for p38α for 72 h. After transfection, cells were treated with 500 μmol/L DIM, or DMSO vehicle control (*CON*) and the cell lysates used for immunoblot analysis. A875 cell lysates were used as a positive control for p75^{NTR} expression. β-Actin was used as the loading control. *B*, activation of the p38 MAPK pathway by DIM. PC-3 cells were treated with 500 μmol/L DIM for 0, 1, 5 min, 1, 4, or 8 h. Cell lysates were prepared for immunoblot analysis using antibodies to phosphorylated p38 MAPK (P-p38 MAPK). Blots for P-p38 MAPK were stripped and reprobed for total p38 MAPK as a loading control.

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