Inhibition of gamma-secretase affects proliferation of leukemia and hepatoma cell lines through Notch signaling

Saipin Suwanjunee[a], Wipawee Wongchana[a] and Tanapat Palaga[a,b]

Notch signaling is a well-conserved pathway playing crucial roles in regulating cell fate decision, proliferation, and apoptosis during the development of multiple cell lineages. Aberration in Notch signaling is associated with tumorigenesis of tissues from various origins. To investigate the role Notch signaling plays in the proliferation of cancer cell lines, the expression profiles of Notch1 in six human cancer cell lines (Jurkat, HepG2, SW620, KATOIII, A375, BT474) were examined. All cell lines differentially expressed Notch1, and only Jurkat and SW620 expressed cleaved Notch1 (Val1744). Among the six cell lines tested, only Jurkat and HepG2 showed a decrease in cell proliferation during 4 days of treatment with a γ-secretase inhibitor (GSI). This is the first report on the anti-proliferative effects of GSI on a human hepatoma cell line. These two cell lines expressed *Notch1-3*, *Jagged1*, *Jagged2*, *Dlk1* and *Hes1*. GSI treatment led to a decrease in *Hes1* expression in both cell lines. Surprisingly, GSI treatment resulted in the accumulation of Notch1 protein upon treatment. During this period, GSI treatment did not induce apoptosis, but caused cell cycle arrest in both cell lines. This was also correlated with decreased c-myc expression. Forced expression of activated intracellular Notch1 completely abrogated GSI sensitivity in both cell lines. These results clearly demonstrate that Notch signaling positively regulates cell proliferation in Jurkat and HepG2 cell lines and that GSI treatment inhibits tumor cell proliferation through the suppression of Notch signaling. *Anti-Cancer Drugs* 19:477–486 © 2008 Wolters Kluwer Health | Lippincott Williams & Wilkins.

Anti-Cancer Drugs 2008, **19**:477–486

Keywords: apoptosis, c-myc, HepG2, Jurkat, Notch signaling, proliferation, tumor, γ-secretase inhibitor

[a]Program in Biotechnology and [b]Department of Microbiology, Faculty of Science, Chulalongkorn University, Bangkok, Thailand

Correspondence to Assistant Professor Tanapat Palaga, PhD, Faculty of Science, Department of Microbiology, Chulalongkorn University, Bangkok 10330, Thailand
Tel: +66 2 218 5070; fax: +66 2 252 7576; e-mail: tanapat.p@chula.ac.th

Received 26 June 2007 **Revised form accepted** 11 February 2008

Introduction

Notch signaling is an evolutionarily conserved signaling pathway that plays an essential role in regulating cell fate determination, differentiation, proliferation, and apoptosis [1]. The Notch receptor is a transmembrane protein expressed on the cell surface as a heterodimer of an EGF-like repeat-rich extracellular domain and an intracellular domain with a single pass transmembrane domain [2]. There are four distinct isoforms of Notch (Notch1-4) in mammals, whereas five different ligands have been identified [3]. Notch signaling is initiated by ligand–receptor interaction, leading to two proteolytic cleavage events of the Notch receptor, by TACE and γ-secretase [4]. This cleavage results in the release of the intracellular domain from the cell surface. Intracellular Notch migrates to the nucleus where it forms a large protein complex with the DNA binding protein CSL and other transcriptional co-activators, such as MAML and p300 [5]. In humans, Notch receptor was first identified in T cell acute lymphoblastic leukemia (T-ALL), where chromosomal translocation resulted in the expression of truncated Notch under regulation of the *TCR* promoter in T lymphocytes [6]. Since then, aberrations in Notch signaling have been linked to various forms of neoplastic formation, such as T cell leukemia, breast cancer, and lung cancer [1,7,8]. However, unlike other genes involved in tumorigenesis, Notch signaling functions in a context-dependent manner. It can function to promote tumor formation in one instance and limit tumor growth in another [7].

The involvement of Notch signaling in tumor formation invoked an intense interest in exploring γ-secretase inhibitor (GSI) as a new therapeutic agent to control tumor growth [1,7]. γ-Secretase is a multi-subunit enzyme with specificity in cleaving intra-membranous substrates, such as Notch, APP, CD44, and Erb4 [9]. Mutations in one of its subunits, *presenilin*, are associated with early onset of familial Alzheimer's disease [10,11]. As all Notch receptors share the feature of activation-based γ-secretase cleavage, and hyperactivation of Notch signaling in many cases is linked to tumor formation, it is therefore logical to try to suppress Notch signaling (of all four subtypes) using inhibitors designed to suppress γ-secretase activity as cancer therapeutics. Many groups have reported that GSI is highly effective in suppressing tumor growth both *in vitro* and *in vivo* [12–15]. Therefore, γ-secretase represents a new target for controlling tumor growth in a molecular target-specific manner. In this study, we report the effect of GSI treatment on cell growth of six human cancer cell lines *in vitro* and identify Notch signaling as a crucial signaling pathway in controlling cell proliferation in a T cell leukemia cell line (Jurkat), and hepatocellular carcinoma-derived cell line

(HepG2) through the regulation of c-myc. In addition, we present findings on the accumulation of Notch receptor upon GSI treatment, which may present a problem for using GSIs as cancer therapeutics.

Materials and methods

Cell lines and reagents

Jurkat (ATCC no. TIB152), HepG2 (ATCC no. HB-8065), SW620 (ATCC no. CCL-227), KATOIII (ATCC no. HTB-103), BT474 (ATCC no. HTB-20), A375 (ATCC no. CRL-1619) were obtained from the American Type Culture Collection (Manassas, Virginia, USA). All cells were maintained in RPMI1640 supplemented with 10% fetal bovine serum (Hyclone, Utah, USA), 100 mmol/l HEPES, 100 mmol/l sodium pyruvate, and 10 mmol/l gentamycin and grew at 37°C in 5% CO_2. *N*-[*N*-(3,5-difluorophenacetyl-L-alanyl)]-*S*-phenylglycine-*t*-butyl ester (DAPT; Calbiochem, San Diego, California, USA) was dissolved in DMSO at 100 mmol/l and stored at −80°C. VP16 was dissolved in DMSO and stored at −20°C (Sigma Aldrich, St Louis, Missouri, USA).

MTT proliferation assay

Cells were treated as indicated and MTT dissolved in PBS (5 mg/ml) was added at 4 h before the end of treatment. To dissolve the formazan crystal generated by the reduction reaction, 200 µl of isopropanol (0.04 N HCl) was added to each well and mixed by pipetting. Absorbance was measured using a microtiter plate reader at 540 nm (Bio-Tek Instruments, Inc., Winooski, Vermont, USA). Fold increase in proliferation after 4 days of culture was calculated using the following formula:

$$\text{Fold increase in proliferation day 4} = (\text{Abs540 at day 4})/(\text{Abs540 at day 0})$$

Western blot and antibodies

Cells lysates from cell lines treated as indicated were prepared as described elsewhere [16]. In brief, cells were washed once each in PBS and ice cold buffer A (50 mmol/l Tris–HCl pH 7.2, 140 mmol/l KCl, 1 mmol/l EGTA, 2.5 mmol/l $MgCl_2$, 1 mmol/l DTT, and protease inhibitor cocktail). Cell lysis was achieved by resuspending the cell pellet in ice-cold buffer B (buffer A containing 1% Nonidet-p40). After centrifugation, lysate supernatants were kept at −80°C before analysis. A protein assay was carried out using a BCA protein assay kit (Pierce, Rockford, Illinois). Thirty micrograms of proteins were equally applied for separation by SDS-PAGE in all experiments, unless otherwise specified. Proteins were transferred onto PVDF membrane (GE Healthcare) using a semi-dry transfer unit (BioRad). Primary antibodies were used as follows: anti-Notch1 Ab, goat anti-Jagged1 Ab, rabbit anti-c-myc Ab (SantaCruz Biotech), and rabbit anti-cleaved Notch1 (Val1744) Ab (Cell Signaling Technology) were all used at a dilution 1 : 1000. Horseradish peroxidase-conjugated donkey anti-rabbit IgG Ab was used at 1 : 4000 and rabbit anti-goat IgG Ab was used at 1:12,000 as secondary antibodies (GE Healthcare). The signals were detected with ECL detection reagents (GE Healthcare).

Plasmids and transfections

Expression vectors for activated Notch1 (pcDNA3-ICN1; a kind gift from Dr Barbara Osborne, U. of Massachusetts at Amherst) were previously described and prepared using a Plasmid Midi Kit (Qiagen) [17,18]. For transfection of plasmids into the HepG2 cell line, Fugene-6 reagent was used according to manufacturer's instruction (Roche). For the transfection of plasmids into the Jurkat cell line, electroporation was carried out using a Gene Pulser (BioRad). Both transient and stable transfections were performed and the expression of ICN1 was confirmed by western blot. To obtain stable clones, G418 was added to culture media at a concentration of 400 µmol/l for both cell lines.

Semi-quantitative real time-PCR

Total RNA was extracted from cells treated as indicated using TriZol reagent (Invitrogen). One microgram of RNA was used to generate cDNA using a random hexamer (New England Biolab) and Revert aid M-MuL V reverse transcriptase (Fermentus). Five microliters of cDNA was used for PCR reactions. All reactions, except for *Dlk1*, which was performed for 30 cycles, were carried out for 30 cycles. Primers used in this study are as follows: *Notch1*, forward 5′-CAGCCTGCACAACCAGACAGA-3′ and reverse 5′-TGAGTTGATGAGGTCCTCCAG-3′; *Hes1*, forward 5′-ACGACACCGGATAAACCAAA-3′ and reverse 5′-CGGAG GTGCTTCACTGTCAT; *β-actin*, forward 5′-ACCAACTGG GACGACATGGACAA-3′ and reverse 5′-GTGGTGGTGA AGCTGT AGCC. PCR products were analyzed on 2% agarose gel electrophoresis. For calculating the expression level, the band intensities of PCR products were measured using QuantityOne software (BioRad) and normalized to that of *β-actin*.

Apoptosis and cell cycle analysis

Cells were treated as indicated, fixed in 1% paraformaldehyde for 2 h in the dark, and stained with Hoechst 33 342 (1 mmol/l). Cells were mounted and directly observed under a fluorescence microscope (Nikon model UfX-II). For measuring apoptosis, apoptotic nuclei and total nuclei were counted in three randomly chosen fields and percentages of apoptotic nuclei were calculated. For positive control, cells were treated with VP16 as indicated. To detect apoptotic cells by Annexin V staining, the Annexin V-FITC Apoptosis Detecting Kit was used according to the manufacturer's instruction (Sigma Aldrich). For cell cycle analysis, cells were fixed in 70% cold ethanol and treated with RNaseA (1 µg/ml) at 37°C for 1 h. After washing, cells were stained with propidium iodide (1 mg/ml) for 30 min and subjected

to FACS analysis using Cell Quest Software (BD Biosciences).

Statistical analysis

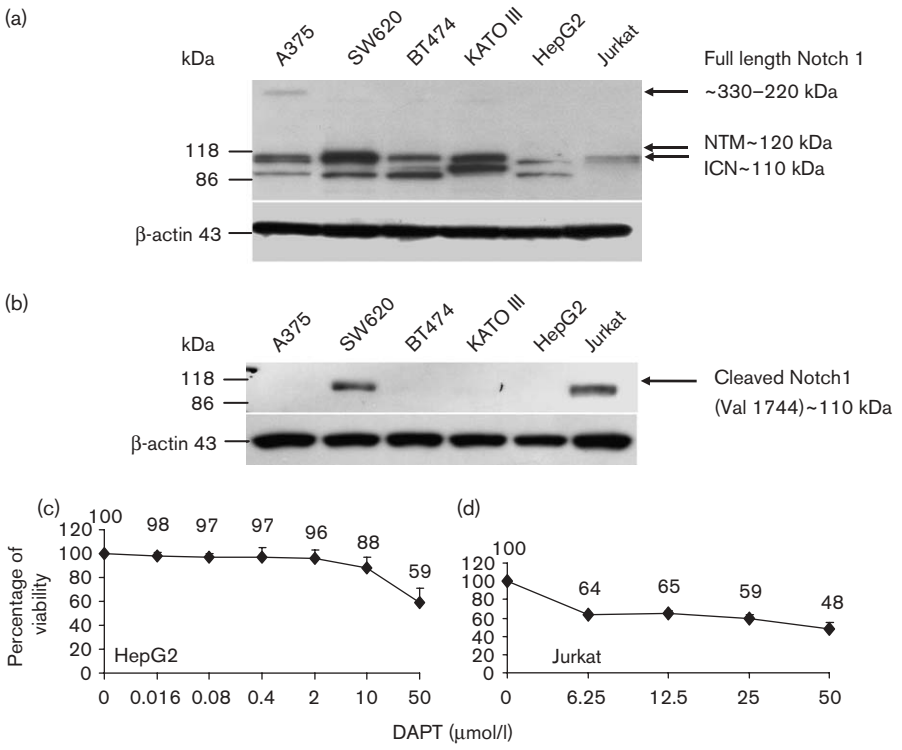
Means ± SD of independent experiments were analyzed. Intergroup comparisons in all experiments were analyzed using an independent *t*-test of SPSS software (version 11.5). A P value of < 0.05 was considered statistically significant.

Results

Expression of Notch receptors/ligands and GSI sensitivity in six human cancer cell lines

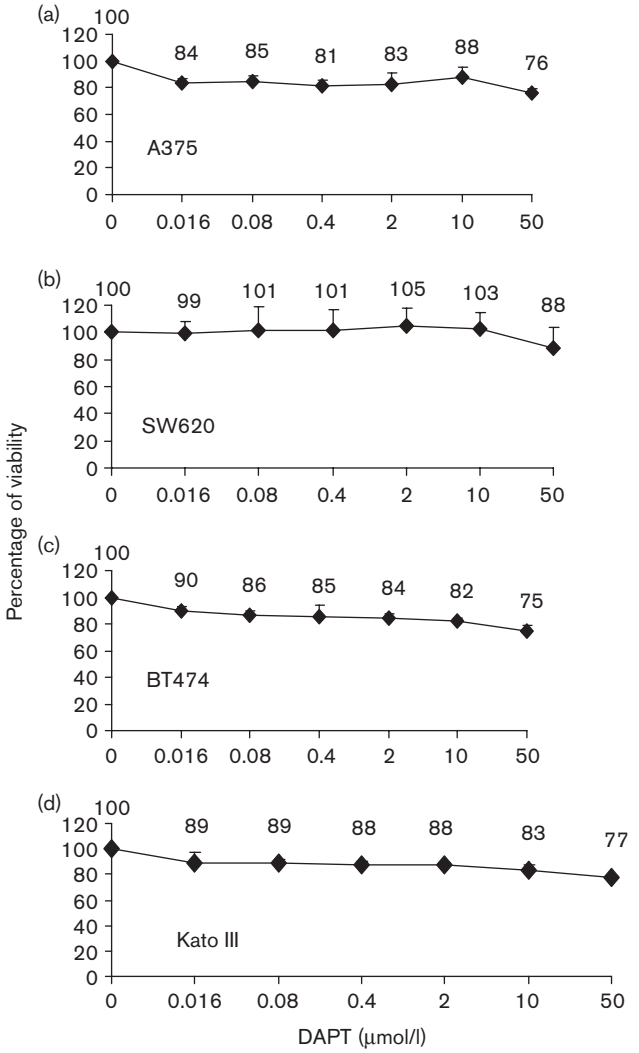
To determine whether human cancer cell lines from different tissue origins express the Notch1 protein, we carried out western blotting using antibodies directed against the intracellular domain of Notch1 and cleaved Notch1 (Val1744). As shown in Fig. 1a and b, all human cancer cell lines examined in this study expressed Notch1 at different levels, with SW620 and KATOIII expressing the highest, while HepG2 and Jurkat expressed the lowest amount of Notch1 protein. Interestingly, all cell lines except Jurkat showed multiple bands of cross-reactivity, which may represent different forms of posttranslational modifications. When antibodies specific for cleaved Notch1 were used, only SW620 and Jurkat expressed the activated form of Notch1, an implication of continuous activation of the Notch1 signaling pathway in these two cell lines. It is not known whether the activation of Notch1 in these cell lines is ligand-dependent. Next, we treated these six cell lines with GSI and DAPT at various concentrations for 4 days and measured cell viability by MTT assay. As shown in Figs 1c, d and 2, only HepG2 and Jurkat cells showed sensitivity to DAPT as cell viability decreased to 59 and 48%, respectively, at a DAPT concentration of 50 μmol/l. This result implies that Notch signaling may play a crucial role in regulating cell proliferation in these two cell lines. By comparing the effective doses that affect cell proliferation, Jurkat is more sensitive to DAPT than HepG2. Surprisingly, SW620, which expresses activated Notch1, did not show sensitivity to DAPT. It is possible that mutations in Notch1 in this cell line bypass γ-secretase cleavage, or that Notch signaling is not involved in proliferation in this cell line [19]. To gain insight into the expression profiles of other Notch receptors, Notch ligands, and the Notch target gene *Hes1* in DAPT sensitive cell lines, we examined expression of *Notch1-4*,

Fig. 1



Expression of Notch1 and cleaved Notch 1 and effect of DAPT treatment on viability of cancer cell lines. Six human cancer cell lines were analyzed for Notch1 and cleaved Notch1 expression by western blot. Antibodies specific for intracellular Notch1 domain (a) and cleaved Notch1 (Val1744) (b) were used. β-actin was used as loading control. HepG2 (c) and Jurkat (d) were treated with various concentrations of DAPT, as indicated, for 4 days. Cell viability was assayed by MTT method. The results represent two independent experiments in triplicate and are shown in triplicate ± SD. ICN, intracellular Notch; NTM, intracellular Notch with transmembrane domain.

Fig. 2



Effects of DAPT treatment on human cancer cell lines. Indicated cell lines were treated with various concentration of DAPT for 4 days. Cell viability was assayed using the MTT method (a–d). The results represent two independent experiments in triplicate and are shown in triplicate ± SD.

Jagged1-2, and *Dlk1* by western blot and RT-PCR. Both HepG2 and Jurkat expressed *Notch2*, *Notch3*, *Hes1*, *Jagged1-2*, and *Dlk1*, but not *Notch4* (data not shown).

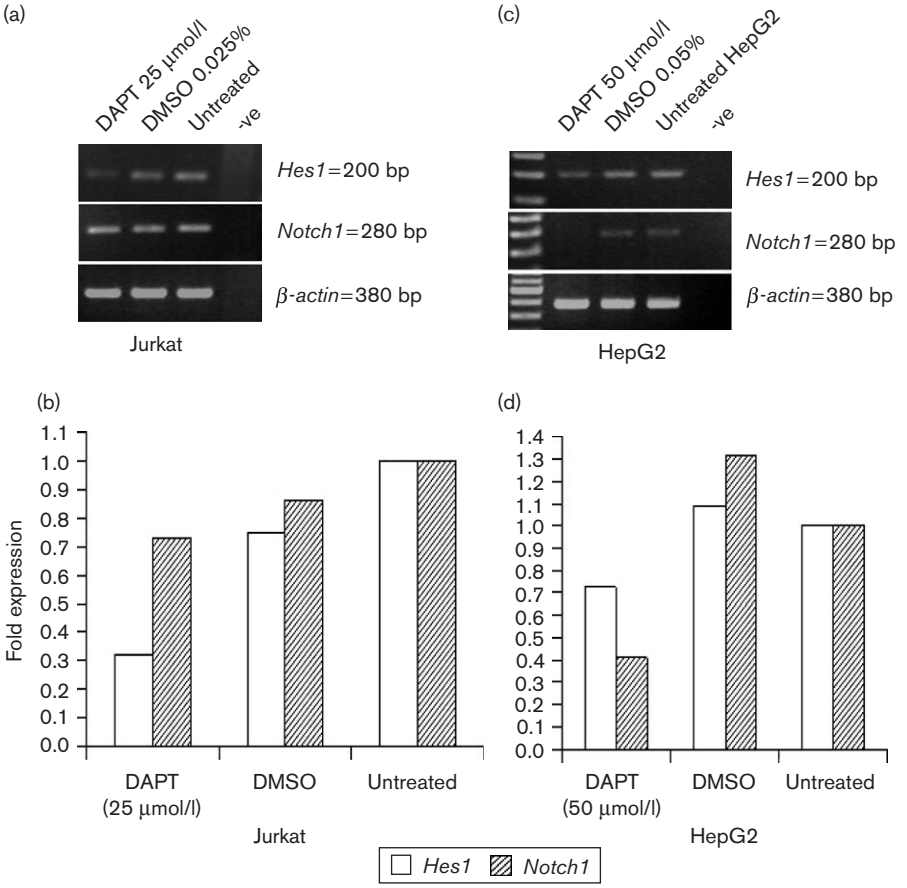
Effects of GSI treatment on Hes1 and Notch1 expression

To determine the effects of GSI treatment on the activation of the Notch signaling pathway, the expression of *Hes1* was examined by real-time PCR. In Jurkat and HepG2, DAPT treatment led to a significant decrease in *Hes1* expression as compared to mock treatment (Fig. 3a to d). These results confirm that the target gene of Notch signaling, *Hes1*, is regulated by the activated Notch signaling pathway and that GSI treatment suppressed Notch activation in both cell lines. On the other hand, expression of *Notch1* was not affected by GSI treatment in Jurkat cells, but was significantly decreased in HepG2, suggesting that different regulatory mechanisms govern *Notch1* expression in these two cell lines. In HepG2, mRNA expression of *Notch1* was negatively affected by DAPT treatment, implying that it may be under self-regulatory control (Fig. 3c and d). When protein expression was examined, increased Notch1 expression was detected in both cell lines upon DAPT treatment (Fig. 4). Increased Notch1 detected upon DAPT treatment may result from the net accumulation of Notch protein on the cell surface as they are not processed by γ-secretase when treated with DAPT. This is the first report on the effect of GSI in increasing Notch1 protein. It is likely that GSI treatment led to the accumulation of cell surface Notch1 in these cells. Accumulation of Notch proteins upon GSI treatment implies that Notch protein is continuously synthesized in these cell lines.

To determine whether decreased viability upon DAPT treatment is because of apoptotic cell death, apoptotic nuclei stained by Hoechst 33 342 were assayed under a fluorescence microscope. As shown in Fig. 5, both cell lines did not show any hallmark of nuclear morphological changes normally observed during apoptosis. Compared with treatment with VP16 as a positive control, GSI treatment of both cell lines, at least during the period of 4 days, did not result in apoptosis. In addition, when Annexin V and propidium iodide staining was used to identify apoptotic population, similar results were obtained. The percentage of Annexin V and propidium iodide-positive (apoptotic) population in untreated, DMSO-treated and DAPT-treated Jurkat are 1.9, 1.79, and 1.65%, respectively, whereas VP16-treated Jurkat contained 68.38% of apoptotic cells. In HepG2 the percentages of apoptotic population in untreated, DMSO-treated and DAPT-treated are 11, 5, and 7%, respectively, whereas VP16-treated HepG2 showed 21.21% of apoptotic cells. Therefore, it is likely that GSI treatment may lead to cell cycle arrest in these two cell lines, and as a result, decrease the number of viable cells as revealed by MTT assay. When cell cycle analysis was performed, significantly higher cell numbers in the G1 phase were detected in DAPT-treated Jurkat and HepG2 cells than mock-treated or untreated cells (Fig. 6a and b). A slight increase in apoptotic sub G1 populations in untreated and vehicle control treatments resulted from exhaustion of the culture after 4 days of incubation.

Many target genes of the Notch signaling pathway have been reported that are involved in cell cycle regulation. Recently, *c-myc* has been identified as one of the genes directly regulated by Notch signaling in T-cell leukemia [20,21]. We therefore examined the expression of c-myc during GSI treatment. In both HepG2 and Jurkat cells treated with GSI, c-myc was dramatically decreased, confirming the essential role of c-myc in promoting tumor

Fig. 3



Effects of GSI treatment on *Notch1* and *Hes1* mRNA expression in Jurkat and HepG2 cells. Jurkat or HepG2 cells were treated with DAPT at indicated concentrations for 4 days and total RNA was isolated. Expression of *Notch1* and *Hes1* mRNA were analyzed by RT-PCR (a and c). Band densities of Notch1 (d) and Hes1 (b) were normalized to loading control *β-actin* using Quantity One software and the results represent two independent experiments.

cell proliferation and tumorigenesis (Fig. 6c and d). Taken together, DAPT treatment led to cell cycle arrest, but not apoptosis in these two cell lines, probably through downregulation of c-myc expression.

Effect of GSI on cell proliferation is Notch signaling-specific

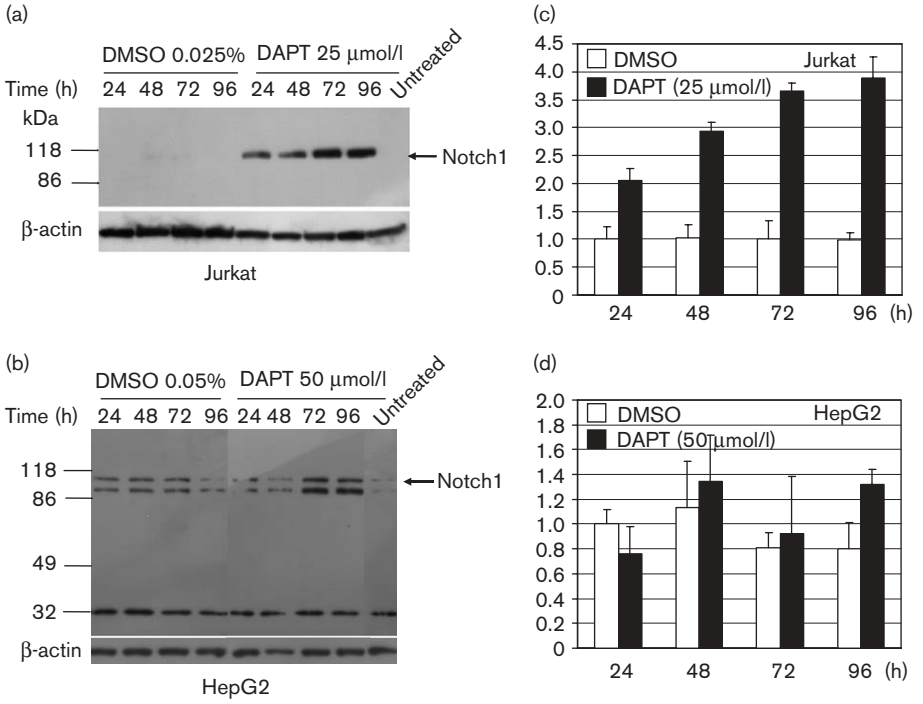
γ-Secretase has multiple substrates and when inhibitors are used to inhibit its activity, it is important to pinpoint those substrate(s) that are the major targets in generating the observed phenotypes [22]. To determine whether the effects seen with DAPT treatment are Notch-specific, we overexpressed ICN1, a truncated Notch1 corresponding to amino acids 1759–2556. ICN1 is constitutively active, bypassing the requirement of ligand binding and γ-secretase cleavage. When ICN1-overexpressing Jurkat and HepG2 cells were treated with effective doses of DAPT (25 and 50 μmol/l, respectively), decreased proliferation was no longer observed (Fig. 7). This result strongly suggests that the anti-proliferative effect of DAPT is specific for Notch signaling and other substrates of γ-secretase are not involved in this phenotype.

Discussion

Aberration in Notch signaling is associated with various types of tumor formation. Targeting Notch signaling using GSI or siRNA represents a novel approach in controlling tumor growth mediated by hyperactivation of Notch signaling. The advantage of using GSI lies in the fact that all Notch receptors require γ-secretase for processing and signaling; thus, by using GSI, the problem of functional redundancy observed among Notch receptors can be avoided [23,24]. On the other hand, γ-secretase has multiple substrates that have been identified to date [9]. Therefore, inhibition of Notch is not always indicative of inhibition of Notch signaling.

We have screened six human cancer cell lines representing six different tissue origins and found that they all

Fig. 4



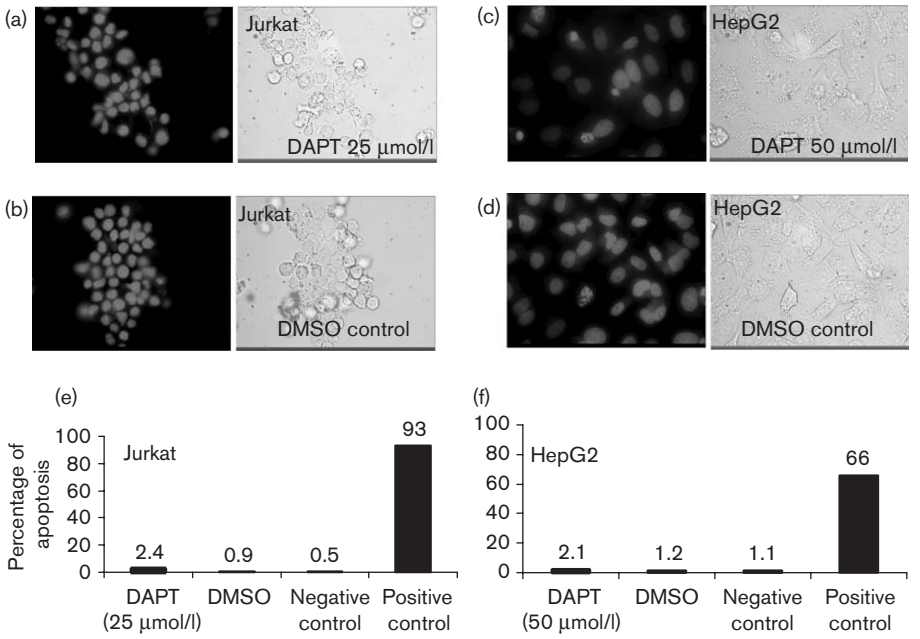
Effects of GSI treatment on Notch1 expression in Jurkat and HepG2 cells. Jurkat (a) or HepG2 (b) cells were treated with DAPT or vehicle control DMSO for indicated durations. Expression of Notch1 was detected by western blot using antibody specific for intracellular Notch1 protein. The results of quantitated protein expression are presented in c and d. The results shown represent three independent experiments with similar results.

expressed Notch1. In addition, Notch 2 and 3 were also detected in these cell lines (data not shown). Among these, only Jurkat and SW620 cells expressed detectable cleaved Notch1. As these two cell lines expressed cleaved Notch1, they were expected to show sensitivity against GSI as well. Surprisingly, however, only Jurkat and HepG2 cells exhibited sensitivity against GSI treatment. Therefore, Notch signaling may not be involved in regulating cell growth in the colon carcinoma-derived SW620 cell line. In the case of HepG2, even cleaved Notch1 was not detected; this line might express cleaved Notch1 at a low level or have other activated Notch receptors, which may be responsible for its sensitivity.

DAPT is a nontransitional analog inhibitor with an IC50 for inhibiting production of β-amyloid 42 of 200 nmol/l. Several studies have previously reported the effect of DAPT on the growth of tumor cell lines with a lower effective dose (in the range of 1–20 μmol/l) [25]. In addition, DAPT induced apoptotic cell death in most studies, although we did not observe cell death upon treatment with DAPT (Fig. 5). Therefore, the possible explanation for discrepancies of doses used in our and others' studies may lie in different cell lines or cell lines with different tissue origins that were used. Furthermore, some studies also observed the effect of GSI on cancer cell growth during varying periods of incubation (in a range of 2–6 days). We have carried out the time point experiments and found that the most profound effects on cell viability could be observed at 4 days. As DAPT did not induce apoptosis in these two cell lines, longer culture periods may be needed to observe anti-proliferative effects.

GSI is reported to inhibit proliferation of T-ALL by inducing cell cycle arrest and apoptosis, which is mediated by direct regulation of *c-myc* expression through the mTOR pathway by Notch signaling [12,15,20,26]. We have confirmed the anti-proliferative effect of GSI on the T-cell leukemia Jurkat cell line through downregulation of *c-myc*. Recently, mutations in a ubiquitin ligase gene, *Fbw7*, were reported to be the cause of GSI resistance in leukemic cells [27]. In this report, no mutations were found in the HD and PEST domains of the *Notch1* gene, but a single-point mutation was identified in the *Fbw7* gene. For the hepatocellular carcinoma cell line HepG2, this is the first reported anti-proliferative effect of GSI. Apoptosis is not induced in both cell lines by GSI treatment for the duration of 4 days in this study, contradicting other reports where apoptosis was observed upon GSI treatment [15,28]. This may be because of differences in doses used and cell lines tested. Notch

Fig. 5



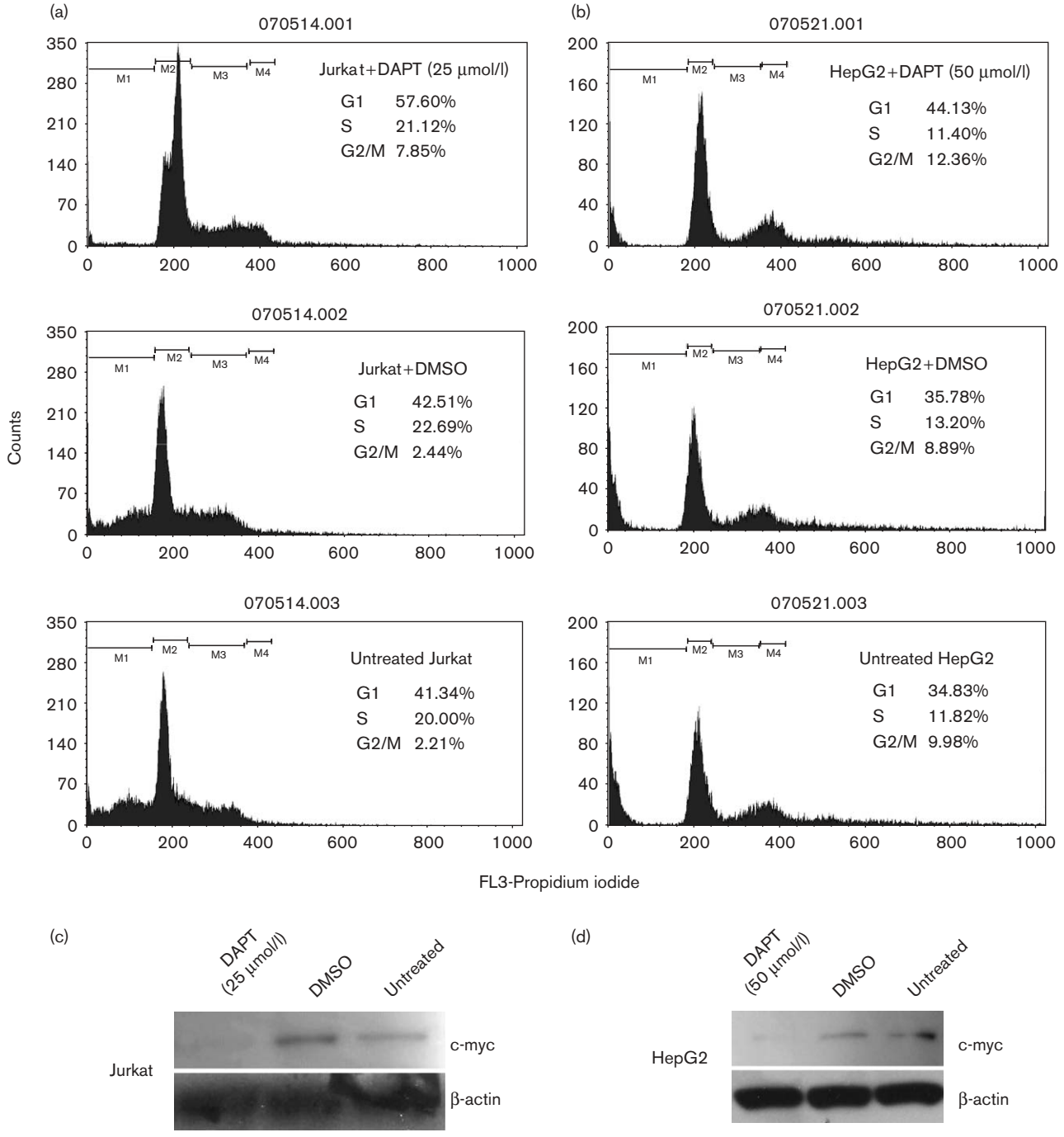
Detection of apoptotic nuclei upon GSI treatment. Jurkat or HepG2 cells were treated with DAPT (a, c), or vehicle control DMSO (b, d) at indicated concentrations for 4 days. Cells were fixed and stained with Hoechst 33 342 before observing under a fluorescence microscope. Apoptotic nuclei were counted in three different randomly chosen fields and presented as a percentage of apoptotic nuclei as shown in e and f. Positive control was carried out by treating cells with VP16 for 12 h and the negative control used untreated cells. The results shown represent two independent experiments.

signaling was identified as a major regulator of cell proliferation in these cell lines, as forced expression of ICN1 restored proliferation in these two cell lines in the presence of GSI. Previously, overexpression of activated Notch was shown to cause cell cycle arrest and apoptosis in a human hepatocellular carcinoma cell line [29]. The discrepancy between our findings and the aforementioned report may result from different cell lines and approaches used.

Interestingly, when these two cell lines were treated with GSI, while mRNA of *Hes1* decreased in both cases, decreased *Notch1* mRNA was detected in HepG2 but not in Jurkat cells. This result suggests that the expression of *Notch1* in HepG2 is autoregulated by Notch signaling itself, but other signaling(s) controls Notch expression in Jurkat cells. Notch signaling is shown to regulate its own expression in stimulated T lymphocytes [16].

Surprisingly, when the expression of Notch1 protein was examined, both cell lines showed increased Notch1 expression upon GSI treatment. This is likely because of the accumulation of cell surface Notch1 when γ-secretase is inhibited. When Notch is continuously processed by γ-secretase, newly synthesized Notch will be continuously turned over and the level of Notch expression at any given time appears to be low. The high turnover nature of Notch protein is predicted as being regulated by a PEST domain in the c-terminus, which targets it for proteasomal degradation. Upon GSI treatment, however, processing of newly synthesized Notch is halted and Notch proteins accumulate on the cell surface. In Jurkat cells where *Notch1* mRNA expression was not altered by GSI treatment, the level of accumulation was striking and started as early as 24 h after treatment. Even in HepG2, where *Notch1* mRNA decreased upon GSI treatment, accumulation of Notch1 was evident by day 3. Others have reported that active form of Notch1 is down-regulated upon GSI treatment as expected when γ-secretase is inhibited [30,31]. In addition, some also reported a reduced overall expression of Notch receptor since an expression of Notch is under its own positive feedback loop [16]. In this study, we observed down-regulation of *Notch1* mRNA expression in HepG2 but not in Jurkat. This led us to speculate that the expression of *Notch1* in the Jurkat cell line may not be under a positive feedback loop of Notch signaling itself. More importantly, however, the accumulation of Notch1 upon DAPT treatment implies that Notch1 is continuously turned over by the γ-secretase cleavage and the proteins accumulate when the activity of this enzyme is inhibited. In the Jurkat cell line where expression of *Notch1* mRNA is not diminished by DAPT treatment, the accumulation of protein Notch1

Fig. 6

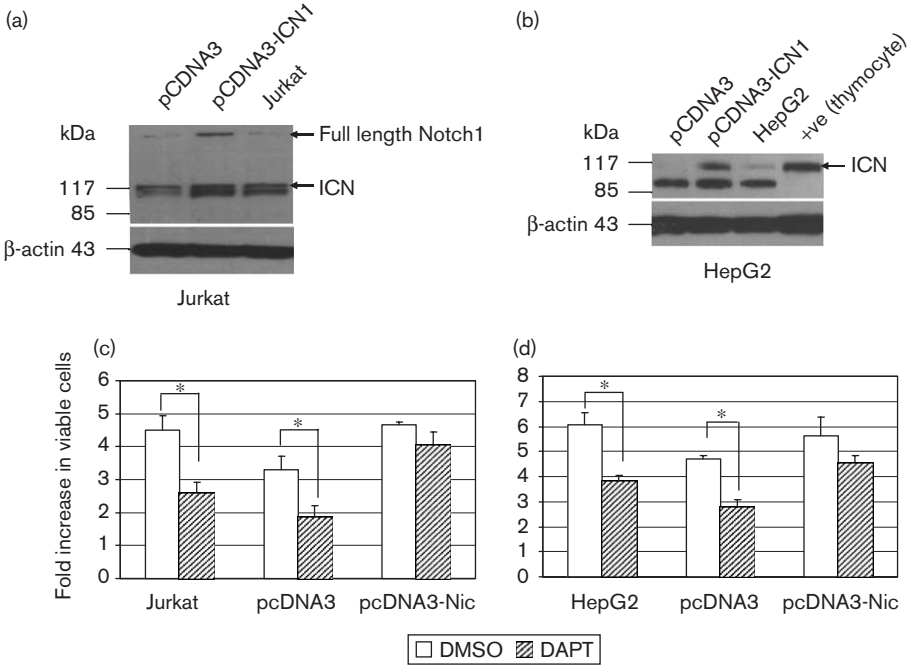


Cell cycle analysis and c-myc expression in DAPT-treated Jurkat and HepG2 cells. Jurkat (a) and HepG2 (b) cells were treated with DAPT, DMSO, or left untreated for 4 days. Cell cycle analysis was performed as described in materials and methods using FACS. Whole-cell lysates were analyzed for c-myc expression by western blot (c and d).

is obvious at 24 h upon treatment. In HepG2, however, a similar accumulation is observed but to a lesser extent, probably because the expression of *Notch1* mRNA decreased upon DAPT treatment in this cell line (Fig. 3c and d).

As we did not detect apoptosis in our system, this accumulation of Notch1 may present potential obstacles for using γ-secretase as a therapeutic target if it is used alone or the duration of treatment is not monitored to induce apoptosis of tumor cells. If GSI is withdrawn from

Fig. 7



Forced expression of ICN1 rescued Jurkat and HepG2 from effects of GSI treatment. Expression of ICN1 was confirmed in empty vector control pcDNA3 or pcDNA3-ICN1-transfected Jurkat (a) and HepG2 (b) by Western blot. Transfected cells were treated with DAPT or DMSO for 4 days and cell viability was assayed by the MTT method. The results represent two independent experiments and are shown in triplicate ± SD. *$P<0.05$ indicates statistical significance.

cells when Notch accumulates at a high level, it may cause transduction of the strong Notch signal by accumulated Notch receptors on the cell surface. In light of the involvement of Notch on cell proliferation of tumor cells, a massive signal from Notch receptors will result in hyper-proliferation of tumor cells. Therefore, a combination of GSI and other apoptosis-inducing agents may be a safer and more realistic approach to controlling tumor growth.

Notch signaling is shown to regulate cell cycle entry in cancer cells via interaction with other signal pathways, such as NF-κB, Ras, and mTOR [32,33]. In addition, Notch-mediated transformation in some instances requires upregulation of cell cycle regulators cyclin D1 and c-myc [20,34]. Currently, we are investigating signaling affected by GSI treatment in these two cell lines.

In conclusion, we report that GSI inhibited proliferation of Jurkat and HepG2 through inhibition of Notch signaling and cell cycle arrest, but apoptosis could not be detected. GSI treatment resulted in decreased *Hes1* and *Notch1* mRNA expression, and accumulation of Notch1 proteins in these two cell lines was, instead, detected. The efficacy of GSI in controlling these two cancer cell lines should be validated further in an in-vivo model.

Acknowledgements

This work was supported by a Graduate Research Grant from Graduate School of Chulalongkorn University. The authors are grateful to the Institute of Biotechnology and Genetic Engineering at Chulalongkorn University and Dr Barbara Osborne at the University of Massachusetts at Amherst for sharing reagents. We also thank Noppadol Sa-Ard-Iam for his help on cell cycle analysis.

References

1 Nickoloff BJ, Osborne BA, Miele L. Notch signaling as a therapeutic target in cancer: a new approach to the development of cell fate modifying agents. *Oncogene* 2003; **22**:6598–6608.
2 Artavanis-Tsakonas S, Rand MD, Lake RJ. Notch signaling: cell fate control and signal integration in development. *Science* 1999; **284**:770–776.
3 Radtke F, Wilson A, Mancini S, MacDonald H. Notch regulation of lymphocyte development and function. *Nat Immunol* 2004; **5**:247–253.
4 Maillard I, Adler SH, Pear WS. Notch and the immune system. *Immunity* 2003; **19**:781–791.
5 Maillard I, Fang T, Pear WS. Regulation of lymphoid development, differentiation, and function by the Notch pathway. *Annu Rev Immunol* 2005; **23**:945–974.
6 Ellisen LW, Bird J, West DC, Soreng AL, Reynolds TC, Smith SD, *et al.* TAN-1, the human homolog of the Drosophila notch gene, is broken by chromosomal translocations in T lymphoblastic neoplasms. *Cell* 1991; **66**:649–661.
7 Roy M, Pear WS, Aster JC. The multifaceted role of Notch in cancer. *Curr Opin Genet Dev* 2007; **17**:52–59.
8 Allenspach EJ, Maillard I, Aster JC, Pear WS. Notch signaling in cancer. *Cancer Biol Ther* 2002; **1**:466–476.
9 Kopan R, Ilagan MX. Gamma-secretase: proteasome of the membrane? *Nat Rev Mol Cell Biol* 2004; **5**:499–504.

10 Selkoe DJ. The cell biology of β-amyloid precursor protein and presenilin in Alzheimer's disease. *Trends Cell Biol* 1998; **8**:447–453.
11 Pollack SJ, Lewis H. Secretase inhibitors for Alzheimer's disease: challenges of a promiscuous protease. *Curr Opin Investig Drugs* 2005; **6**:35–47.
12 Weng AP, Ferrando AA, Lee W, Morris JPt, Silverman LB, Sanchez-Irizarry C, *et al*. Activating mutations of NOTCH1 in human T cell acute lymphoblastic leukemia. *Science* 2004; **306**:269–271.
13 Paris D, Quadros A, Patel N, DelleDonne A, Humphrey J, Mullan M. Inhibition of angiogenesis and tumor growth by beta and gamma-secretase inhibitors. *Eur J Pharmacol* 2005; **514**:1–15.
14 Kimura K, Satoh K, Kanno A, Hamada S, Hirota M, Endoh M, *et al*. Activation of Notch signaling in tumorigenesis of experimental pancreatic cancer induced by dimethylbenzanthracene in mice. *Cancer Sci* 2007; **98**: 155–162.
15 Curry CL, Reed LL, Nickoloff BJ, Miele L, Foreman KE. Notch-independent regulation of Hes-1 expression by c-Jun N-terminal kinase signaling in human endothelial cells. *Lab Invest* 2006; **86**:842–852.
16 Palaga T, Miele L, Golde T, Osborne B. TCR-mediated Notch signaling regulates proliferation and IFN-gamma production in peripheral T cells. *J Immunol* 2003; **171**:3019–3024.
17 Jeffries S, Capobianco AJ. Neoplastic transformation by Notch requires nuclear localization. *Mol Cell Biol* 2000; **20**:3928–3941.
18 Shin HM, Minter LM, Cho OH, Gottipati S, Fauq AH, Golde TE, *et al*. Notch1 augments NF-kappaB activity by facilitating its nuclear retention. *Embo J* 2006; **25**:129–138.
19 Das I, Craig C, Funahashi Y, Jung KM, Kim TW, Byers R, *et al*. Notch oncoproteins depend on gamma-secretase/presenilin activity for processing and function. *J Biol Chem* 2004; **279**:30771–30780.
20 Weng AP, Millholland JM, Yashiro-Ohtani Y, Arcangeli ML, Lau A, Wai C, *et al*. c-Myc is an important direct target of Notch1 in T-cell acute lymphoblastic leukemia/lymphoma. *Genes Dev* 2006; **20**:2096–2109.
21 Liao WR, Hsieh RH, Hsu KW, Wu MZ, Tseng MJ, Mai RT, *et al*. The CBF1-independent Notch1 signal pathway activates human c-myc expression partially via transcription factor YY1. *Carcinogenesis* 2007; **28**: 1867–1876.
22 Wolfe MS. The gamma-secretase complex: membrane-embedded proteolytic ensemble. *Biochemistry* 2006; **45**:7931–7939.
23 Saxena MT, Schroeter EH, Mumm JS, Kopan R. Murine notch homologs (N1-4) undergo presenilin-dependent proteolysis. *J Biol Chem* 2001; **276**:40268–40273.
24 Kitamoto T, Takahashi K, Takimoto H, Tomizuka K, Hayasaka M, Tabira T, *et al*. Functional redundancy of the Notch gene family during mouse embryogenesis: analysis of Notch gene expression in Notch3-deficient mice. *Biochem Biophys Res Commun* 2005; **331**:1154–1162.
25 O'Neil J, Calvo J, McKenna K, Krishnamoorthy V, Aster JC, Bassing CH, *et al*. Activating Notch1 mutations in mouse models of T-ALL. *Blood* 2006; **107**:781–785.
26 Chan SM, Weng AP, Tibshirani R, Aster JC, Utz PJ. Notch signals positively regulate activity of the mTOR pathway in T-cell acute lymphoblastic leukemia. *Blood* 2007; **110**:278–286.
27 O'Neil J, Grim J, Strack P, Rao S, Tibbitts D, Winter C, *et al*. FBW7 mutations in leukemic cells mediate NOTCH pathway activation and resistance to gamma-secretase inhibitors. *J Exp Med* 2007; **204**: 1813–1824.
28 Lewis HD, Leveridge M, Strack PR, Haldon CD, O'Neil J, Kim H, *et al*. Apoptosis in T cell acute lymphoblastic leukemia cells after cell cycle arrest induced by pharmacological inhibition of notch signaling. *Chem Biol* 2007; **14**:209–219.
29 Qi R, An H, Yu Y, Zhang M, Liu S, Xu H, *et al*. Notch1 signaling inhibits growth of human hepatocellular carcinoma through induction of cell cycle arrest and apoptosis. *Cancer Res* 2003; **63**:8323–8329.
30 Palomero T, Sulis ML, Cortina M, Real PJ, Barnes K, Ciofani M, *et al*. Mutational loss of PTEN induces resistance to NOTCH1 inhibition in T-cell leukemia. *Nat Med* 2007; **13**:1203–1210.
31 Palaga T, Buranaruk C, Rengpipat S, Fauq AH, Golde TE, Kaufmann SH, *et al*. Notch signaling is activated by TLR stimulation and regulates macrophage functions. *Eur J Immunol* 2008; **38**:174–183.
32 Yao J, Duan L, Fan M, Yuan J, Wu X. Notch1 induces cell cycle arrest and apoptosis in human cervical cancer cells: involvement of nuclear factor kappa B inhibition. *Int J Gynecol Cancer* 2007; **17**:502–510.
33 Weijzen S, Rizzo P, Braid M, Vaishnav R, Jonkheer SM, Zlobin A, *et al*. Activation of Notch-1 signaling maintains the neoplastic phenotype in human Ras-transformed cells. *Nat Med* 2002; **8**:979–986.
34 Stahl M, Ge C, Shi S, Pestell RG, Stanley P. Notch1-induced transformation of RKE-1 cells requires up-regulation of cyclin D1. *Cancer Res* 2006; **66**:7562–7570.