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ORIGINAL ARTICLE

γ-secretase inhibitors exerts antitumor activity via down-regulation of Notch and Nuclear factor kappa B in human tongue carcinoma cells

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OBJECTIVE: To investigate the effect of the γ-secretase inhibitors (GSIs) on the growth of human tongue carcinoma cells and to provide the molecular mechanism for potential application of GSIs in the treatment of tongue carcinoma.

MATERIALS AND METHODS: Human tongue carcinoma Tca8113 cells were cultured with the GSI L-685 458. Cell growth was determined by the methylthiazole tetrazolium method. Cell cycle and apoptosis were analyzed by flow cytometry and/or confocal microscopy. RT-PCR and Western blot were employed to determine the intracellular expression levels. Nuclear factor kappa B (NF-κB) activation was examined by electrophoretic mobility shift assay.

RESULTS: L-685,458 dose-dependently inhibited the growth of human tongue carcinoma Tca8113 cells by inducing G0–G1 cell cycle arrest and apoptosis. The mRNA and protein levels of Hairy/Enhancer of Split-1, a target of Notch activation, were decreased dose-dependently by L-685,458. Furthermore, L-685,458 down-regulated cyclin D1, B-cell lymphocytic-leukemia proto-oncogene 2 and c-Myc expressions, which are regulated by the transcription factor NF-κB. Coincident with this observation, L-685,458 induced a dose-dependent reduction of constitutive NF-κB activation in Tca8113 cells.

CONCLUSIONS: The GSI L-685,458 may have a therapeutic value for the treatment of human tongue carcinoma. Moreover, the effects of L-685,458 in tumor inhibition may act partially via the modulation of Notch and NF-κB.

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Introduction

Notch receptors (Notch 1–4) are highly conserved transmembrane proteins. They regulate many cell fate decisions during the development of many organs, including cell proliferation and carcinogenesis (Artavanis-Tsakonas *et al*, 1999; Hayashi *et al*, 2004). Notch signaling is initiated through contact between the receptor and one of its several ligands, of which mammals have five: Jagged1, Jagged2, Delta-like1, Delta-like3, and Delta-like4. Receptor–ligand interaction is followed by two successive cleavage events, the first mediated by a disintigrin and metalloprotease, and the second, by the γ-secretase complex that contains the membrane proteins, presenilin and nicastrin. The second cleavage event releases the soluble intracellular domain of Notch (ICN) (Baron, 2003). Thus, inhibition of γ-secretase prevents Notch signaling (Hadland *et al*, 2001). Following cleavage, ICN translocates to the nucleus, forms a complex with a transcription factor, CCAAT-binding factor 1, and acts as a transcription coactivator, activating transcription of target genes, including 'Enhancer of Split' basic helix-loop-helix (bHLH) transcription factors in Drosophila, their mammalian homologues Hes (Hairy/Enhancer of Split) (Artavanis-Tsakonas *et al*, 1999), Hes-related repressor protein (HERP) (Maier and Gessler, 2000; Iso *et al*, 2001), which modulate differentiation by regulating other bHLH proteins. Other Notch targets include $p21^{CIP1/WAF1}$ (Rangarajan *et al*, 2001), transcription factors of the nuclear factor κB (NF-κB) (Cheng *et al*, 2001), poly (ADP-ribose) polymerase families (Garces *et al*, 1997) and so on. The set of directly and indirectly Notch-regulated genes and proteins is probably very large and context dependent.

Notch has been identified as a substrate of γ-secretase. Notch signaling mediates cell fate decisions in mammalian cells during embryogenesis and has become a therapeutic target for investigators interested in treating cancer (Nickoloff *et al*, 2003). Notch receptors are activated by intramembrane proteolysis catalyzed by γ-secretase (Nickoloff *et al*, 2003). Because γ-secretase is needed for all four Notch receptors activation, by targeting this enzyme, it is possible to simultaneously interfere with all Notch receptor-mediated signaling events. γ-Secretase inhibitors (GSIs) have the most immediate therapeutic potential. The GSI cbz–IL–CHO has Notch1-dependent antineoplastic activity in Ras-transformed fibroblasts (Weijzen *et al*, 2002). The GSI z-Leu-leu-Nle-CHO induces apoptosis in melanoma cell lines and melanoma xenografts, but not normal melanocytes, via p53-independent up-regulation of NADPH oxidase (NoxA) (Qin *et al*, 2004). GSI compound E causes growth arrest in T-cell acute lymphoblastic leukemia cells (Weng *et al*, 2004). Like other small-molecule agents, GSIs have multiple effects. γ-secretase not only cleaves all Notch receptors and some ligands but also ErbB4, syndecan, CD44, and other proteins (Nickoloff *et al*, 2003; Kopan and Ilagan, 2004). Therapeutically, this may actually be advantageous because many cancers coexpress two or three different Notch homologues. Some GSIs may also affect proteases other than γ-secretase. Pharmacologic studies with GSIs need to carefully address target specificity, and a complementary transcriptional silencing approach is necessary in each model. The pathways affected by Notch inhibition are likely to be context dependent, and rational combinations of GSIs with other antineoplastic drugs will require mechanistic studies in individual models. Accumulating knowledge about cross-talk between target pathways will guide therapeutic choices.

Additionally, recent studies have shown that antisense RNA or small interfering RNA (siRNA) approach for the inactivation of Notch1 has striking antineoplastic effect *in vitro* and *in vivo* (Weijzen *et al*, 2003). These studies strongly support the hypothesis that Notch signaling is a novel therapeutic target for human malignancies (Nickoloff *et al*, 2003). However, the usefulness of antisense RNA or siRNA approach for the inactivation of Notch1 in a therapeutic setting is not available at the present time. Therefore, we tested whether GSIs, which can down-regulate the Notch signaling, had the similar effect as antisense RNA or siRNA approach, and then our approach would be useful therapeutically. More recent studies have indicated that GSIs, which attenuate Notch signaling, resulted in cell cycle arrest and/or apoptosis in carcinoma cells (O'Neil *et al*, 2006).

In the present study, we found that L-685,458, a potent GSI, could inhibit the growth of human tongue carcinoma Tca8113 cells through induction of cell cycle arrest and apoptosis. One plausible mechanism of the growth inhibition might be the regulation of Notch and NF-κB signaling, suggesting a cross-talk between Notch and NF-κB in Tca8113 cells. This led to down-regulation of expression of gene products regulated by Notch and NF-κB directly/indirectly, thus suppressing proliferation, and inducing cell cycle arrest and apoptosis in tongue carcinoma cells.

Materials and methods

Cell line

Human tongue carcinoma cell line Tca8113 (China Center for Type Culture Collection, Wuhan University, Hubei, China) was cultured in Dulbeco's Modified Eagle's Medium (DMEM), supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 50 units ml^{-1} of penicillin, and 50 $\mu g\ ml^{-1}$ of streptomycin.

L-685,458 treatment of human tongue carcinoma cell line Tca8113

L-685,458 (Sigma, St Louis, MO, USA) was dissolved in 100% dimethyl sulphoxide (DMSO) to make a stock solution of 1.0 mM, which was then diluted in culture medium to obtain the desired concentrations. The final concentrations ranged from 0.5 to 20.0 μM. Control cells were treated with DMSO in amounts equal to the concentration of DMSO necessary to solubilize L-685,458. This treatment was administered for 48 h.

Analysis of cell growth in vitro

The *in vitro* growth rate of Tca8113 cells treated with L-685,458, was measured by the methylthiazole tetrazolium (MTT) method. Briefly, Tca8113 cells were seeded in 96-well plates. On the day of harvest, 100 μl of spent medium was replaced with an equal volume of fresh medium containing 10% MTT 5 mg ml^{-1} stock. Plates were incubated at 37°C for 4 h, then 100 μl of DMSO was added to each well, and then plates were shaken at room temperature for 10 min. The absorbance was measured at 570 nm.

Semiquantitative RT-PCR detection of Hes-1

Total RNA from Tca8113cells was isolated using TRIzol reagent (Invitrogen, Grand Island, NY, USA) according to the manufacturer's instructions. Reverse transcription was performed on 1 μg of total RNA from each sample using oligo (dT)18 primers and 200 units of avian myeloblastosis virus reverse transcriptase (AMV-RT) for extension. PCR amplification was performed with 1.25 units Ex Taq polymerase (TaKaRa Schuzo, Tokyo, Japan). Primers used in the present study were designed using PRIMER3, based on human sequences published in the NCBI data base. The primer sequences used to amplify Hairy/Enhancer of Split-1 *Hes-1* were: forward 5′-TGG AAA TGA CAG TGA AGC ACC T-3′, reverse 5′-GTT CAT GCA CTC GCT GAA GC -3′. *β-actin* was used as the internal control. The primer sequences for *β-actin* were: forward 5′-CGC CGC GCT CGT CGT CGA CA-3′, reverse 5′-GTC ACG CAC GAT TTC CCG CT-3′. The amplified PCR products were subjected to 2.5% agarose gels, and then photographed by a digital scanning system (Bio-Rad Laboratories, Hercules CA, USA).

Flow cytometry analysis
Cells were cultured in 60-mm tissue-culture dishes. The culture medium was replaced with fresh medium when the cells were 80% confluent and then cells were exposed to various concentrations of L-685,458 for 48 h. Later, adherent and floating cells were pooled, washed with phosphate buffered saline (PBS), then fixed in ice-cold 70% ethanol, and stored at −20°C. Prior to analysis, cells were washed and resuspended at 1×10^6 cells ml^{-1} in PBS buffer, and incubated with 0.1 mg ml^{-1} RNase A and 40 μg ml^{-1} propidium iodide at 37°C for 30 min. Samples were analyzed by a fluorescence activated cell sorting (FACS) scanner (Becton Dickinson, San Joes, CA, USA).

Nuclear staining with Hoechst 33342
Tca8113 cells (1×10^8 ml^{-1}) were cultured in 60 mm dishes in Dulbecco's Modified Eagle's Medium (DMEM) containing 1% fetal bovine serum in the absence or presence of L-685,458 (0.5–20 μM). The samples were stained in 4 μg ml^{-1} Hoechst 33342 (Sigma, USA) for 30 min at 37°C, fixed for 10 min in 4% paraformaldehyde and observed by confocal microscopy. For statistical analysis, 200 cells were counted in eight random microscopic fields at 400 × magnification.

Western blotting
Medium was aspirated from the plate, and the cell monolayer was washed with PBS. Cellular total protein extraction was carried out using M-Per Mammalian Protein Extraction Reagent (Pierce, Rockford, IL, USA). Cell extracts were colleted and centrifuged at 14 000 × *g* in a microcentrifuge for 20 min at 4°C. The supernatants were collected, and the protein concentration was determined using Bradford's method. Cell extracts were either used immediately or stored at −70°C. For polyacrylamide gel electrophoresis (PAGE), 20 μg of proteins from each extract were loaded into each well of the gel and electrophoresed at 98 V. Proteins on the gel were transferred onto a polyvinylidene difluoride membrane by half-dry electrotransfer for 1 h. The membrane was blocked in 3% bovine serum albumin (BSA) in tris-buffered saline (TBS) containing 0.05% Tween 20 for 1 h. The primary antibody against Hes-1, Notch1, cyclin D1, B-cell lymphocytic-leukemia proto-oncogene 2 (Bcl-2), c-Myc (Santa Cruz Biotechnology, Santa Cruz, CA, USA), or β-actin (Sigma, USA) was added, and incubated with the membrane overnight at 4°C before the membrane was washed three times with TBS/Tween20. Secondary antibody conjugated to horseradish peroxidase was added to the membrane and was incubated at room temperature with gentle agitation. 1 h later, the membrane was washed with four changes of TBS/Tween 20, at 5 min per wash. Differences in protein expression were determined by densitometry analysis using Scion Image Software (Scion Corporation, Frederick, MD, USA). Western blot experiments were repeated at least twice to confirm the results.

Electrophoretic mobility shift assay
Electrophoretic mobility shift assay (EMSA) was performed using a commercial kit (Gel Shift Assay System; Promega, Madison, WI, USA) as previously described (Cheng *et al*, 2001). The NF-κB oligonucleotide probe (5′-AGT TGA GGG GAC TTT CCC AGG C-3′) was end-labeled with (γ-^{32}P) ATP (Free Biotech, Beijing, China) with T4-polynucleotide kinase. Nuclear protein (2 μg) was preincubated in binding buffer, consisting of 10 mM Tris–Cl, pH 7.5, 1 mM $MgCl_2$, 50 mM NaCl, 0.5 mM ethylene diamine tetra acetic acid, 0.5 mM dithiothreitol (DTT), 4% glycerol, and 0.05 g l^{-1} of poly (deoxyinosinic deoxycytidylic acid) for 15 min at room temperature. After the addition of 1 μl ^{32}P-labeled oligonucleotide probe, incubation was continued for 30 min at room temperature. The reaction was stopped by adding 1 μl of gel loading buffer, and the mixture was subjected to non-denaturing 4% PAGE in 0.5 × tris-borate-EDTA (TBE) buffer. The gel was vacuum-dried and exposed to X-ray film at −70°C. The specificity of NF-κB DNA binding activity was also examined by competition with the unlabeled oligonucleotide.

For supershift assays, nuclear extracts prepared from L-685,458-treated cells were incubated with antibodies against either p50 or p65 of NF-κB for 30 min at room temperature before the complex was analyzed by EMSA. Antibodies against preimmune serum were included as negative controls. The dried gels were visualized, and radioactive bands were quantitated by a PhosphorImager (Molecular Dynamics, Sunnyvale, CA, USA) using Imagequant software.

Statistical analysis
Data are expressed as the means ± s.d. Statistical differences were determined by ANOVA or Student's *t*-test. A $P < 0.05$ represented a statistically significant difference.

Results

L-685,458 inhibits cell growth of Tca8113 cells
The treatment of Tca8113 cells with L-685,458 led to cell growth inhibition. DMSO, which was used for dissolving L-685,458, served as the control. As shown in Figure 1, L-685,458 dose-dependently (0.5–5.0 μM) inhibited the cell growth of human tongue carcinoma cell line Tca8113. However, the growth inhibitory action of L-685,458 was not found to be enhanced at much higher concentrations (10.0–20.0 μM), suggesting that some growth factors may protect cells from Notch inactivation. Inhibition of cell growth observed by MTT 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 L-685,458.

L-685,458 induces G0–G1 arrest in Tca8113 cells
In order to examine whether the growth inhibitory effect of L-685,458 was related to induction of cell cycle arrest or apoptotic process in Tca8113 cells, we used flow cytometry to analyze cell cycle. As shown in Figure 2,

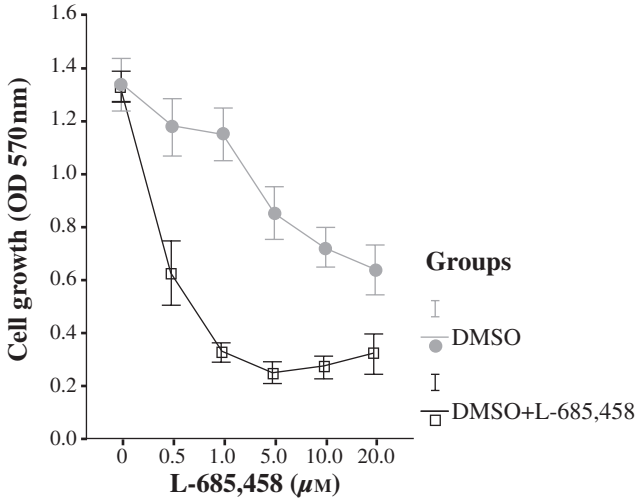

Figure 1 Growth inhibitory effect of L-685,458 in cultured Tca8113 cells. Cells were treated with the indicated concentrations of L-685,458 for 48 h, and determined the cell growth with methylthiazole tetrazolium method. At least three independent experiments were carried out and each was performed in triplicate in 96-well plates. The cells cultured with L-685,458 at concentrations ranging from 0 to 20 μM

L-685,458 dose-dependently induced G0–G1 arrest at concentrations ranging from 0.5 to 5.0 μM for 48 h, which, however, started diminishing at much higher concentrations ranging from 10.0 to 20.0 μM.

L-685,458 induces apoptosis in Tca8113 cells

To further clarify whether the growth inhibitory effect of L-685,458 was associated with apoptosis, flow cytometry was also used to analyze apoptosis. L-685,458-treated cells demonstrated a sub-G1 (M1) DNA in a dose-dependent manner (Figure 3a and b). At 0.5 μM of L-685,458, the cells started to show evident sub-G1 (M1) peaks, and 54.68% and 70.38% of total cells were observed in the apoptotic region after treatment with 1.0 and 5.0 μM L-685,458, respectively. The induction of apoptosis was dose-dependent and was found to be most pronounced at the concentration of 5.0 μM. However, it should be noted that considerably higher concentrations of L-685,458 (10.0–20.0 μM) were relatively less effective in inducing apoptosis in these cells, which was just consistent with the results of MTT and cell cycle analysis by flow cytometry.

Cells undergoing apoptosis display profound structural changes, including nuclear disintegration and membrane blebbing formation. After treatment with L-685,458 for 48 h, nuclei were stained with Hoechst 33342 dye and observed by confocal microscopy in order to study the change in nuclear morphology. 5.0 μM L-685,458 significantly induced the increase of chromatin condensation and fragmentation (Figure 3c) compared with DMSO control, suggesting the occurrence of apoptotic changes in the cells.

Effects of L-685,458 on Hes-1 and Notch1 expressions

To examine the effect of the GSI on expression of Hes-1, a target of Notch activation (Jarriault *et al*, 1998), we carried out a semi-quantitative RT-PCR and Western blot analysis. Hes-1 was constitutively expressed in freshly prepared Tca8113 cells. In the presence of L-685,458, a dose-dependent reduction in the levels of mRNA (Figure 4a) and the cellular total protein of Hes-1 (Figure 4b) was observed in L-685,458-treated cells. However, the cellular total protein levels of Notch1 remained unchanged after L-685,458 treatment (Figure 4b), suggesting L-685,458 could inhibit Notch activation rather than affect the expression levels of Notch1 in Tca8113 cells.

L-685,458 down-regulates cyclin D1, Bcl-2, and c-Myc expressions

As cyclin D1, Bcl-2 and c-Myc play an important role in cell cycle arrest and apoptosis, we assayed the protein expressions of cyclin D1, Bcl-2 and c-Myc with Western blot to understand the molecular basis for effects of L-685,458. As shown in Figure 5, when the Tca8113 cells were treated with various concentrations of L-685,458 for 48 h, the cellular total protein levels of cyclin D1, Bcl-2, and c-Myc were gradually decreased in a dose-dependent manner. Consistent with the above results of MTT, cell cycle and apoptosis analysis, a little increase in the protein levels of cyclin D1, Bcl-2 and c-Myc was observed in Tca8113 cells treated with 10.0–20.0 μM L-685,458, suggesting down-regulation of cyclin D1, Bcl-2, and c-Myc expressions might be involved in cell cycle arrest and apoptosis induced by L-685,458.

L-685,458 inhibits constitutive NF-κB activation

Because cyclin D1, Bcl-2, and c-Myc have all been shown to be regulated via NF-κB signaling pathway (Pahl, 1999), we investigated whether the down-regulation of cyclin D1, Bcl-2 and c-Myc is via NF-κB signaling pathway in Tca8113 cells. To examine the effect of L-685,458 on constitutive NF-κB activation, we prepared the nuclear extracts of Tca8113 cells treated with various concentrations of L-685,458, and examined NF-κB DNA binding activity by EMSA. Gel shift assays demonstrated that NF-κB DNA binding activity was observed to significantly decrease at concentrations of 0.5–20.0 μM, and be most pronounced at concentration of 5.0 μM (Figure 6a). Consistent with above results, the inhibition of NF-κB DNA binding activity was not promoted at concentration of 10.0–20.0 μM, indicating that L-685,458 might down-regulate the protein levels of cyclin D1, Bcl-2 and c-Myc partially by inhibiting NF-κB activation, and led to the induction of growth inhibition, cell cycle arrest and apoptosis in Tca8113 cells, suggesting that NF-κB is responsible for down-regulation of cyclin D1, Bcl-2 and c-Myc expressions.

Various combinations of Rel/NF-κB proteins can constitute an active NF-κB heterodimer that binds to a specific sequence in DNA (Ghosh *et al*, 1998). To show that the retarded band visualized by EMSA was indeed NF-κB in L-685,458-treated cells, we incubated nuclear extracts from 5.0 μM L-685,458-treated cells with antibody to either the p50 or the p65 subunit of NF-κB. Only NF-κB/p65 shifted the band to a higher molecular mass (Figure 6b), thus suggesting that the L-685,458-

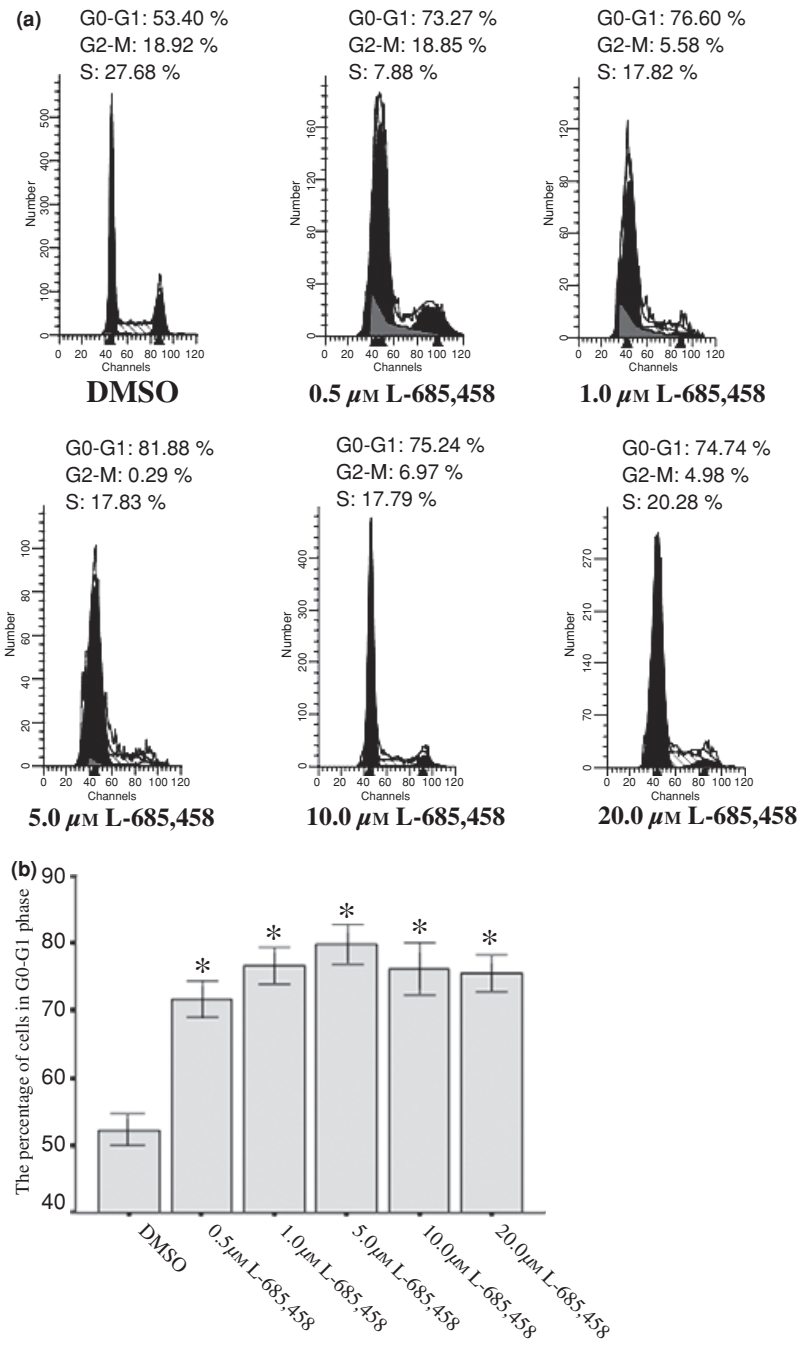

Figure 2 Effect of L-685,458 on cell cycle progression. Cells were treated with the indicated concentrations of L-685,458 for 48 h, and then the distribution of cell cycle was analyzed by flow cytometry. A representative result (**a**), and data (**b**) are mean ± s.d. of two independent experiments in triplicate. Significant differences from control
$^*P < 0.05$

treated complex consisted of p65 subunits. These results provide evidence for a potential cross-talk between Notch1 and NF-κB signaling pathways during cell growth inhibition, cell cycle arrest and apoptosis are induced by L-685,458 in Tca8113 cells.

Discussion

Here, we report that down-regulation of Notch-1 by the GSI L-685,458 can inhibit the growth of human tongue squamous cell carcinoma cell line Tca8113, accompanied by cell cycle arrest and apoptosis, as shown by MTT assay, flow cytometry analysis and confocal microscopy, respectively. Furthermore, besides inhibition of Notch activation, inhibition of NF-κB activation partially contributes to cell cycle arrest and apoptosis induced by L-685,458 in human tongue carcinoma Tca8113 cells, and thus provide the possibility and molecular mechanism for potential application of GSIs in the treatment of tongue carcinoma.

Based on the antiproliferative activity of L-685,458, we investigated the possible mechanism. L-685,458 could inhibit the growth of Tca8113 cells *in vitro*. Significantly, the concentrations of L-685,458 (0.5–

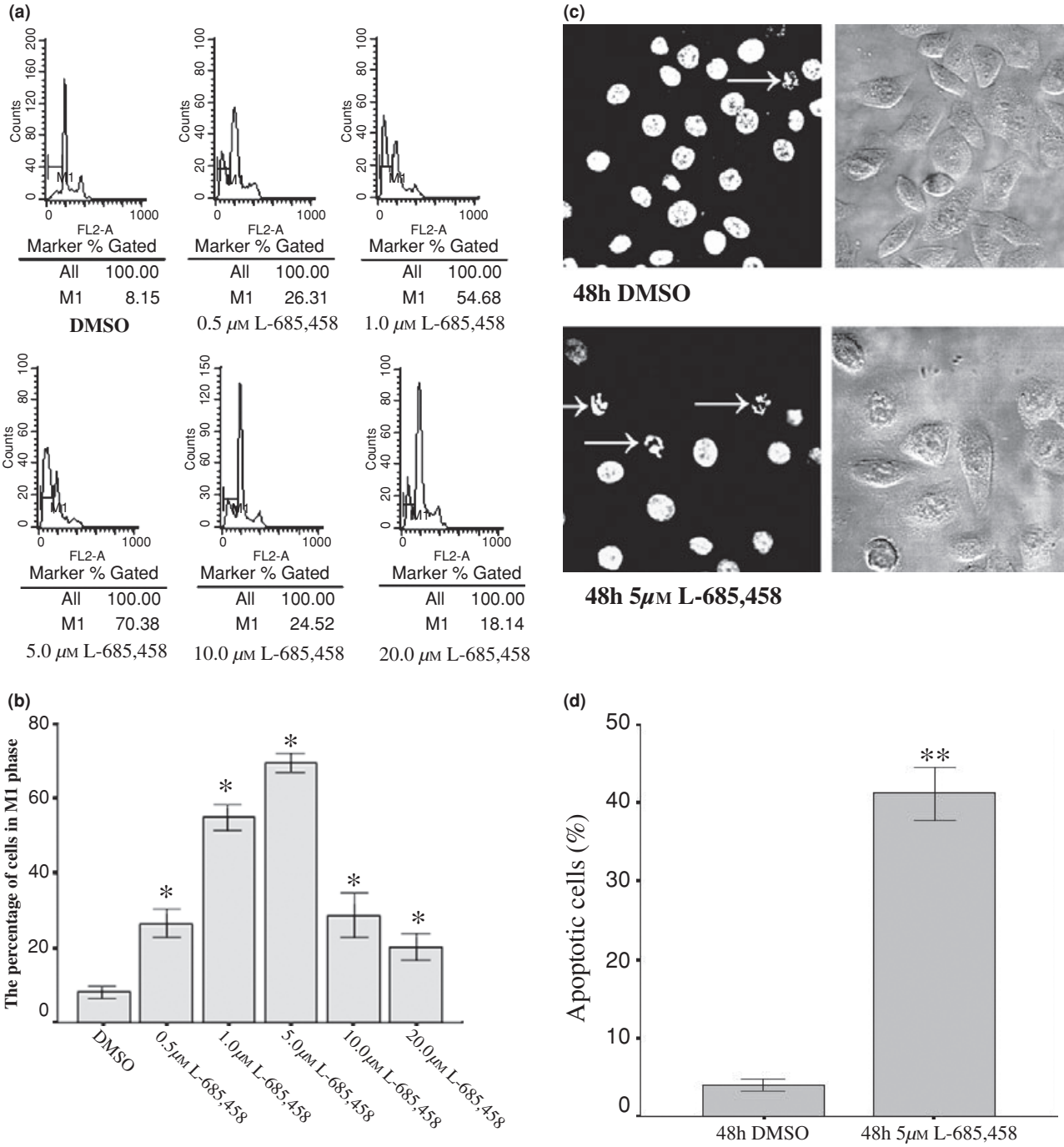

Figure 3 Effect of L-685,458 on apoptosis. Analysis of apoptosis in Tca8113 cells incubated with 0–20.0 μM L-685,458 for 48 h. After incubation, cells were stained with propidium iodide and analyzed by flow cytometry (**a**) as described in materials and methods. Percentage of cells with sub-G1 (M1) is expressed as mean ± s.d. from three independent experiments (**b**). Tca8113 cells treated by dimethyl sulphoxide, and 5 μM L-685,458 for 48 h were stained with Hoechst 33342 fluorescent dye (**c**). Cells in the stages of apoptosis are indicated by arrows. Fluorescent-stained nuclei of apoptotic cells were analyzed morphologically and expressed as the percentage of total nuclei, as described in methods (**d**). Significant differences from control $^{**}P < 0.01$

5.0 μM) 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 much higher concentrations (10.0–20.0 μM), L-685,458 did not enhance cell growth suppression, cell cycle arrest and apoptotic activity. The exact mechanism of action of GSIs in the cells has been still unclear exactly but most likely would have involved more than one single molecular target. γ-secretase has numerous substrates, including all four Notch receptors, several Notch ligands, ErbB4, syndecan, CD44, and several other molecules (Nickoloff *et al*,

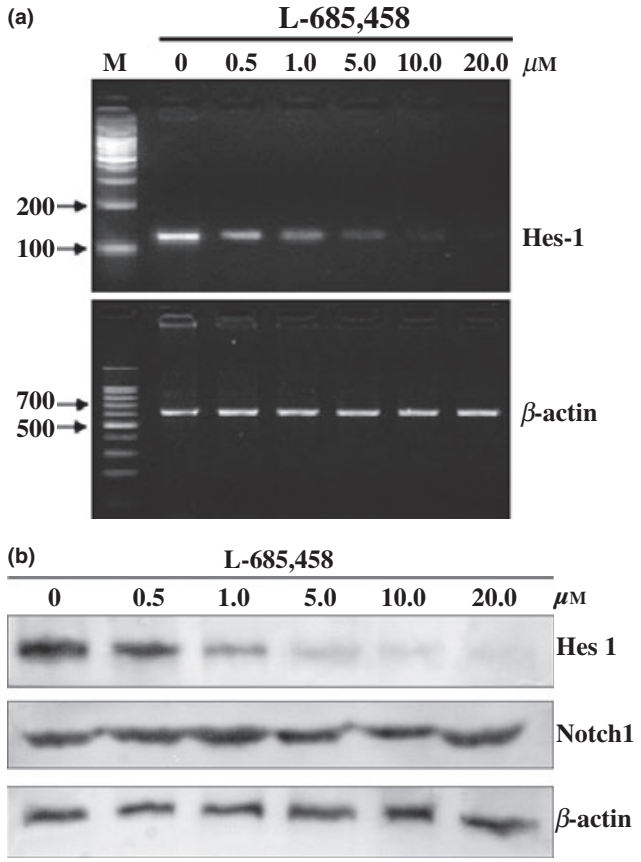

Figure 4 Effects of L-685,458 on the expression of Hairy/Enhancer of Split-1 (Hes-1) and Notch1. Hes-1 mRNA expression (115 bp) in Tca8113 cells was detected by a semi-quantitative RT-PCR analysis, and mRNA levels of Hairy/Enhancer of Split (Hes-1) were normalized by β-actin (619 bp) mRNA expression (**a**), Protein levels of Hes-1 and Notch1 were detected by Western blot analysis (**b**). Each of the blots shown was demonstrated to have equal protein loading by reprobing with the monoclonal antibody for β-actin

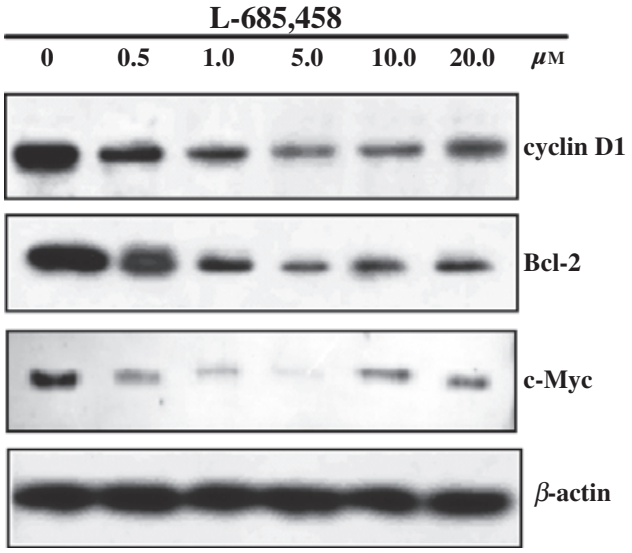

Figure 5 Effects of L-685,458 on cyclin D1, B-cell lymphocytic-leukemia proto-oncogene 2 (Bcl-2) and c-Myc expressions in Tca8113 cells. The protein levels of cyclinD1, Bcl-2 and c-Myc were detected by Western blot analysis. Each of the blots shown was demonstrated to have equal protein loading by reprobing with the antibody for β-actin

2003). Moreover, we cannot rule out that the GSI may have additional targets besides γ-secretase (Hartmann *et al*, 1997), or exclude the possibility of its action on undefined substrates of γ-secretase (Paris *et al*, 2005). It is not surprising that the same pharmacological agents may exert different, even reverse effects at different doses. Therefore, it is logic to speculate that L-685,458 may dose-dependently induce cell cycle arrest and apoptosis in certain concentration range via some pathways, such as Notch and NF-κB, while too high concentrations of L-685,458 may trigger other pathways to resist the antiproliferative effect to survive the cells, and a negative feedback circuit may exist between them. However, further studies are needed to systematically explore this possibility.

Plausible mechanisms for effects of Notch signaling in transformed cells include growth promotion (Jehn *et al*, 1999; Shelly *et al*, 1999; Jundt *et al*, 2002). Different mechanisms may predominate in different cell types. Regulation of the cell cycle via Notch signaling involves the coordination of different, and sometimes antagonizing pathways in a highly cell context-dependent manner. In this study, Blocking Notch activation with L-685,458 resulted in cell growth suppression of human tongue carcinoma cells. A marked reduction was observed in the levels of cyclin D1 and c-Myc expression in L-685,458-treated cells, which were strongly correlated with the altered cell cycle distribution (Zhu *et al*, 2006). Besides the role of Notch on cell cycle, Notch may also play a role in apoptosis (Artavanis-Tsakonas *et al*, 1999). To explore the molecular mechanism by which L-685,458 results in the induction of apoptosis in Tca8113 cells, we examined the expression of the antiapoptotic protein Bcl-2, which plays a very important role in oral carcinogenesis (Loro *et al*, 2005), and observed a marked reduction in the levels of Bcl-2 protein in L-685,458-treated cells. Because Bcl-2 can protect cells from apoptosis, our findings suggested that decreased Bcl-2 expression might participate in apoptosis induced by L-685,458 in Tca8113 cells.

Accumulating knowledge about cross-talk between Notch and NF-κB pathways (Bellavia *et al*, 2000; Wang *et al*, 2001; Aguilera *et al*, 2004) will provide novel explanations to some of the elusive effects of the cascade. NF-κB acts at the crossroads of many signaling pathways. Inappropriate or excessive activation of NF-κB can lead to various cancers (Dobrovolskaia and Kozlov, 2005). In recent years, increasing evidence indicates that activation of NF-κB plays an important role in coordinating the control of apoptosis (Srivastava and Singh, 2004). Both Notch and NF-κB signaling play a key role in embryogenesis, regulation of cell proliferation, motility, and cell fate, and aberrant Notch and NF-κB signaling have been shown to contribute to a variety of cancers (Oakley *et al*, 2003; Axelson, 2004; Moynagh, 2005). NF-κB can be regulated by Notch1 (Cheng *et al*, 2001; Wang *et al*, 2001;

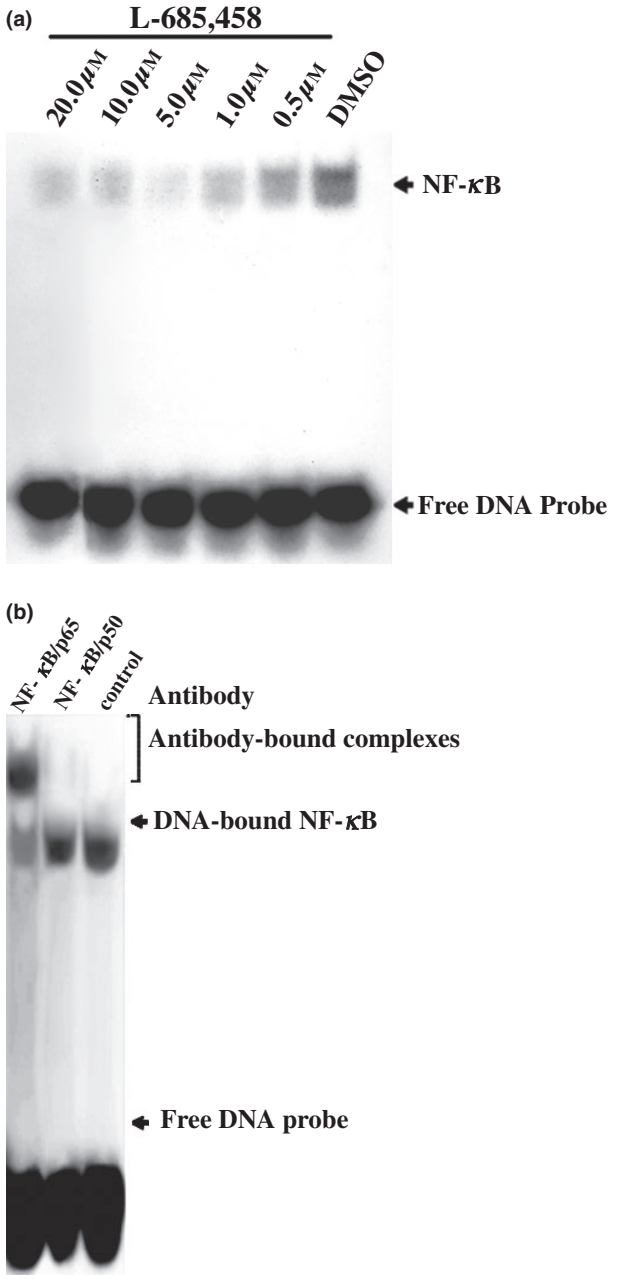

Figure 6 Effect of L-685,458 on Nuclear factor kappa B (NF-κB) DNA binding. Nuclear extracts from Tca8113 cells incubated with 0–20.0 μM L-685,458 for 48 h were subjected to electrophoretic mobility shift assay to assess NF-κB DNA binding (**a**). Supershift assay of 5.0 μM L-685,458 treated Tca8113 cells with specific NF-κB antibody (**b**)

Jang *et al*, 2004) and exert anti or proapoptotic functions that are cell type and context dependent (Srivastava and Singh, 2004; Dobrovolskaia and Kozlov, 2005) Consistent with the previous findings, blocking Notch activation with the GSI L-685,458 led to NF-κB inhibition in Tca8113 cells. Moderate or endogenous levels of Notch1 activation are accompanied by rapid and transient induction of NF-κB activity (Bellavia *et al*, 2000; Nickoloff *et al*, 2002; Lathion *et al*, 2003; Palaga *et al*, 2003; Jang *et al*, 2004; Pear and Aster, 2004), suggesting that Notch activation partially contributes to induction of NF-κB activity in those cells. Conversely, overexpression of Notch1 has been shown to inhibit NF-κB activity (Guan *et al*, 1996; Wang *et al*, 2001). If both theories are correct, we would expect both down-regulation and overexpression of Notch to result in lower NF-κB activity.

In summary, our studies show that L-685,458, a potent GSI, inhibits the growth of human tongue carcinoma cells by inducing cell cycle arrest and apoptosis, with reduced protein levels of Hes-1, cyclin D1, Bcl-2, and c-Myc. Furthermore, the regulation of Notch and NF-κB signaling pathways might be involved in the antiproliferative mechanism of L-685,458 in Tca8113 cells. This report, therefore, may provide a novel chemical class of antitumor agents targeting Notch and NF-κB functionality, and also indicate the possible mechanism of this type of compound on the control of tongue carcinoma cell growth. Further studies are in progress to investigate the additional antiproliferative and antitumor mechanism of novel γ-secretase inhibitors *in vitro* and *in vivo* experimental systems for devising novel preventive and therapeutic strategies for human tongue carcinoma.

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