

Increased expression and activity of p75^{NTR} are crucial events in azacitidine-induced cell death in prostate cancer

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Abstract. The high affinity nerve growth factor (NGF) NGF receptor, p75^{NTR}, is a member of the tumor necrosis factor (TNF) receptor superfamily that shares a conserved intracellular death domain capable of inducing apoptosis and suppressing growth in prostate epithelial cells. Expression of this receptor is lost as prostate cancer progresses and is minimal in established prostate cancer cell lines. We aimed to verify the role of p75^{NTR} in the azacitidine-mediated antitumor effects on 22Rv1 and PC3 androgen-independent prostate cancer cells. In the present study, we reported that the antiproliferative and pro-apoptotic effects of 5-azacytidine (azacitidine) were more marked in the presence of physiological concentrations of NGF and were reduced when a blocking p75^{NTR} antibody or the selective p75^{NTR} inhibitor, Ro 08-2750, were used. Azacitidine increased the expression of p75^{NTR} without interfering with the expression of the low affinity NGF receptor TrkA and induced caspase 9-dependent caspase 3 activity. Taken together, our results suggest that the NGF network could be a candidate for future pharmacological manipulation in aggressive prostate cancer.

Introduction

Prostate cancer (PCa) is one of the most common malignancies in men (1). This tumor is androgen-dependent and is treated using surgery, radiotherapy and hormone therapy (2).

However, PCa frequently progresses into a hormone-independent, highly aggressive and invasive disease (3) presenting with multiple drug resistance. This neoplasia is also the most common malignancy in which the chromosome 17q21 is segregated (4-6) suggesting that genes located in the immediate vicinity of 17q21 can be important in PCa development/progression (5). p75^{NTR} is a 75-kDa glycoprotein receptor that belong to the tumor necrosis factor (TNF) receptor superfamily and binds mainly to nerve growth factor (NGF) and has structural and sequential similarity to the TNF receptor (7). Notably, the human p75^{NTR} gene locus has been mapped distal to 17q21-q22 (6). The prostate gland is one of the most abundant sources of NGF controlling cell proliferation and apoptosis (8,9). However, p75^{NTR} is progressively lost during the progression of PCa (10-12). Loss of expression of p75^{NTR} protein is correlated with increased Gleason's score of organ-confined pathological prostate tissues (12-14), and is completely absent in prostate tumor cells derived from metastases (11). The re-expression of p75^{NTR} was previously shown in PCa to retard cell cycle progression by inducing accumulation of cells in the G1 phase with a concomitant reduction in cells in the S phase thus inducing apoptosis (15) and reverting androgen-independent growth (16). p75^{NTR} can be upregulated by prolonged treatment with NGF (17) or GnRH treatment (18) indicating epigenetic mechanisms of protein expression.

DNA hypermethylation plays an important role in the downregulation of genes important for cell death in PCa. Demethylating agents have been shown to prevent tumorigenesis and delay androgen-independent disease (19,20) in a TRAMP mouse model of PCa as well as to revert androgen independence (19,21,22) and chemoresistance in prostate tumor cells (23).

In the present study, we demonstrated that azacitidine treatment induced a dose-dependent increase in p75^{NTR} expression. This was in agreement with a recent report showing that azacitidine and estrogen treatment induced p75^{NTR} (24) in 22rv1 cells. Blocking NGF antibodies or specific p75^{NTR} inhibitors reverted this effect. Taken together, our results suggest that the

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NGF network could be a candidate for future pharmacological manipulation in PCa therapy.

Materials and methods

Reagents. All the materials for the tissue culture were purchased from EuroClone S.p.A. (Milan, Italy). Antibodies: Bcl-2 (sc-509), Bax (N20) (sc-493), Bad (C20) (sc-943), p-Bad ser112 (sc-7998), Bcl-xL (H2) (sc-8392), TrkA (C20) (sc-20537), p-TrkA (E6) (sc-8058), p75^{NTR} (H92) (sc-5634), TRADD (A-5) (sc46653), RIP (H207) (sc 7881), TRAF2 (D-3) (sc-136997), DR4 (C20) (sc-6823) and DR5 (N19) (sc-7192) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). DNMT1, DNMT3a and DNMT3b antibodies were purchased from BioCarta LLC (San Diego, CA, USA). Plasticware was obtained from Nunc (Roskilde, Denmark). Azacitidine (Vidaza[®]) was obtained from Celgene Corporation (Summit, NJ, USA). The p38 MAPK inhibitor SB202190 and ibuprofen were purchased from Sigma-Aldrich Italy (Milan, Italy). Ro 08-2750, a selective p75^{NTR} inhibitor, was purchased from Tocris Bioscience (Bristol, UK).

Cell lines. Two aggressive PCa models (PC3 and 22rv1 cell lines) were obtained from the American Tissue Culture Collection (ATCC; Rockville, MD, USA) and DSMZ (Braunschweig, Germany), respectively, and were grown as recommended.

Growth assays. Cells were seeded at a density of 2×10^4 cells/ml into 24-well plates. Cells were left to attach and grow in 5% FCS and Dulbecco's modified Eagle's medium (DMEM). Next, the cells were cultured under appropriate experimental conditions. Morphological controls were performed every day with an inverted phase-contrast photomicroscope (Nikon Diaphot, Tokyo, Japan) before cell trypsinization and counting by the NucleoCounter[™] NC-100 automated cell counter system (Chemotec, Gydevang, Denmark). The effect on cell proliferation was measured by taking the mean cell number with respect to the controls over time for the different treatment groups.

Cell cycle and apoptosis analysis. Adherent cells were trypsinized, pooled with the culture supernatant containing the apoptotic cells already detached from the dish and centrifuged. Cells (1×10^6) were washed in phosphate-buffered saline (PBS) and fixed for 30 min by the addition of 1 ml of 70% ethanol. Apoptosis and cell cycle analyses were performed using the Tali Cell Cycle kit and the Tali apoptosis kit, Annexin V-Alexa Fluor 488 and propidium iodide-based staining (Life Technologies Italia, Monza, Italy). Apoptosis was further confirmed by cytofluorimetric analysis following the manufacturer's instructions. Stained cells were then measured on a Tali Image-Based Cytometer. Caspase 8 and -9 activities were evaluated using enzymatic assays from GenScript (Piscataway, NJ, USA).

Western blot analysis. Cells (1×10^6) were washed with cold PBS and immediately lysed with 100 μ l of lysis buffer (50 mM HEPES, pH 7.5, 150 mM NaCl, 10% glycerol, 1% Triton X-100, 1 mM EDTA, 1 mM EGTA, 50 mM NaF, 1 mM

sodium orthovanadate, 30 mM p-nitrophenyl phosphate, 10 mM sodium pyrophosphate, 1 mM phenylmethylsulfonyl fluoride, 10 μ g/ml aprotinin and 10 μ g/ml leupeptin). Cell lysates were electrophoresed in 7% SDS-PAGE, and separated proteins were transferred to a nitrocellulose membrane and probed with the appropriate antibodies as indicated.

In vivo treatments. Male CD1 nude mice (Charles River, Milan, Italy) were maintained under the guidelines established by our institution (University of L'Aquila, Medical School and Science and Technology School Board Regulations, complying with the Italian government regulation no. 116, January 27, 1992, for the use of laboratory animals). All mice received s.c. flank injections of 1×10^6 PC3 and 22rv1 cells. Tumor growth was assessed by a bi-weekly measurement of tumor diameters with a Vernier calliper (length \times width). Tumor weight was calculated as previously indicated (22). Treatments were started when tumor volumes reached ~ 80 mm³ (day 0) and were stopped after 28 days. Mice were grouped as follows: group 1, 10 mice received intraperitoneal (i.p.) injections of 100 l PBS for a consecutive 7 days; group 2: 10 mice received i.p. injections of 100 l of azacitidine (Aza-CR) (0.8 mg/kg) for a consecutive 7 days. Tumors were fixed in paraformaldehyde for histochemical and immunohistochemical analyses. Indirect immunoperoxidase staining of tumor xenografts was performed on paraffin-embedded 4- μ m tissue sections. Briefly, the sections were incubated with the primary antibodies overnight at 4°C. Next avidin-biotin assays were carried out using the Vectastain Elite kit (Vector Laboratories, Burlingame, CA, USA). Mayer's hematoxylin was used as a nuclear counterstain.

Statistical analysis. Continuous data are presented as the mean \pm standard deviation (SD), and were compared using an unpaired Student's t-test.

Results

Azacitidine restores the expression of p75^{NTR} in PC3 and 22rv1 cell models. In the present study, we demonstrated that the antiproliferative and pro-apoptotic effects induced by azacitidine at non-toxic concentrations (ranging between 0.1 and 0.5 μ M) were enhanced in the presence of 10 ng/ml NGF. We observed that NGF-induced growth inhibition was significantly higher when compared to data observed following treatment with the demethylating agent alone both in the PC3 (Fig. 1A) and 22rv1 cells (Fig. 1B). The addition of NGF triggered a significant and early apoptosis in both cell lines (Fig. 1C and D). Next, we analyzed the molecular arrangement induced by treatments using western blotting. We observed that expression of the high affinity p75^{NTR}, but not expression of the low affinity receptor (TrkA) was increased after *in vitro* administration of azacitidine in a time-dependent manner in the PC3 and 22rv1 cells (Fig. 2A and B). As a positive control we used the androgen receptor (AR) which was widely demonstrated to be induced after azacitidine treatment (19,25). Western blot analyses were confirmed by immunocytochemical analyses (Fig. 2C) in the aggressive 22rv1 cell line. The same tumor cell models were examined for expression levels of downstream components proximal to p75^{NTR} (TRADD, RIP, DR4, DR5 and TRAF2) in both

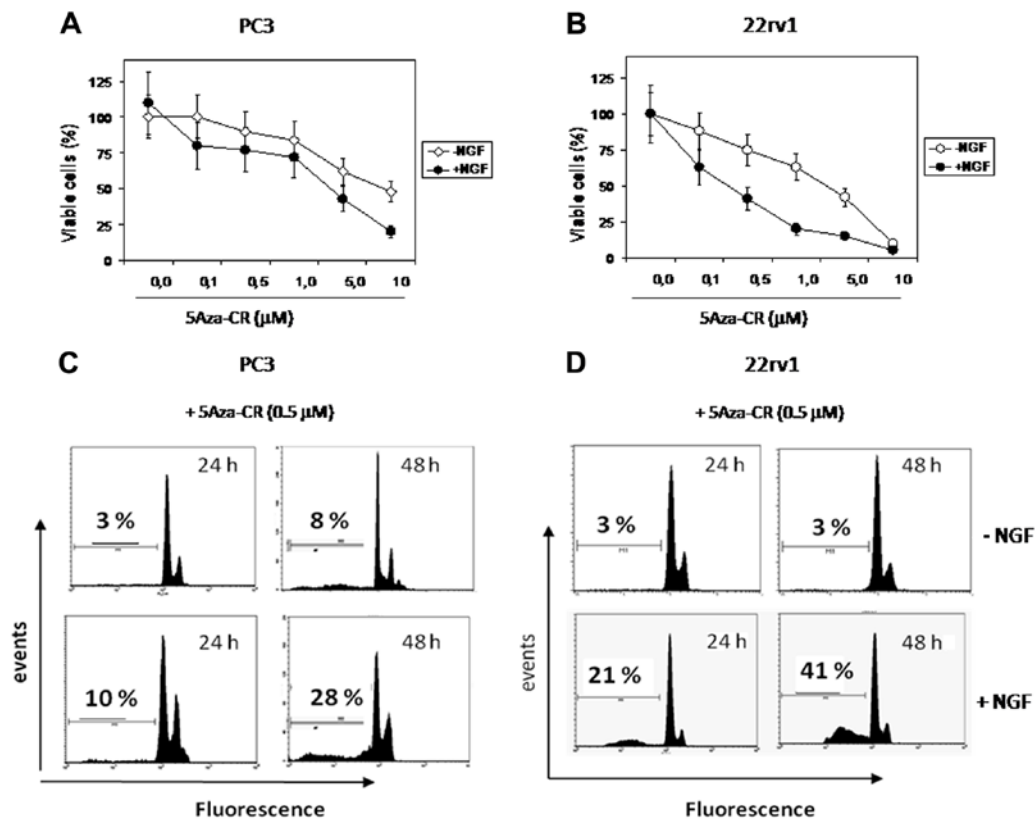


Figure 1. Antiproliferative and pro-apoptotic effects induced by azacitidine (5Aza-CR) at different concentrations in the presence or not of 10 ng/ml NGF. (A) (PC3) and (B) (22rv1) cells are subjected to the cytotoxic effects of different doses of azacitidine with or without NGF. (C) (PC3) and (D) (22rv1) cells undergo time-dependent apoptosis after treatment with a non-toxic concentration (0.5 μ M) of azacitidine in combination with NGF. Data are representative of three independent replicates.

the PC3 (Fig. 2D) and 22rv1 cells (Fig. 2E). Previously, we demonstrated that an intraperitoneal administration of azacitidine (Vidaza[®], 0.8 mg/kg for 7 consecutive days) in nude male mice subcutaneously inoculated with 22rv1 and PC3 cells was able to reduce tumor growth both in the PC3 and 22rv1 xenograft models (22). In the present study, we demonstrated that reduction in the tumor mass after Aza-CR treatment was associated with p75^{NTR} re-expression *in vivo* (Fig. 2F). Cell death was dependent on activation of the caspase 9 pathway which we also verified *in vivo* by analyzing FasL and TRAIL protein expression in the control and Aza-CR-treated tumors.

Azacitidine induces p75^{NTR}-mediated cell death. It has been previously demonstrated that p38 MAPK activation, associated with ibuprofen treatment, is related to induction of p75^{NTR} in PCa cells (26). Therefore, considering that the antitumor effects of azacitidine are associated with increased p38 MAPK activity necessary for the induction of cell cycle arrest in the G2 phase (27,28) and apoptosis (29), we verified whether the p38 MAPK inhibitor, SB202190, was able to prevent the induction of p75^{NTR} using as a positive control, treatment with ibuprofen (1 μ M). In agreement with the literature data, we demonstrated that: i) NGF reduced ERK activation, but not p38 MAPK (Fig. 3A), and ii) ibuprofen induced p75^{NTR} (Fig. 3B) whereas p38 MAPK inhibition was able to reduce p75^{NTR} expression (Fig. 3B). Similar results were obtained using P38 MAPK siRNA (data not shown) indicating that p75^{NTR} expression was under p38 MAPK-

dependent epigenetic control. Thus, we analyzed the role of p75^{NTR} in the azacitidine-induced cell death. For this aim we used a blocking antibody for p75^{NTR} as well as the non-peptide antagonist of NGF, Ro 08-2750 [able to bind to the NGF dimer and inhibit selectively p75^{NTR} at submicromolar concentrations whereas both p75^{NTR} and TrkA receptors were blocked at >5 μ M (29)]. Both anti-NGF agents significantly increased the cell viability (Fig. 3C) and apoptosis (Fig. 3D) induced by co-treatment with azacitidine and NGF. Conversely the p75^{NTR} induction by Aza-CR treatment was partial after SB202190 (data not shown). Increased p75^{NTR} observed after azacitidine treatment was related to increased expression of Bax and PARP cleavage as well as to reduced levels of Bcl2, XIAP and survivin (Fig. 4A) with caspase 8-dependent caspase 3 activation (Fig. 4B and C) in agreement with our *in vivo* effects (30).

Discussion

The low-affinity neurotrophin receptor p75^{NTR}, a member of the tumor necrosis factor (TNF) receptor superfamily, is expressed in prostate gland (6-11) and has been implicated in promoting cell apoptosis and death through a conserved intracellular death domain (31-34). In the present study, we demonstrated that azacitidine was able to restore the expression of p75^{NTR} in p75^{NTR}-negative and aggressive prostate cancer (PCa) cell lines. This was dependent on increased p38 MAPK activation and the inhibition of DNMT activity (35,36), and this was in agreement with a recent study in which estrogen and

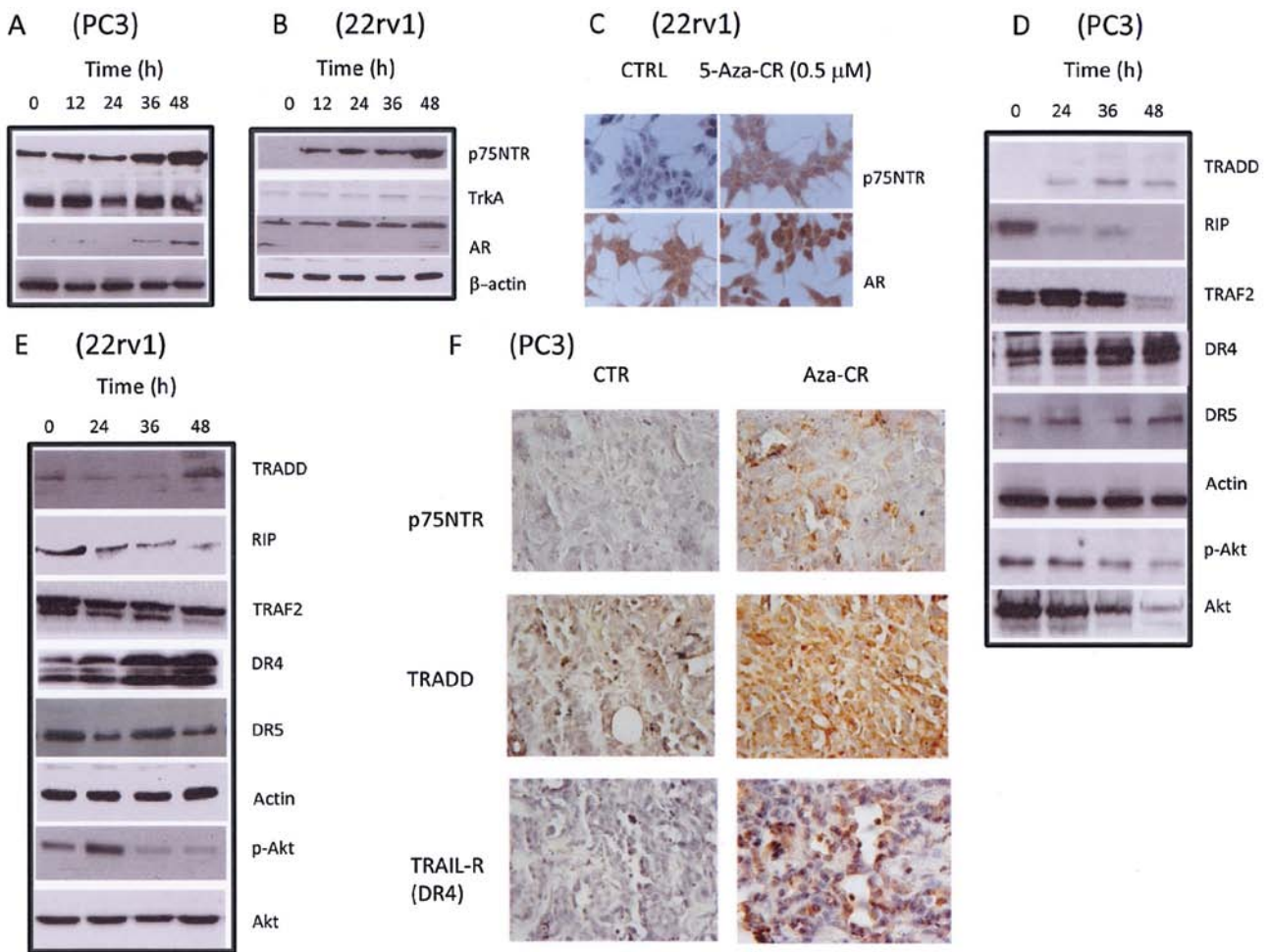


Figure 2. Western blot analyses show a time-dependent induction of p75^{NTR} and AR, but not TrkA expression, after *in vitro* administration of 0.5 μ M azacitidine (Aza-CR) in PC3 (A) and 22rv1. (C) Immunocytochemical expression of p75^{NTR} and AR in 22rv1 cells. (D) PC3 and (E) 22rv1 cells were examined for expression levels of downstream components proximal to p75^{NTR} (TRADD, RIP, DR4, DR5 and TRAF2) after treatment with 0-5 μ M azacitidine and 10 ng/ml NGF. (F) Immunohistochemical evaluation of p75^{NTR}, TRADD and TRAIL-R (DR4) in PC3 cell tumor-bearing nude mice treated or not with azacitidine (Vidaza 0.8 mg/kg/7 consecutive days). Each lane was loaded with 100 μ g of proteins from the cell extracts obtained from the control and treated cells.

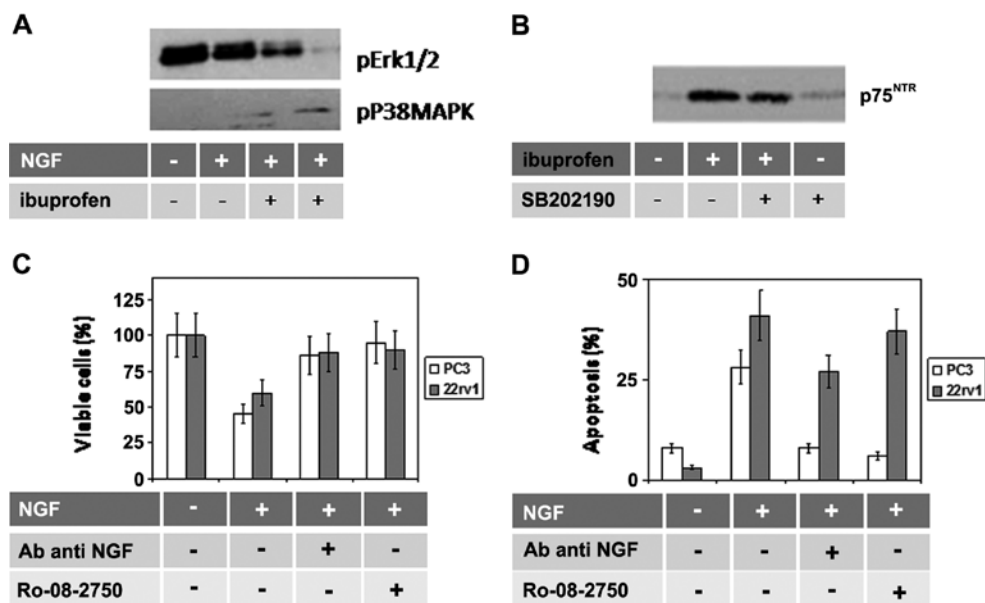


Figure 3. (A) Induction of pErk and p38 MAPK by ibuprofen in PC3 cells treated with 10 ng/ml NGF. (B) The p38 MAPK inhibitor, SB202190, reduced p75^{NTR} expression. (C) Thus, we analyzed the role of p75^{NTR} in azacitidine-induced cell death. For this aim we used a blocking antibody for p75^{NTR} as well as the non-peptide antagonist of NGF, Ro 08-2750. Both anti-NGF agents significantly increased the cell viability (C) and apoptosis (D) induced by co-treatment of azacitidine (0.5 μ M) and NGF (10 ng/ml). Each lane was loaded with 100 μ g of proteins from the cell extracts obtained from control and treated cells. Data are representative of three independent replicates.

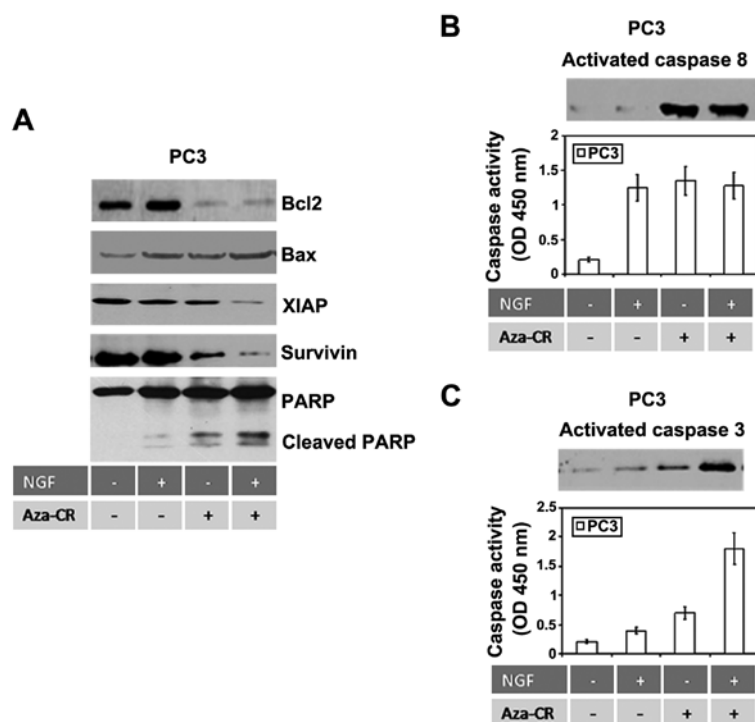


Figure 4. (A) Combination treatment of NGF and azacitidine induced Bax and PARP cleavage and reduced Bcl2, XIAP and survivin expression. This was associated with activation of (B) caspase 8 and (C) caspase 3. Each lane was loaded with 100 μ g of proteins from cell extracts obtained from the control and treated cells. Data are representative of three independent replicates.

azacitidine modulated the increased expression of p75^{NTR} and apoptosis in 22rv1 cells (24). Previously, it has been shown that treatment with nonsteroidal anti-inflammatory drugs induces p75^{NTR} expression leading to p75^{NTR}-mediated decreased survival through a p38 MAPK-mediated mechanism (26-28). p38 MAPK could be a complementary mechanism by which prostate tumor cells re-express p75^{NTR}. The re-expression of p75^{NTR} tumor suppressor activity is associated with reduced tumor cell growth and activation of caspase 9-dependent caspase 3 activation. Since the re-expression of p75^{NTR} does not alter levels of TrkA, these changes in cell cycle progression can be directly attributed to the changes in levels of the p75^{NTR} protein. These effects were increased in the presence of NGF and indicate a TRAIL-mediated crosstalk with TRAILR (DR4) (37,38) or FAS:FASL signaling (39,40). In addition, cell cycle arrest, differentiation and apoptosis after p75^{NTR} activation could involve further intracellular signals. It has been demonstrated that anti-inflammatory compounds modulate DNMT expression/activity (41,42) via phospholipase A2, able for example to induce cell arrest and differentiation in rat NK cells (43), and PPAR γ activation (43) but not PPAR β , involved in differentiation of neuronal cells (44,45). In the absence of a ligand, p75^{NTR}-dependent cell cycle arrest was accompanied by a rank-order increase in the apoptosis of PCa cells. NGF increased the apoptosis, via mitochondrial activation of a caspase cascade, induced by p75^{NTR} re-expression. Taken together, it seems clear that p75^{NTR} expression selectively alters specific cell cycle regulatory molecules that retard progression. In conclusion, the present study showed that p75^{NTR} retards cell cycle progression and induces NGF-mediated apoptosis and suggests that the NGF network could be a candidate for future pharmacological manipulation for aggressive PCa.

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