DAPT Enhances the Apoptosis of Human Tongue Carcinoma Cells

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Abstract

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Aim To investigate the effect of DAPT (γ-secretase inhibitor) on the growth of human tongue carcinoma cells and to determine the molecular mechanism to enable the potential application of DAPT to the treatment of tongue carcinoma.

Methodology Human tongue carcinoma Tca8113 cells were cultured with DAPT. Cell growth was determined using Indigotic Reduction method. The cell cycle and apoptosis were analyzed by flow cytometry. Real-time PCR and Immuno-Fluorescence (IF) were employed to determine the intracellular expression levels.

Results DAPT inhibited the growth of human tongue carcinoma Tca8113 cells by inducing G_0–G_1 cell cycle arrest and apoptosis. The mRNA levels of Hairy/Enhancer of Split-1 (Hes-1), a target of Notch activation, were reduced by DAPT in a dose-dependent manner. Coincident with this observation, DAPT induced a dose-dependent promotion of constitutive Caspase-3 in Tca8113 cells.

Conclusion DAPT may have a therapeutic value for human tongue carcinoma. Moreover, the effects of DAPT in tumor inhibition may arise partly via the modulation of Notch-1 and Caspase-3.

Keywords DAPT, human tongue carcinoma cells, Notch, Caspase-3

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Introduction

Notch signalling is known as an evolutionarily conserved mechanism involved in cell proliferation, differentiation and apoptosis (Katoh and Katoh, 2007; Hansson *et al.*, 2004). Nowadays, it has been studied as an important factor in multiple contexts such as organ development (Hansson *et al.*, 2004), tissue regeneration (Lovschall *et al.*, 2005) and carcinogenesis (Liao *et al.*, 2007; Maliekal *et al.*, 2008; Talora *et al.*, 2008). Briefly, it serves a fundamental role in many cell fate decisions, maintaining the balance between cell differentiation and proliferation (Katoh and Katoh, 2007).

Notch signalling is initiated through interactions between a receptor and one of its several ligands between neighbouring cells. In mammalian cells, there are four Notch receptors (Notch-1, Notch-2, Notch-3 and Notch-4) and five type Ⅰ transmembrane ligands (Jagged-1, Jagged-2, Delta- like-1, Delta-like-3, and Delta-like-4). Although similar,

the four receptors show differences in structure that are likely to be responsible for their different expression patterns (Baldi *et al*., 2004) and unique functions (Artavanis-Tsakonas *et al*., 1999). Once bound with a ligand, a Notch receptor is cleaved away first by a disintigrin and metalloprotease, TACE (tumor necrosis factor-α (TNF-α) converting enzyme) and then by a γ-secretase complex that contains the membrane proteins, presenilin and nicastrin. The second cleavage event releases the soluble intracellular fragment of Notch, called NICD (Notch intracellular domain) (Baron *et al.*, 2003).

NICD translocates to the nucleus and binds a CCAAT-binding protein, CBF-1 (also called CSL or RBPJκ). As a consequence, NICD displaces the repressor complex of CBF-1/SMRT–sin3–HDAC-1 and recruits nuclear co-activators, such as MAML1 (mastermind-like 1) (Mitsiadis *et al*., 2005; Kadesch *et al*., 2004) and histone acetyltransferases, converting CBF-1 into a transcriptional activator. Notch activation through CBF-1–NICD interactions can then activate transcription of various target genes, including Hes (Hairy/Enhancer of Split) (Artavanis-Tsakonas *et al*., 1999), HERP (Hes-related repressor protein) (Iso *et al*., 2001; Iso *et al*., 2003), NF-κB (nuclear factor-κB) (Ohazama *et al*., 2004) and PPAR (peroxisome-proliferator-activated receptor) (McKenzie and Sabin, 2005) families, and genes of cell cycle regulators such as p21CIP1/WAF1 and cyclin D (Joshi *et al*., 2008). The set of directly and indirectly Notch-regulated genes and proteins is probably very large and context dependent. Thus, DAPT (N-[N-(3,5-difluorophenacetyl)-L-alanyl]-S-phenylglycine t-butyl ester), an inhibitor of γ-secretase (GSI) can efficiently block the γ-secretase complex and as a consequence, efficiently prevents Notch signaling (Cheng and Kopan, 2005).

Aberrant Notch signalling can be detected in a wide variety of human tumors, such as mammary carcinoma and leukemia (Imatani and Callahan, 2000; Callahan and Egan, 2004; Grabher *et al*., 2006). Notch can either suppress or promote tumors depending on the cell type and context (Fan *et al*., 2004; Mansour *et al*., 2006; Vacca *et al*., 2006; Zhu *et al*., 2006; O'Neill *et al.*, 2007). Not only are there multiple Notch receptors and ligands (each with a unique expression pattern), but the large number of target genes and potential cross-talk between Notch and other signalling cascades further complicate the system. Given the complexity of Notch signalling, it is understandably difficult to predict the outcome of Notch activation. Much more remains to be unraveled about the cross-talk between Notch signalling and other signalling pathways, which will provide explanations for some of the elusive effects of the cascade.

In the present study, we found for the first time that DAPT, a potent γ-secretase inhibitor, could strongly suppress proliferation of Tca8113 cells by inducing cell cycle arrest and apoptosis. One plausible mechanism of this growth inhibition might involve down-regulation of Notch-1 and Caspase-3 signalling, suggesting an across-talk between them in Tca8113 cells. Our findings may provide important clues to aid our understanding of the pathogenesis and modulation of the state of oral squamous cell carcinomas.

Materials and methods

Cancer cell line

Human tongue carcinoma cell line Tca8113 (State Key Laboratory of Oral Diseases, Sichuan University, China) was cultured in α-MEM, supplemented with 10% fetal bovine serum (FBS).

DAPT treatment of human tongue carcinoma cell line Tca8113

DAPT (Sigma-Aldrich, USA) was dissolved in 100% dimethylsulphoxide (DMSO) to make a stock solution of 1 mmol/L, which was then diluted in culture medium to obtain the desired concentrations of 1, 2, 5 and 10 μmol/L. Control cells were treated with 0.5% DMSO for 48 hours.

Analysis of cell growth and G_0-G_1 cell cycle arrest *in vitro*

The *in vitro* growth rate of Tca8113 cells treated with DAPT was measured with the SunBio™ Am-Blue Cell proliferation and activation detection kit (SBO™, China). Briefly, Tca8113 cells were seeded in 96-well plates. On the day of harvest, 10 μL of SunBio™ Am-Blue were added to

each well. Plates were incubated in a cell incubator at 37℃ for 4 hours. When the medium color changed from blue into pink, the absorbance was measured (excitation 540 nm, emission 595 nm) using a HTS 7000 Plus Bio Assay Reader (PERKIN ELMER, PE Co., USA). Cells for flow cytometry analysis were cultured in medium-sized culture flasks. The culture medium was replaced with fresh medium when the cells were 80% confluent and then cells were exposed to various concentrations of DAPT for 48 hours. Later, adherent and floating cells were pooled, washed with phosphate buffered saline (PBS), then fixed in ice-cold 70% ethanol, and stored at −20℃. Prior to analysis, cells were washed and resuspended at 1×10^6 cells/mL in PBS buffer, and incubated with 0.1 mg/mL RNase A and 40 μg/mL 4',6-diamidino-2-pheny-lindole dihydrochloride (DAPI; Roche, Switzerland) at 37℃ for 30 minutes. Samples were analyzed using a fluorescence activated cell sorting (FACS) scanner (Becton Dickinson, USA).

Real-time polymerase chain reaction detection of Notch-1, Hes-1, Caspase-3, Bcl-1

Total RNA from Tca8113 cells was extracted using Total Tissue/cell RNA Extraction Kits (Watson, China), according to the manufacturer's protocol. RNA was reversely transcribed into cDNA in a 10 μL reverse transcription system (Takara, Japan) according to the manufacturer's instructions. To establish the standard curve of home-keeper or target genes, cDNA samples were amplified using a reverse transcriptase polymerase chain reaction (RT-PCR) kit (Tiangen, China). Primers used in the present study were designed using the Primer-BLAST tool from the NCBI website online (http://www.ncbi.nlm.nih.gov/tools/primerblast/index.cgi?LINK_LOC=BlastHomeAd), based on a human mRNA sequence database. The primer sequences used to amplify these genes are shown in Table 1. All PCR products were resolved on a 2% agarose gel and underwent agarose electrophoresis, generating bright and condensed target bands. Expression of certain genes in ASCs was then quantified by real-time PCR using the SYBR® Premix Ex Taq™ (Perfect Real Time) kit (Takara, Japan). Reactions were carried out on an ABI 7300 system (ABI, USA), under the following conditions: cDNA was denatured for 10 seconds at 95℃, followed by 40 cycles, consisting of 5 seconds at 95℃ and 34 seconds at 60℃. For each reaction, a melting curve was generated to test for primer dimmer formation and false priming. Reactions were carried out on an ABI 7300 system (ABI, USA), under the following conditions: relative quantification of target genes was carried out according to the two-standard-curve method (Jorgensen and Leser, 2007).

Immunofluorescence staining

Tca8113 cells cultured in ordinary medium were seeded on glass coverslips in six-well plates before staining. After 48 hours of incubation with 0.5% DMSO-added medium or DAPT-added medium, cells were washed briefly with PBS, fixed in cold acetone for 10 minutes at room temperature, and then permeabilized and blocked in 5% bovine serum albumin (BSA) for 30 minutes at room temperature. Slips were subsequently incubated overnight at 4℃ with rabbit polyclonal antibodies against Caspase-3 (AbCam, USA). Sequentially, slides were incubated with secondary antibodies conjugated to fluorescein isothiocyanate (FITC; Pierce, USA), and nuclei were stained with DAPI. After rinsing in PBS,

Table 1 Primers used in the present study were designed using the Primer-BLAST tool from the NCBI website online based on a human mRNA sequence database

Genes	NM	Forward (5'–3')	Reverse (5'–3')	Product (bp)
Notch-1	017617.3	TACAAGTGCAACTGCCTGCT	TCATAGTCCTCGGATTGCCT	116
Hes-1	005524.2	TCAACACGACACCGGATAAA	TCAGCTGGCTCAGACTTTCA	111
Bcl-1	053056.2	CGTGGCCTCTAAGATGAAGG	CCACTTGAGCTTGTTCACCA	127
c-Myc	002467.3	TAGTGGAAAACCAGCAGCCT	TCGTCGCAGTAGAAATACGG	109
Caspase-3	004346.3	CCTCTTCCCCCATTCTCAT	GAGTCCATTGATTCGCTTCC	119
GAPDH	002046.3	CCGCATCTTCTTTTGCGT	AGTTAAAAGCAGCCCTGGTG	120

cells were observed and imaged using a fluorescence microscope (Leica, DMi 6000 B, Germany).

Statistic analysis

Data were summarized as means values ± SD ($\bar{\chi} \pm s$). Statistical differences were determined using ANOVA or Student's *t*-test. A $P<0.05$ represented a statistically significant difference.

Results

DAPT inhibits the growth of Tca8113 cells

The treatment of Tca8113 cells with DAPT led to cell growth inhibition. DMSO, which was used for dissolving DAPT, served as the control. DAPT dose-dependently (1, 2, 5 and 10 μmol/L) inhibited the cell growth of Tca8113 (Figure 1). Inhibition of cell growth observed by Indigotic Reduction method could also be attributable to the induction of cell cycle arrest and/or apoptosis. Therefore, we further explored whether the inhibition of cell growth was also accompanied by the induction of cell cycle arrest and/or apoptosis induced by DAPT.

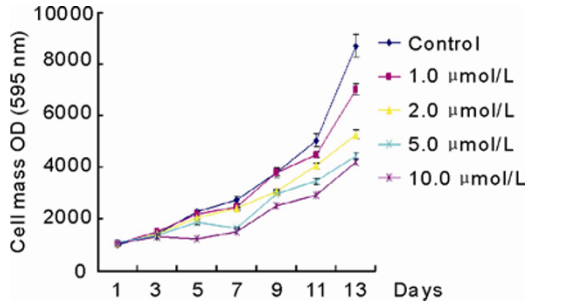

Figure 1 Effect of DAPT on Tca8113 proliferation

Cells were cultured with various concentrations of DAPT ranging from 0 to 10 μmol/L for 48 hours, and then cell growth was determined using the Indigotic Reduction method. Three independent experiments were carried out and each was performed in triplicate in 96-well plates.

DAPT induces G_0–G_1 arrest and apoptosis in Tca8113 cells

To examine whether the growth inhibitory effect of DAPT was related to the induction of cell cycle arrest or an apoptotic process in Tca8113 cells, we used flow cytometry to analyze the cell cycle. DAPT dose-dependently induced G_0–G_1 arrest at concentrations ranging from 1 to 5.0 μmol/L for 48 hours (Figure 2). To further clarify whether the growth inhibitory effect of DAPT was associated with apoptosis, flow cytometry was also used to analyze apoptosis. DAPT-treated cells demonstrated a sub-G_1 (M_1) DNA in a dose-dependent manner. At 1 μmol/L of DAPT, the cells started to show sub-G_1 (M_1) peaks, and 47.01%, 44.24% 32.55% and 30.05% of total cells were observed in the apoptotic region after treatment with 10, 5.0, 2.0 and 1.0 μmol/L DAPT (Figures 2A, 2B, 2C, 2D), respectively. The induction of apoptosis was dose-dependent and was found to be most pronounced at a concentration of 10 μmol/L. Cells undergoing apoptosis displayed profound structural changes, including nuclear disintegration and membrane bleb formation.

Effects of DAPT on Hes-1 and Notch-1 expressions

To examine the effect of DAPT on the expression of Hes-1, a target of Notch activation (Jarriault *et al.*, 1998), we carried out a real-time PCR analysis. Hes-1 was constitutively expressed in freshly prepared Tca 8113 cells. A dose-dependent reduction in the levels of mRNA (Figure 3A) was observed in DAPT-treated cells. The mRNA levels of Notch-1 were also reduced by 40%–78% after DAPT treatment (Figure 3B), suggesting DAPT could both inhibit Notch activation and affect the expression levels of Notch-1 in Tca8113 cells.

DAPT up-regulates Caspase-3 and down-regulates Bcl-1 expression

As Caspase-3 and Bcl-1 play important roles in cell cycle arrest and apoptosis, we assayed the mRNA expression of Caspase-3 and Bcl-1 using real-time PCR to understand the molecular basis of the effects of DAPT. Consistent with the above results from FACS, cell cycle and apoptosis analysis, when the Tca8113 cells were treated with various concentrations of DAPT for 48 hours, the cellular mRNA level of Bcl-1 gradually decreased in a dose-dependent manner (Figure 3C). However, the mRNA level and immunoactivation of Caspase-3

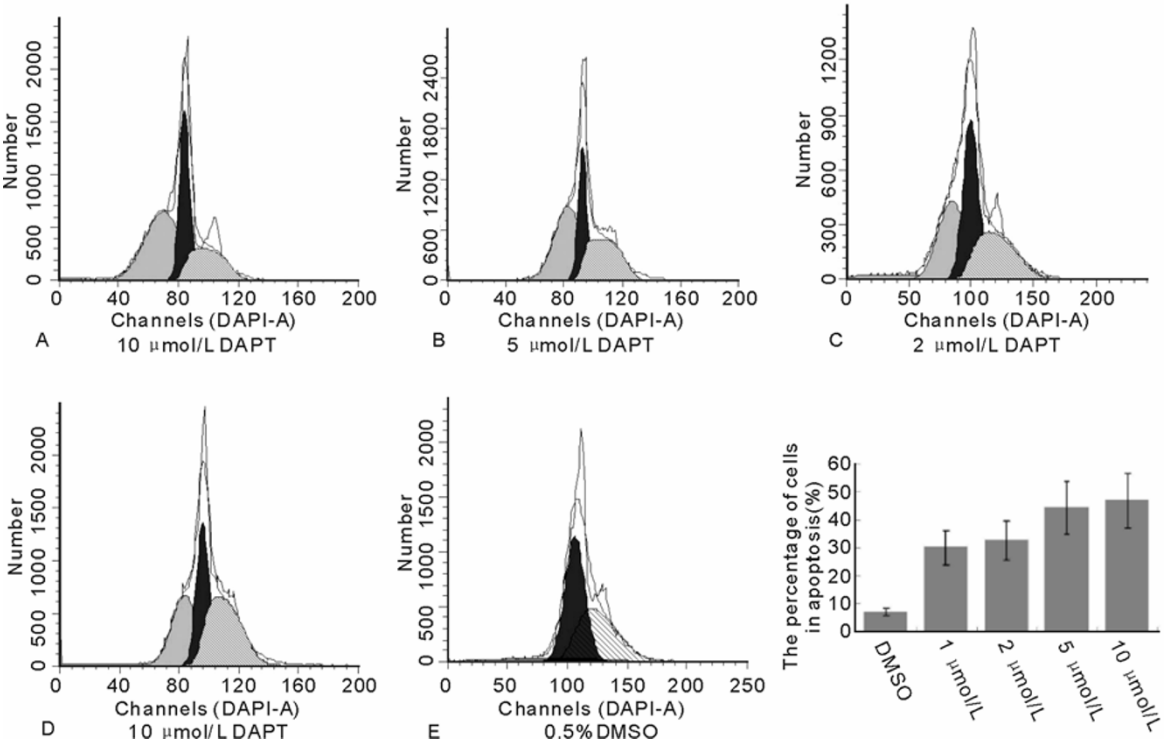

Figure 2 Effect of DAPT on cell cycle and apoptosis

Cells were treated with various concentrations of DAPT for 48 hours, and then analyzed using FACS. The results show G_2/G_1 and Total S phase (A–E), and data (F) of percentages of cells in apoptosis showing the mean ± S.D. from two independent experiments carried out in triplicate. A: Apoptosis 47.01%, G_2/G_1 1.48, Total S phase 39.68%; B: Apoptosis 42.24%, G_2/G_1 1.44, Total S phase 42.02%; C: Apoptosis 32.55%, G_2/G_1 1.38, Total S phase 46.73%; D: Apoptosis 30.05%, G_2/G_1 1.29, Total S phase 56.56%; E: Apoptosis 7.03%, G_2/G_1 1.20, Total S phase 65.10%. There was a significant difference in apoptosis rate between the DAPT-treated group and the control group ($P< 0.005$).

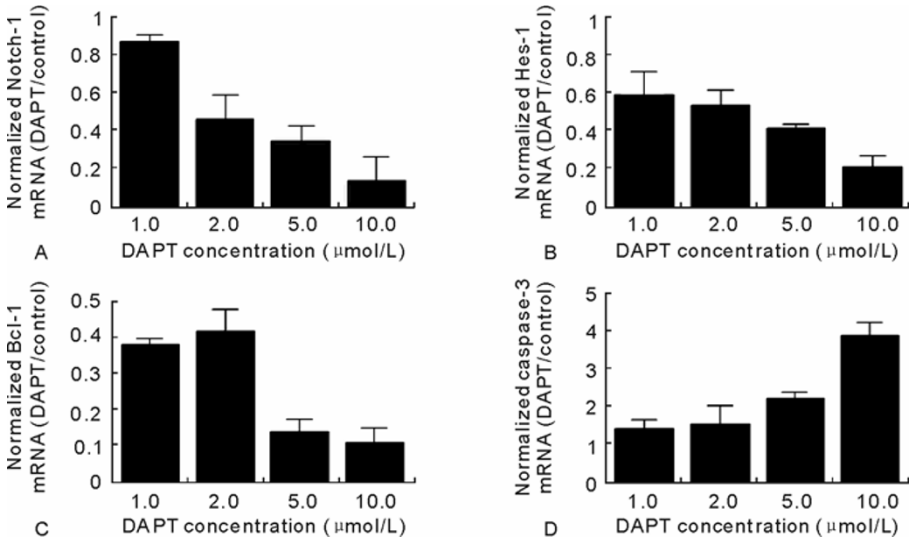

Figure 3 Effects of indicated concentration of DAPT ranging from 1.0 to 10.0 μmol/L on the mRNA expression of Notch-1, Hes-1, Bcl-1 and Caspase-3

The mRNA expression of these genes in Tca8113 cells was detected by real-time PCR analysis, and mRNA levels were normalized by control mRNA expression. Each DAPT concentration was tested in at least three independent experiments. There was a significant difference between each treated group and the control group (0.5% DMSO) ($P<0.05$).

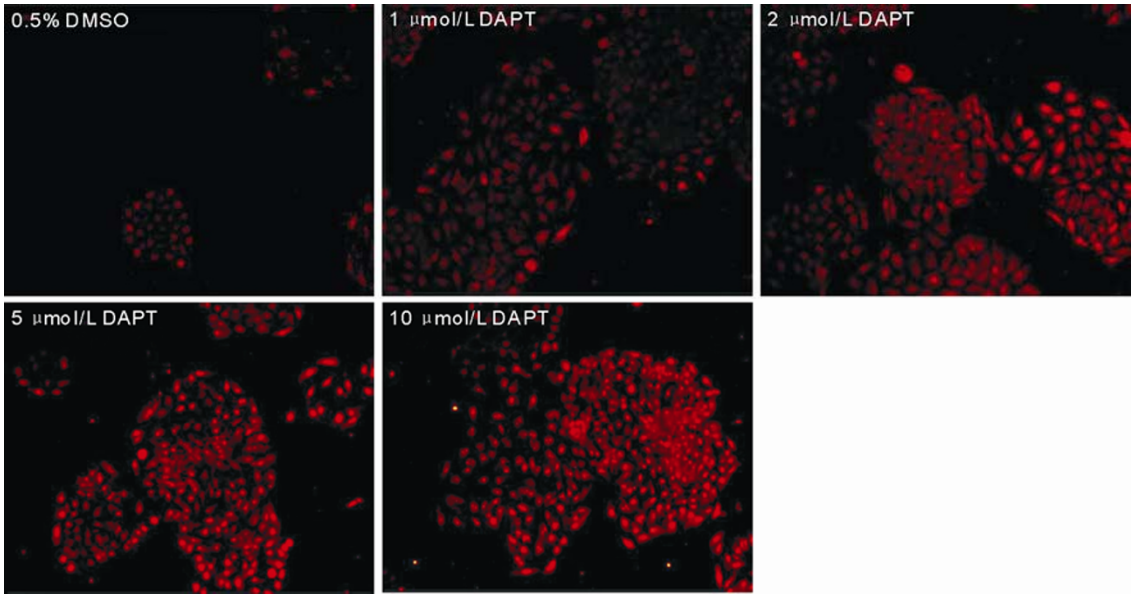

Figure 4 Immunofluorescent detection of caspase-3 antigen in DAPT-treated Tca8113 cells

Immunoactivation of caspase-3 in nuclei was significantly enhanced when the DAPT concentration was increased from 0 to 10 μmol/L.

(Figure 3D and Figure 4) increased significantly in DAPT-treated cells. This suggests that down-regulation of Bcl-1 expression and up-regulation of Caspase-3 might be involved in cell cycle arrest and apoptosis induced by DAPT.

Discussion

Activation of Notch-1 in mammalian epithelial carcinoma has been well studied in mouse models and human carcinoma (Farnie and Clarke, 2007; Yao *et al.*, 2007; Liu *et al.*, 2008). Not only is over-expression of Notch detected in some epithelial derived carcinoma such as mammary, prostate and so on, but truncated NICD protein can induce carcinogenesis *in vitro* (Capobianco *et al.*, 1997). In mouse mammary epithelial cells, Notch-1, -3 and -4 block mammary gland development leading to mammary tumorigenesis. Although in human tongue cancer, the potential role of Notch is still unclear, activated Notch signaling may be common during tumorigenesis. To understand the biological function of Notch-1 in human oral squamous cell carcinoma, we examined the effects of Notch-1 inhibition on a human tongue cancer cell line, Tca8113 cells, using a GSI reagent, DAPT. Our results indicate that DAPT can induce cell cycle arrest and apoptosis. More importantly, we find that Caspase-3, a central apoptosis protein, is up-regulated in Tca8113 cells.

Regulation of the cell cycle via Notch signalling involves the coordination of different, and sometimes antagonizing pathways in a highly cell context-dependent manner. In addition to its role in the cell cycle, Notch may play a role in apoptosis (Artavanis-Tsakonas *et al.*, 1999).

Notch signalling has been implicated in tumorigenesis (Callahan and Raafat, 2001; Wu and Griffin, 2004; Axelson *et al.*, 2006; Kimura *et al.*, 2007), and Notch can either promote or suppress tumors depending on signalling strength, timing, cell type and context (Radtke and Raj, 2003; Lefort and Dotto, 2004). The Notch-1 signaling pathway plays a major role in maintaining the balance of cell proliferation, differentiation and apoptosis, and is closely associated with tumorigenesis. Aberrant increases in Notch-1 signalling are frequently present in colorectal carcinoma and a tendency for increased expression was observed when going from well to poorly differentiated carcinomas (Chu *et al.*, 2008). A constitutively activated Notch-1 signaling pathway was observed in an esophageal squamous cell carcinoma (ESCC) cell line (Lu *et al.*, 2008). The activated Notch-1 signaling pathway gave rise to proliferation suppression of the cells, accompanied by cell cycle inhibition at the G_0/G_1 phase and apoptosis. Furthermore, recent studies have shown

that an antisense RNA or small interfering RNA (siRNA) approach for the inactivation of Notch-1 has a striking anti-neoplastic effect *in vitro* and *in vivo* (Weijzen *et al.*, 2003), strongly supporting the potential role of Notch-1 as a novel therapeutic target for human malignancies. However, some studies suggest that activated Notch-1 and/or Notch-2 inhibit growth and induce cell cycle arrest in small-cell lung cancer (Sriuranpong *et al*., 2001) and hepatocellular carcinoma (Qi *et al.*, 2003).

In this study, inhibition of cell growth observed using the Indigotic Reduction method suggested the induction of cell cycle arrest and/or apoptosis. Significantly, different concentrations of DAPT (1.0–5.0 μmol/L) were found to be effective in the suppression of cell growth, accompanied by cell cycle arrest and apoptosis, in a dose-dependent manner. However, when administered at a much higher concentration (10.0 μmol/L) (Figure 1), DAPT did not enhance cell growth suppression, cell cycle arrest and apoptotic activity. Furthermore, a marked reduction was observed in the levels of Bcl-1 expression in DAPT-treated cells, which was strongly correlated with the altered cell cycle distribution.

To explore the potential molecular mechanism by which DAPT results in the induction of apoptosis in Tca8113 cells, we further examined the expression of Caspase-3, a critical pro-apoptosis protein, and observed a marked increase in the levels of this protein in DAPT-treated cells. Activation of Caspase-3 is a critical event during cell apoptosis, which can be activated by upstream signaling from, for example, Caspase-8 and -9 (Janicke, 2008). However, simultaneous deregulation of Caspase-3 and -8 is a frequent event in tongue squamous cell carcinoma (Andressakis *et al*., 2008). Activation of Caspase-3, which is predominantly down-regulated, may be a crucial process for induction of apoptosis and response to therapeutic strategies. Because Caspase-3 can promote cells into apoptosis, our findings suggested that Caspase-3 overexpression and partial inactivation of Notch-1 appear to be relevant events that are somehow coupled to alterations in DAPT-treated Tca8113 cells.

More recent studies have indicated that GSIs, which attenuate Notch signalling, result in cell cycle arrest and/or apoptosis in carcinoma cells. The GSI cbz–IL–CHO has Notch1-dependent anti-neoplastic activity in Ras-transformed fibroblasts (Weijzen *et al*., 2002). The GSI L-685, 458 can dose-dependently inhibit the growth of human tongue carcinoma cells by inducing G_0–G_1 cell cycle arrest and apoptosis (Yao *et al*., 2007). However, the exact mechanism of action of GSIs in the Tca8113 cells has not been exactly revealed. Further studies are in progress to investigate the additional antiproliferative and antitumor mechanisms of novel γ-secretase inhibitors in *in vitro* and *in vivo* experimental systems for devising novel preventive and therapeutic strategies for human tongue carcinoma.

Conclusion

In summary, our studies show that DAPT inhibits the growth of human tongue carcinoma cells by inducing cell cycle arrest and apoptosis, with reduced levels of proteins Hes-1 and Bcl-1, and increased constitutive expression of Caspase-3. These results may suggest a novel chemical class of antitumor agents targeting Notch functionality, and also indicate the possible mechanism of action of this type of compound in the control of tongue carcinoma cell growth. Further in-depth experiments are needed to investigate the precise molecular mechanisms with regard to the cause and effect relationship between Notch-1 and Caspase-3 in oral squamous cell carcinoma.

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