

Full Length Research Paper

Down-regulation of Notch-1 by γ -secretase inhibitor suppress the proliferation and migration of prostate cancer cells

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Notch-1 signaling is crucial for stem cell maintenance and in a variety of tissues. Previous research has demonstrated that Notch-1 activity plays a key role in prostate tumorigenesis. However, the function of Notch-1 signaling in tumorigenesis can be either oncogene or suppressor gene. In our paper, γ -secretase inhibitor (N-[N-(3,5-difluorophenacetyl) -L-alanyl] -S-phenylglycine t-butyl ester, (DAPT) was used to block the release of Notch-1 intracellular domain (NICD). We investigated whether DAPT plays a role in the regulation of the proliferation and migration of prostate cancer cells through down-regulation of the Notch-1 activation. Here, we reported that DAPT treatment inhibited the PC cells proliferation and migration in dose- and time- dependent manner. The expression of Notch-1 was decreased significantly. MT1-MMP and its target-molecule MMP2, which function in cell migration-related behavior, also decreased in accordance with NICD. DAPT treatment for 24 h also down-regulated the binding between NICD and hes-1 promoter by chromatin immunoprecipitation assay (ChIP). Taken together, we demonstrate that DAPT inhibited the proliferation and migration of PC cells through down-regulation of the Notch-1 activation and its targeted genes.

Key words: Prostate cancer, γ -secretase activation, Notch signaling, MT1-MMP.

INTRODUCTION

Notch-1 belongs to the Notch family of single-pass type I transmembrane receptors and is predominantly expressed in many cancer cells, but the mechanism for its function as oncogene or suppressor has not been elucidated (Schwanbeck, 2011; Kopan, 2009; Duan, 2006). Synthesized Notch receptors are proteolytically cleaved during translocation to the cell surface, creating heterodimer mature receptors comprising noncovalently associated extracellular (NEC) and transmembrane (NTM) subunits (Kopan, 2009). Notch activation is always ligand dependent in malignancy (Yunsun et al., 2002). Upon binding to ligands, the Notch receptor undergoes two proteolytic cleavages. The release of NICD is mainly

mediated by γ -secretase protease, a member of the presenillin family (Pratt et al., 2010). Released NICD then translocates to the nucleus and interacts with the CSL family of transcription factors (CBF-1/RBP-Jk, Su (h) and LAG-1) to modulate the expression of target genes, such as Hes-1 and Hey-1, which regulate cell differentiation and survival. Many reports have shown that down-regulation of the Notch-1 activation contributed to cancer cell growth inhibition and apoptosis onset. Recent data indicated that Notch-1 signaling pathway and its target gene Hes-1 correlate with prostate cell proliferation (Signoretti, 2007). Therefore, we hypothesize that down-regulation of the Notch-1 activation by γ -secretase inhibitor may inhibit prostate cell growth and invasion. Our results indicate that down-regulation of Notch-1 activation significantly inhibited cell growth and invasion in prostate cancer cells. This may help us to further explore the function of Notch-1 signaling in different cell

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types.

MATERIALS AND METHODS

Experimental reagents

Primary antibodies against Notch-1, MT1-MMP, MMP2 and β -actin were purchased from Santa Cruz Biotechnology (Santa Cruz, USA). HN-[N-(3,5-Difluorophenacetyl)-L-alanyl]-S-phenylglycine t-butyl ester (DAPT, Sigma), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were from Solarbio, China. Q-PCR kits were purchased from Promega Company.

Drugs

γ -secretase inhibitor (DAPT), provided by Sigma Company, was dissolved in DMSO, stored at -20°C , and diluted in media. The final concentration of DMSO was 0.1% or less in all experiments.

Cell culture

Human prostate cancer cell lines PC3 and DU145 were cultured in RPMI 1640 (Invitrogen) supplemented with 10% fetal bovine serum (FBS), 100 units/ml penicillin and 100 $\mu\text{g}/\text{ml}$ streptomycin. All cells were cultured in a 5% CO_2 humidified atmosphere at 37°C . In some experiments, different concentration of DAPT was added to the medium whenever necessary as indicated in the figure legend.

γ -secretase activity assay

The γ -secretase activity kit (R&D Systems, USA) was used to measure the γ -secretase activity, following the manufacturer's instructions. DU145 and PC3 cells were treated with different concentrations of DAPT (0, 2, 4, 8 and 16 μM) or with DMSO (control) for 24 h. Cells were then washed twice with ice-cold phosphate-buffered saline (PBS), harvested in the cell extraction buffer, and incubated on ice for 30 min. Whole cell lysates were centrifuged at 13200 rpm for 10 min and supernatants were collected. The protein concentration was determined with BCA Protein Assay (Pierce, USA) in each sample.

Total protein (50 μg) was incubated with the γ -secretase fluorescent substrate for 2 h at 37°C and fluorescence intensity was measured at 355/460 nm.

Cell viability studies by MTT assay

PC3 and DU145 cells (5×10^3 per well) were seeded with 100 μl medium in 96-well plate. DAPT was added to each well in different concentrations, and the MTT assay was performed after 48 h. In addition, 8 μM of DAPT was added to each well and the MTT assay was performed at 24, 48, 72, and 96 h time points. MTT reagent (5 mg/ml) was added to each well, and incubated for 4 h at 37°C . The resulting formazan crystals were solubilized by the addition of 150 μl DMSO to each well.

The optical density at 570 nm was measured and cell viability was determined by the formula: cell viability (%) = (absorbance of the treated wells - absorbance of the blank control wells) / (absorbance of the negative control wells - absorbance of the blank control wells) $\times 100\%$. All MTT experiments were performed in triplicate and repeated at least three times.

Wound healing assay

DU145 cells were plated into 24-well plates and grown to confluence. The monolayer was artificially wounded using the tip of a sterile 200- μl pipette. Cell debris was removed by washing with PBS. The cells were then incubated with DAPT-containing basal media for 0, 12, 24, 36 and 48 h. The cells migrated into the wounded areas were photographed. Wound closure was photographed at the indicated times with an inverted microscopy equipped with a digital camera. The extent of healing was defined as the ratio of the difference between the original and the remaining wound areas compared with the original wound area.

Western blot

DU145 cells were treated as previously described, followed by lysis in 50 μl RIPA buffer (50 mM Tris (pH 7.4), 150 mM NaCl, 1% Triton X-100, 0.1% SDS, 1% sodium deoxycholate, 5 mM EDTA, 100 mM NaF, and 1 mM Na_3VO_4) containing protease inhibitor cocktail for 20 min. Cell lysates were centrifuged at 13200 rpm for 30 min, and the supernatants were collected.

The protein concentration was determined with the BCA method (Pierce, USA). Total proteins were fractionated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene difluoride (PVDF)S membrane using a wet transblot system (Bio-Rad, Hercules, CA). Blots were then blocked for 1 h at room temperature with 5% nonfat milk. Membranes were incubated overnight at 4°C with antibodies against C-terminal Notch-1, MT1-MMP, MMP2 and β -actin (1:1000, Santa Cruz Biotechnology, USA). After subsequent washing by PBST (PBS-Tween), the membranes were incubated for 1 h with horseradish peroxidase conjugated goat anti-rabbit or anti-mouse secondary antibody, diluted 1:5,000 in PBST.

After washing, the membrane was visualized using enhanced chemiluminescence (ECL, Pierce, USA), followed by exposure to Fujifilm LAS3000 Imager (Fuji, Japan). The band densities of the western blots were normalized relative to the relevant β -actin band density with Image J Analyst software (NIH).

ChIP assay

PC3 and DU145 cells were cultured in complete cell medium, followed by treatment with different concentrations of DAPT (0, 2, 4, 8 and 16 μM) or with DMSO. After 24 h, all the cells were fixed with 4% formaldehyde and nuclei were isolated, and sonicated to shear DNA length to 200 to 500 bp. DNA fragments were precleared in 50% protein G slurry (Millipore). Precleared chromatin was immunoprecipitated overnight with 6 μg of antibody for Notch-1 (specific for the intracellular portion of human Notch-1 sc-6014, Santa Cruz Biotechnology) and normal rabbit IgG (CST). After the complexes were captured by protein G slurry and centrifugation, beads were washed four times as described previously and immune complexes were eluted.

Then, cross-linked chromatin was reversed and purified; it was used as templates in PCR. PCR was carried out using primer sets specific for *hes-1* promoter (forward, 5'-CTGAAAGTTACTGTGGG-3'; reverse, 5'-TGAGCAAGTGCTGAGGG-3').

Statistical analysis

All the results were expressed as mean \pm standard deviation (SD). Student's t-test was used for the statistical analyses with SPSS 11.0. Differences with $P < 0.05$ were considered statistically significant.

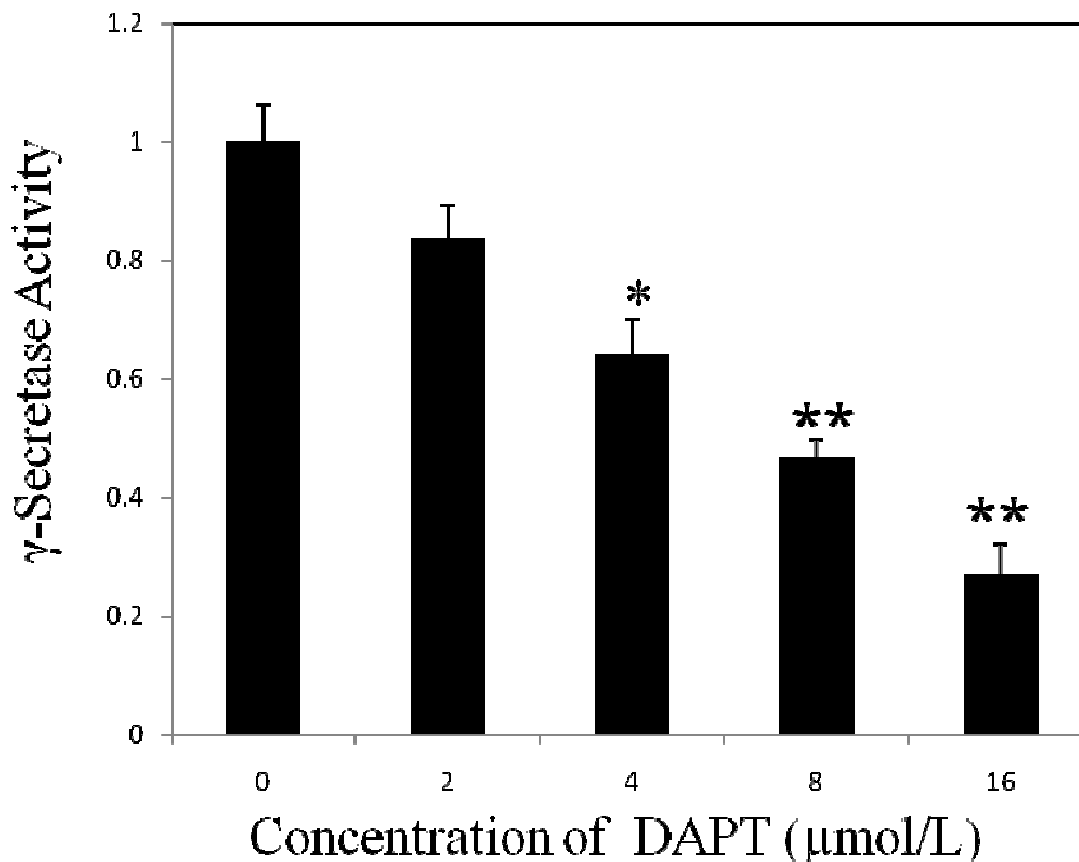


Figure 1. γ -Secretase activity inhibition upon different concentration DAPT. Log-phase growth DU145 cells were treated with different concentrations of DAPT for 48 h, followed by cell whole protein collection and incubation with γ -secretase substrate, then fluorescence intensity was measured at 355/460 nm. For the control group, cells were treated with same volume of DMSO, and its activity was set at 100% (* $p < 0.05$, ** $p < 0.01$, $n = 5$).

RESULTS

γ -secretase activity was inhibited significantly in DU145 and PC3 cells

To verify whether γ -secretase activity was inhibited by DAPT treatment in DU145 and PC3 cells, we first detected γ -secretase activity upon DAPT incubation. The log-phase cells were treated with 0, 2, 4, 8, and 16 $\mu\text{mol/l}$ DAPT for 24, 48 and 96 h, and cell whole lysates were collected for γ -secretase activity detection. As Figure 1 indicates, γ -secretase activity was inhibited in dose- and time- dependent manner.

DU145 and PC3 cell growth and migration were inhibited upon DAPT incubation

To test our hypothesis that γ -secretase activity regulates the proliferation of DU145 and PC3, we detected the cell viability upon DAPT treatment. DU145 and PC3 cells

were treated with special concentration of DAPT for indicated time followed by MTT assay. As we expected, DU145 and PC3 cells growth was significantly inhibited in both dose- and time-dependent manner (Figure 2). Significant inhibitory effect was noted between the doses of 4 and 16 $\mu\text{mol/l}$ ($p < 0.01$). No statistical difference of inhibitory effect was observed between DU145 and PC3.

In addition, we wanted to know if DAPT could down-regulate cell migration and invasion, so we used cell wound healing assay to detect the ability of migration. Our results show that cell migration was indeed inhibited in DU145 cells on treatment with high dose of DAPT (Figure 3). Similar effect was shown in PC3 cells (data not shown).

Expression of NICD, MT1-MMP and MMP2 decreased upon DAPT treatment

To test whether DAPT could regulate the expression of Notch-1 intercellular domain (NICD), we used western

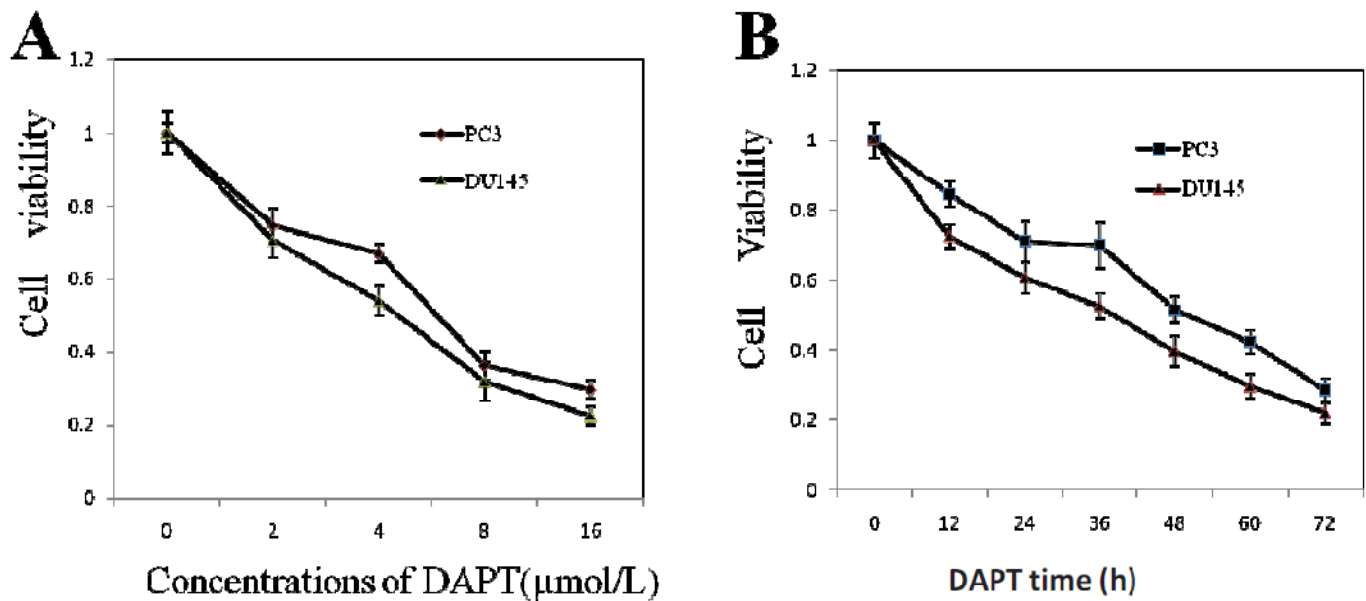


Figure 2. Regulation of cell proliferation upon DAPT incubation in PC3 and DU145 cells. Log-phase cells were incubated with different concentrations of DAPT for 48 h (A); or with 8 μmol/l DAPT for different times (B), followed by MTT assay detection of cells viability. Standard deviations are indicated (n=5).

blotting to detect the level of NICD in DU145 cells upon DAPT treatment. Our study revealed that different concentrations of DAPT (4, 6 and 8 μmol/l) resulted in significantly decreased expression of NICD. Many protein molecules which include MT1-MMP and MMP2 have been involved in regulation of the cell migration and invasion behaviors. To verify the alteration of NICD whether it ultimately results in the alternation of the protein expression of MT1-MMP and MMP2, we detected the level of MT1-MMP and MMP2 at the same time. As expected, the expression was decreased in accordance with the alternation of NICD (Figure 4). These results suggest that DAPT could down-regulate the expression of NICD, MT1-MMP and MMP2.

Down-regulation of the binding of Notch-1 to the Hes-1 promoter upon DAPT treatment

After successive cleavage by Furin, TACE and γ-secretase, Notch-1 NICD is released from the plasma membrane and transported to the nucleus where it associates with the DNA-binding protein CSL (CBF1/RBPJ-κ) and induces transcription of multiple effector genes, including Hes-1. To test if DAPT could inhibit the binding of Notch-1 to the Hes-1 promoter, we performed CHIP assays. Before DAPT treatment, a large amount of Hes-1 was detected on the CSL binding site of Hes-1, and this progressively decreased following different concentrations of DAPT treatment for 30 min (Figure 5a). To better quantify changes in CSL binding to the Hes-1 CHIP promoter, we performed quantitative-PCR assays on the

samples. Notch-1 binding to Hes-1 again decreased after DAPT treatment for 30 min (Figure 5b).

DISCUSSION

The molecular mechanism of prostate cancer is still unknown. Abnormal regulation of genes or signaling pathways involved in prostate epithelium renewal and maintenance such as Wnt, Notch and Hedgehog occurs in carcinogenesis (Wang, 2006). Previous researches have indicated that Notch-1 signaling is involved in prostate stem cell renew and cell differentiation (Chhipa, 2011). Notch signaling pathway plays many important roles in cell biological behaviors, such as stem cell maintenance, differentiation, cell-fate determination and carcinogenesis in malignant tumors (Muller, 2007; Yin, 2010). However, it is confusing that Notch-1 function as oncogene or suppressor in different cells dependent on cell type and context. Abnormal Notch-1 activation has been associated with cancer onset and offers a variety of treatments.

A number of substrate proteins, including the Notch family, and the amyloid precursor protein (APP) are preceded by γ-secretase-dependent proteolytic cleavage and release the activated intracellular/cytoplasmic domain (Sangram, 2001; Iwatsubo, 2004). Notch-1 precursor must be proteolysed by sequential proteolytic events; the activated Notch-1 then translates into cell nucleus to regulate its targeted gene expression. Regulated intramembrane proteolysis requires the activity of distinct proteases that mediate Notch-1 activation.

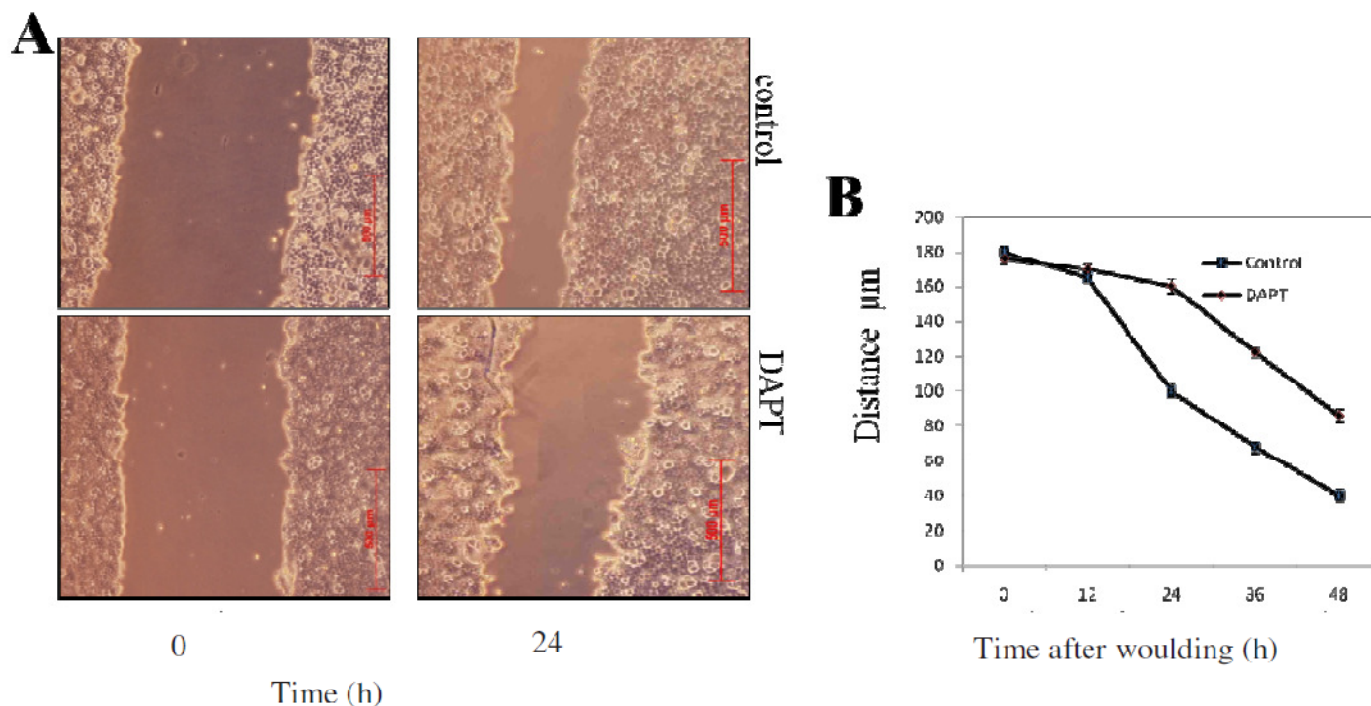


Figure 3. DAPT mediation of the migration of DU145 cells. (A) DU145 cells were treated as before, and the migration after monolayer wounding was detected by microscopy; (B) quantification of the migration was shown as the mean values (\pm SEM) of five measurements for each time point and condition. Standard deviations are indicated ($n=5$).

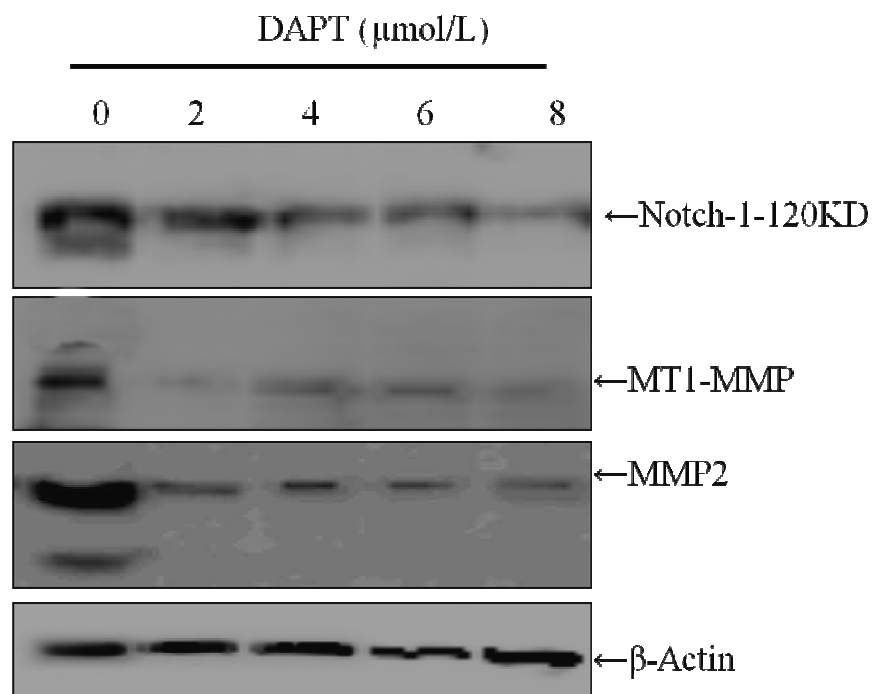


Figure 4. Expression of Notch-1, MT1-MMP and MMP2 regulation by the DAPT. DU145 cells were treated with different concentrations of DAPT for 24 h. Whole cell proteins were extracted by analysis of the expression of Notch-1, MT1-MMP and MMP2 by Western blot assay. β -Actin was used as an internal control to show the equal protein loading.

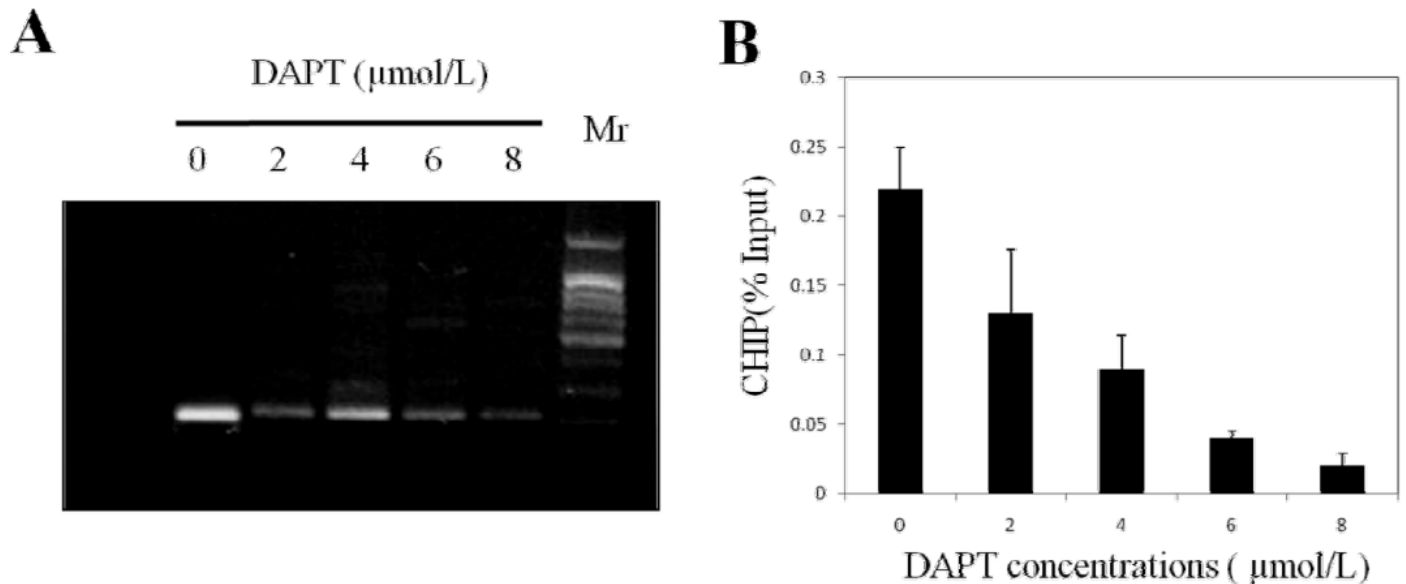


Figure 5. DAPT inhibition of the binding between Notch-1 and Hes-1 promoter. (A) DU145 cells were treated as previous and chromatin samples were immunoprecipitated with Notch-1 antibody and normal rabbit IgG and amplified by 35 cycles of PCR; (B) Q-PCR analysis of CHIP DNA samples showing that DAPT inhibits the association of Notch-1 with Hes-1 promoter. Standard deviations are indicated (n=3).

Regulation of γ -secretase activity has become a potential tumor therapy target that has key relation to Notch-1 signaling pathway (Yunsun et al., 2002; Blat, 2002). In finding out whether the activation of Notch-1 is abnormal in prostate cancer, we first investigated whether regulation of γ -secretase activity could mediate the prostate cancer cell growth and migration by using DAPT. Our results suggest that DAPT treatment significantly inhibited the cells growth and migration in dose- and time- dependent manner. Then, we detected the level of NICD, MT1-MMP and MMP2. MT1-MMP is one of the most important factors involved in cancer cells migration and invasion (Bourboulia, 2010). Once activated, it also activates other protease like pro-MMP2 and pro-MMP13, which are all necessary for invasion machinery (Bravo-Cordero, 2007). The results indicate that the level of NICD, MT1-MMP and MMP2 decreased evidently. Upon entry into nucleus, NICD forms a triprotein complex with RBP-J and the co-activator mastermind that activates the expression of various target genes, including Hes (hairy/enhancer of split), NF- κ B (nuclear factor- κ B) and PPAR (peroxisome-proliferator-activated receptor) families of transcription factors, and cell cycle regulators such as p21CIP1/WAF1 and cyclin D (Jarriault, 1995; Nishimura, 1998; Maier, 2000; Rangarajan, 2001; Iso and Kedes, 2003). So, the production of NICD has vital role in regulation of its effector genes. Our CHIP assay indicate that the Notch-1 target gene Hes-1 was down-regulated, which corresponded for cell growth and invasion.

Our study also show that cell invasion related protein MT1-MMP and its down-target protein MMP2 decreased significantly in accordance with NICD, this suggest that

there may exit a cross-talk between Notch-1 signaling and MT1-MMP-MMP2. Our results indicate that blocking of the Notch1 activity by γ -secretase inhibitors represents a potentially attractive strategy of targeted therapy for prostate cancer.

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