In vitro validation of γ-secretase inhibitors alone or in combination with other anti-cancer drugs for the treatment of T-cell acute lymphoblastic leukemia

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The online version of this article contains a supplemental appendix.

ABSTRACT

Background

Activating *NOTCH1* mutations are common in T-cell acute lymphoblastic leukemia. Inhibition of NOTCH1 signaling with γ-secretase inhibitors causes cell cycle block, but only after treatment for several days. We further documented the effects of γ-secretase inhibitor treatment on T-cell acute lymphoblastic leukemia cell lines and tested whether combining γ-secretase inhibitors with other anti-cancer drugs offers a therapeutic advantage.

Design and Methods

The effect of γ-secretase inhibitor treatment and combinations of γ-secretase inhibitors with chemotherapy or glucocorticoids was assessed on T-cell acute lymphoblastic leukemia cell lines. We sequenced *NOTCH1* in T-cell acute lymphoblastic leukemia cases with *ABL1* fusions and tested combinations of γ-secretase inhibitors and the ABL1 inhibitor imatinib in a T-cell acute lymphoblastic leukemia cell line.

Results

γ-secretase inhibitor treatment for 5-7 days reversibly inhibited cell proliferation, caused cell cycle block in sensitive T-cell acute lymphoblastic leukemia cell lines, and caused differentiation of some T-cell acute lymphoblastic leukemia cell lines. Treatment for 14 days or longer was required to induce significant apoptosis. The cytotoxic effects of the chemotherapeutic agent vincristine were not significantly enhanced by addition of γ-secretase inhibitors to T-cell acute lymphoblastic leukemia cell lines, but γ-secretase inhibitor treatment sensitized cells to the effect of dexamethasone. *NOTCH1* mutations were identified in all T-cell acute lymphoblastic leukemia patients with *ABL1* fusions and in a T-cell acute lymphoblastic leukemia cell line expressing *NUP214-ABL1*. In this cell line, the anti-proliferative effect of imatinib was increased by pre-treatment with γ-secretase inhibitors.

Conclusions

Short-term treatment of T-cell acute lymphoblastic leukemia cell lines with γ-secretase inhibitors had limited effects on cell proliferation and survival. Combinations of γ-secretase inhibitors with other drugs may be required to obtain efficient therapeutic effects in T-cell acute lymphoblastic leukemia, and not all combinations may be useful.

Key words: *NOTCH1*, γ-secretase, inhibitor, T-cell, oncogene.

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Introduction

T-cell acute lymphoblastic leukemia (T-ALL) is associated with several molecular defects including deregulated expression of a variety of transcription factors such as *TLX1, TLX3, TAL1, LYL1,* and *LMO1*, deletion of *CDKN2A* (*p16*), the episomal *NUP214-ABL1* fusion, duplication of the *MYB* gene and mutation of *PTEN* and *FBXW7* genes.[1-7] In addition, activating mutations in NOTCH1 were identified in more than 50% of T-ALL patients.[8-10]

The mature NOTCH1 receptor consists of an extracellular and a transmembrane subunit, which are non-covalently kept together by the heterodimerization domain.[11,12] Binding of delta-serrate-lag2 (DSL) family ligands to the extracellular subunit results in activation of the NOTCH1 receptor by removal of the extracellular subunit and by initiating a cascade of proteolytic cleavages of the transmembrane subunit. The final cleavage is catalyzed by the γ-secretase complex and generates active intracellular NOTCH1. This intracellular NOTCH1 subsequently translocates to the nucleus where it associates with other proteins to form a transcription activator complex. Actived intracellular NOTCH1 has a short half-life, being subject to ubiquitination and degradation via mechanisms involving the C-terminal proline, glutamate, serine and threonine (PEST) domain.[13]

The heterodimerization domain of NOTCH1 is mutated in 29-44% of T-ALL samples, causing destabilization of the association between the NOTCH1 extracellular and transmembrane subunits, resulting in increased NOTCH1 rates of activated intracellular NOTCH1 production in the absence of ligand stimulation.[5-7] In addition, 5% to 30% of the tumors display PEST domain mutations, which prolongs the half-life of the intracellular NOTCH1-containing transcriptional activation complex. Combined hetero-dimerization domain and PEST domain mutations cause synergistic activation of NOTCH1 signaling pathways and are observed in 1 to 18% of analyzed tumors.[5-7] Apart from mutations in the heterodimerization and PEST domains of NOTCH1, rare mutations in the NOTCH1 transactivation domain and in its ankyrin repeat regions have been described.[10]

The discovery of these very frequent NOTCH1 mutations in T-ALL instigated several research groups to elucidate the signaling network downstream of NOTCH1 in the context of T-ALL. These studies revealed important connections between NOTCH1 and MYC, NFKB and the PI3K/AKT signaling pathways.[5,14-16]

At the moment, up to 75% of T-ALL patients are cured by very intensive chemotherapy;[17] however, less toxic, more efficacious drug combinations are desirable. The discovery of frequent NOTCH1 mutations in T-ALL has important therapeutic implications. Mutant NOTCH1 still requires γ-secretase activity to generate critical downstream signals.[8] Because of the involvement of γ-secretase in the production of amyloidogenic peptides in Alzheimer's disease, efforts have already been made to develop potent and selective γ-secretase inhibitors. Unfortunately, long-term therapy of Alzheimer's disease with γ-secretase inhibitors (GSI) does not seem appropriate because of the side effects of the current inhibitors, such as disturbance of lymphocyte development and gut epithelial cell differentiation.[18] In T-ALL these compounds could provide a rational, molecularly targeted therapy with an acceptable level of toxicity, but this still needs to be investigated in clinical trials.[18]

The first proof of principle of the potential of GSI in the context of T-ALL was provided by the observation that five out of 30 tested T-ALL cell lines went into G_0/G_1 cell cycle arrest after 4 to 8 days of γ-secretase inhibition.[8] Several studies confirmed these initial observations and described induction of cell cycle arrest and reduced cell proliferation after treating sensitive NOTCH1 mutation-positive T-ALL cell lines for several days with GSI.[15,19-21] In this work, we further document the short- and long-term effects of GSI on the growth of T-ALL cell lines and tested whether combinations of GSI with other anti-cancer agents could offer any therapeutic advantage over single agent therapy.

Design and Methods

NOTCH1 mutation detection

Genomic DNA was prepared from T-ALL cell lines and samples from patients with *ABL1* fusion positive T-ALL using the Wizard genomic DNA purification kit (Promega, Madison, WI, USA). The detection of mutated *NOTCH1* sequences encoding the heterodimezation domain and PEST domains was described previously.[22] This study was approved by the Ethical Committee of the Medical Faculty of the University of Leuven. Informed consent was obtained from all subjects.

Cell cultures

DND-41, HSB-2, RPMI-8402, KARPAS-45, ALL-SIL, MOLT-4, LOUCY (DSMZ, Braunschweig, Germany) and JURKAT (ATCC CRL 8163) cells were cultured in RPMI-1640 medium supplemented with 20% fetal calf serum. In order to assess cell growth, 0.4×10^6 cells/mL were seeded in triplicate and different concentrations of GSI were added. After 2 to 3 days, the cells were subcultured by centrifugation and resuspension in fresh medium containing a GSI. Viable cells were counted at various time points on a Vi-cell XR cell viability analyzer (Beckman Coulter, Fullerton, CA, USA). In order to determine dose-response curves, 0.4×10^6 cells were seeded in 1 mL medium containing inhibitors and were incubated for 24 to 72 h, depending on the doubling

time of the cell lines. Compound E (γ-secretase inhibitor XXI), L-685458 (γ-secretase inhibitor X), vincristine, daunorubicin, dexamethasone and corticosterone were obtained from Calbiochem (San Diego, CA, USA).

Western blotting

Total cell lysates were analyzed by standard procedures using the following antibodies: anti-cleaved NOTCH1 (Val1744), (Cell Signaling, Beverly, MA, USA), anti-NOTCH1 (C-20) (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and anti-mouse/anti-rabbit peroxidase-labeled antibodies (AP Biotech, Uppsala, Sweden).

Fluorescence activated cell sorting analysis

Quantification of apoptosis, analysis of CD3, CD4 and CD8 expression and cell cycle analysis were performed on 0.5-1×10^6 cells after 2-3, 7 and 14 days of treatment with 0.1 or 1 μM of Compound E, using the Annexin-V-FLUOS Staining kit (Roche, Penzberg, Germany), the TriTEST CD4 FITC/CD8 PE/CD3 PerCP Reagent kit (Becton Dickinson, San Jose, CA, USA) and

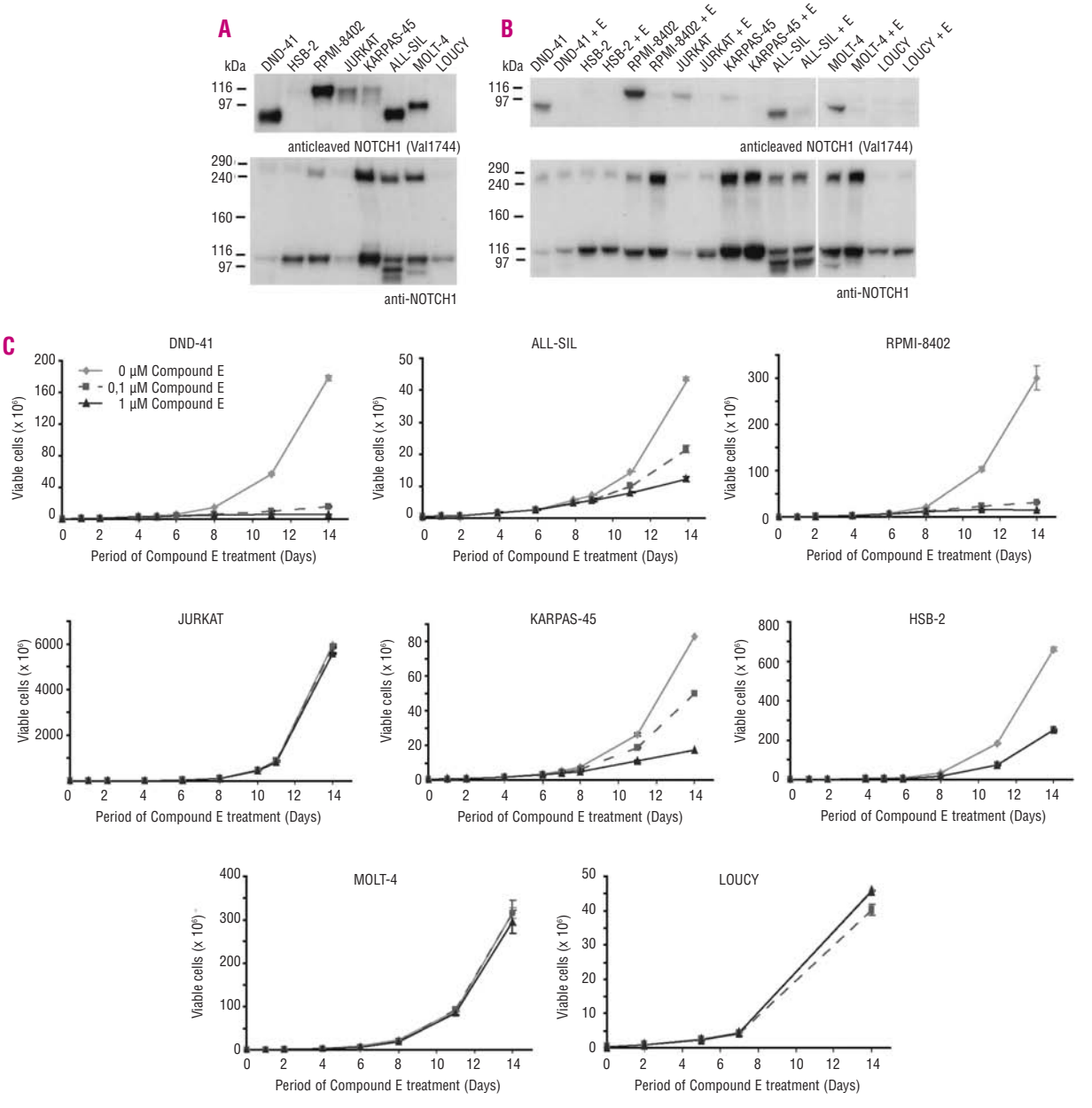

Figure 1. Inhibition of hyperactive NOTCH1 signaling in T-ALL cell lines by γ-secretase inhibition affects cell proliferation. (A) The activity of the NOTCH1 pathway in T-ALL cell lines was evaluated by western blot detection on whole cell lysates with anti-cleaved NOTCH1 (Val1744) antibody followed by stripping and reprobing the blot with anti-NOTCH1 antibody. (B) Western blot detection of active NOTCH1 (anti-cleaved NOTCH1 (Val1744) antibody) in T-ALL cell lines that were treated for 2 days with DMSO or with 1 μM Compound E. The blot was stripped and reprobed with anti-NOTCH1. (C) The indicated T-ALL cell lines were grown in the presence of DMSO, 0.1 or 1 μM Compound E and the total number of viable cells in the culture was measured on the Vi-cell XR cell viability analyzer at the indicated time points.

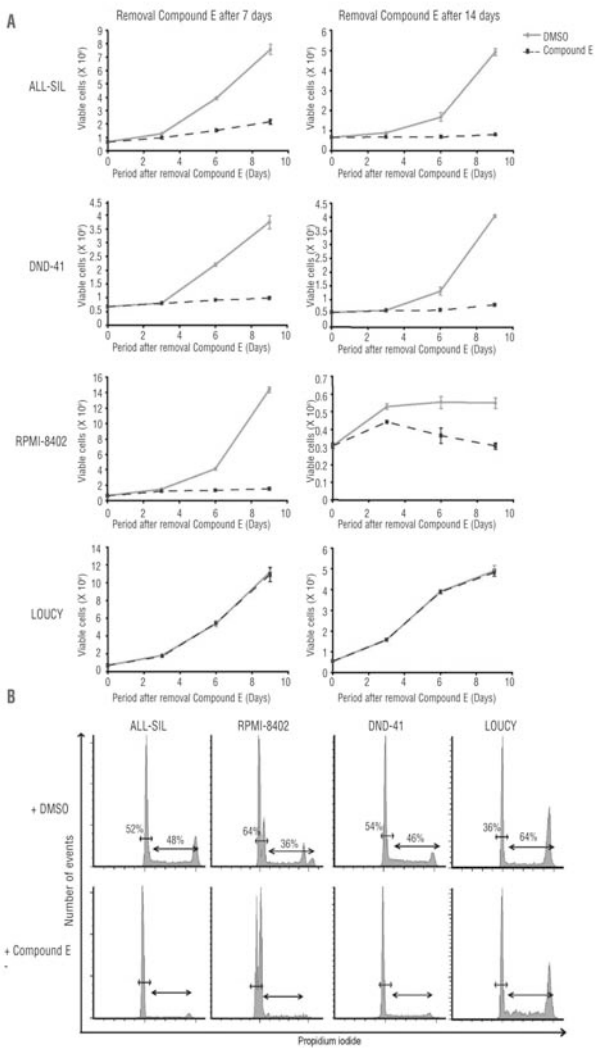

Figure 2. γ-secretase inhibitor-induced inhibition of proliferation and cell cycle block is reversible. **A.** ALL-SIL, DND-41, RPMI-8402 and LOUCY cells were pre-treated for 7 or 14 days with 1 μM of Compound E. On day 7 (left column) or 14 (right column) cells were washed; for half of the cells the treatment with 1 μM Compound E was continued, while the other half was treated with DMSO for the remaining days of the experiment. The graphs represent the number of cells in each treatment group, day 0 on the graphs corresponds to the day Compound E was washed away after the pre-treatment period. **B.** The reversibility of the G_0/G_1 cell cycle block after 7 days of pre-treatment with 1 μM Compound E was evaluated by propidium iodide staining of DNA content of the cells. The experiment was performed 9 days after stopping the pre-treatment with Compound E. The upper row of graphs correspond to the cells that were treated with DMSO after the Compound E pre-treatment, the lower row corresponds to the cells that continued to be exposed to Compound E after the pre-treatment period. The percentages of cells in G_0+G_1 phase (left peak in the graphs) and in S + M + G_2 phase are indicated in the graphs.

the CycleTEST™ PLUS DNA Reagent Kit (Becton Dickinson), respectively. For some cell lines staining to assess apoptosis was also done after 20 and 28 days of treatment, as indicated in *Online Supplementary Figure S1*. After staining, cells were detected on a FACSCanto Flow Cytometer (Becton Dickinson) and the data were analyzed with the BD FACSDiva software (Becton Dickinson). Unstained cells and cells treated with dimethyl sulfoxide were used as controls. The expression of CD4, CD8 and CD3 was evaluated in the gated population of viable cells.

Statistics

The t-test was used to evaluate the differences in effect between GSI treated and untreated samples for each tested concentration of imatinib, vincristine or glucocorticoid. A *p*-value of 0.05 was used as the cut-off below which results were considered statistically significant.

Results

γ-secretase inhibitor treatment reversibly inhibits the growth of T-ALL cell lines and induces cell cycle block

We monitored the effect of continuous treatment with a GSI as a single agent on the proliferation of eight T-ALL cell lines: DND-41, HSB-2, RPMI-8402, JURKAT, KARPAS-45, ALL-SIL, MOLT-4 and LOUCY. DNA sequencing confirmed the presence of *NOTCH1* mutations in DND-41, RPMI-8402, KARPAS-45, ALL-SIL, and MOLT-4 (*Online Supplementary Table S1*). No mutations were found in HSB-2, JURKAT and LOUCY. Western blot analysis showed detectable levels of γ-secretase cleaved, activated intracellular NOTCH1 in the cell lines with *NOTCH1* mutations (Figure 1A). In addition, we detected activate intracellular NOTCH1 in JURKAT cells, suggesting that the JURKAT cells harbor a *NOTCH1* mutation outside the domains we sequenced or in another gene that would result in aberrant NOTCH1 activation.[10] Treatment of the cells with 1 μM Compound E, a non-transition state analog GSI, resulted in a strong reduction of detected levels of activated intracellular NOTCH1, confirming that NOTCH1 cleavage was efficiently inhibited by Compound E (Figure 1B).

We next determined the effect of continuous Compound E treatment of the cell lines during 14 days (Figure 1C). At concentrations of 0.1 μM and 1 μM Compound E, inhibition of proliferation was clearly observed in four of the six cell lines with detectable NOTCH1 activation (DND-41, RPMI-8402, KARPAS-45 and ALL-SIL) (Figure 1C). The period required to obtain a 50% inhibition of growth varied between 7 to 18 days with 0.1 μM Compound E and between 7 to 12 days with 1 μM Compound E. Although the HSB-2 cell line did not have a mutation in *NOTCH1*, nor did it have detectable NOTCH1 activation by western blot analysis, the proliferation of this cell line was also inhibited by GSI treatment (Figure 1C). MOLT-4 cells and JURKAT cells were not sensitive to GSI treatment although activated *NOTCH1* was detectable in these cell lines. We conclude that MOLT-4 and JURKAT cell lines have become independent of mutant *NOTCH1* for their proliferation and cell cycle progression.

The Compound E-induced inhibition of cell proliferation was not immediately associated with increased cell

death, as trypan blue staining and annexin V/propidium iodide staining did not reveal significant differences in the number of dead cells between treated and untreated cultures after 7 days of treatment (*Online Supplementary Figure S1*). We found that continuous GSI treatment for 14 days or longer was required to induce significant apoptosis in the GSI-sensitive cell lines. In RPMI-8402 cultures, 30% apoptotic cells and 15% dead cells were detected after 14 days of gamma-secretase inhibition and this effect became even more pronounced after longer treatment. The other GSI-sensitive cell lines had to be treated for more than 14 days before apoptosis became evident (*Online Supplementary Figure S1*).

It was previously reported that γ-secretase inhibition results in G_0/G_1 cell cycle arrest in some T-ALL cell lines after 4 to 8 days of treatment.[8,15,20,21] By flow cytometric analysis of the DNA content of propidium iodide stained cell populations, we confirmed induction of G_0/G_1 cell cycle arrest after 7 days of GSI treatment in all cell lines that expressed mutant NOTCH1 and displayed a GSI induced proliferation defect (*data not shown*).

To investigate whether the GSI-induced proliferation and cell cycle defects were reversible, Compound E was removed after 7 or 14 days of treatment for the GSI-sensitive cell lines ALL-SIL, DND-41 and RPMI-8402. For ALL-SIL and DND-41, the inhibition of proliferation was reversible when the GSI-treatment was stopped (Figure 2A). For the RPMI-8402 cell line, the effect of treatment for 7 days was reversible, but cell growth did not recover when the GSI was removed after 14 days (Figure 2A). As we found that induction of apoptosis started in RPMI-8402 when the cells were treated for 14 days (*Online supplementary Figure S1*), these results indicate that GSI treatment does not cause irreversible changes in the cells as long as apoptosis is not induced. Recovery of proliferation upon removal of the GSI was associated with restoration of the cell cycle (Figure 2B).

Telomerase activity of untreated and GSI-treated cells was compared in a telomeric repeat amplification protocol (TRAP) assay. In this assay, active telomerase in a cell lysate causes extension of a synthetic primer with the TTAGGG telomere repeat sequence after which this mixture of elongated products is amplified by polymerase chain reaction. The amount of generated polymerase chain reaction product can then be used as a measure for telomerase activity and can be compared between samples by gel electrophoresis or by ELISA. We found that GSI treatment for 12 days reduced telomerase activity in DND-41, ALL-SIL and RPMI-8402, three cell lines showing proliferation defects upon GSI treatment (*Online Supplementary Figures S2A-B*). This decrease was, to a lesser extent, also detectable after 2 and 6 days of treatment (*data not shown*). In the remaining cell lines, telomerase activity was absent (KARPAS-45) or not significantly changed by GSI treatment. However, we could not correlate this reduction in telomerase activity to a reduction in telomerase reverse transcriptase (TERT) expression (*Online Supplementary Figure S2C*) or to a shortening of telomeres (*Online Supplementary Figure S2D*).

Gamma-secretase inhibitor treatment can induce differentiation of T-ALL cell lines

Aberrant activation of NOTCH1 is known to affect T-cell differentiation.[23] The effect of GSI treatment on differentiation of the T-ALL cell lines was tested by determining expression of the cell-surface antigens CD3, CD4 and CD8. GSI treatment induced differentiation of the MOLT-4 cell line from $CD4^-/CD8^{+/-}$ to $CD4^+/CD8^+$ (Figure 3). Changes in CD3 expression became apparent for the DND-41 and RPMI-8402 cells, which showed a change from $CD3^{+/-}$ to $CD3^-$ and from $CD3^-$ to $CD3^+$, respectively (Figure 3). For the ALL-SIL cell line, GSI

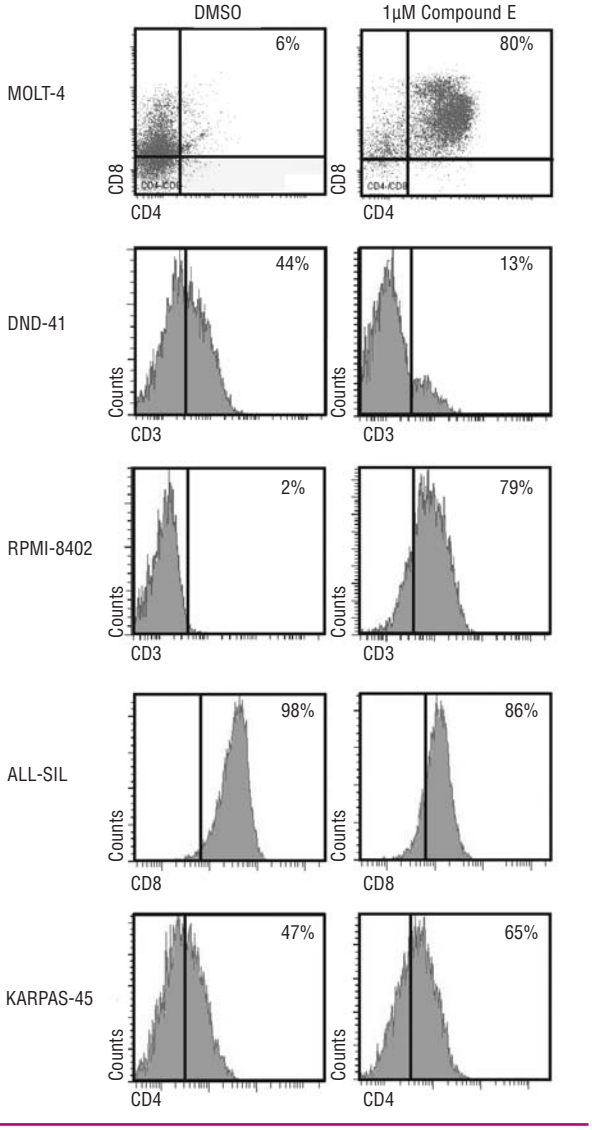

Figure 3. Treatment with γ-secretase inhibitors can cause changes in CD4/CD8/CD3 expression in T-ALL cell lines. FACS comparison of cell surface staining for CD4/CD8 (MOLT-4), CD3 (DND-41 and RPMI-8402), CD8 (ALL-SIL) or CD4 (KARPAS-45) in cells treated for 14 days with DMSO or with 1 μM of Compound E.

treatment decreased CD8 expression and for KARPAS-45 cells we detected increased CD4 expression upon GSI treatment. GSI treatment did not result in detectable changes in the CD3/CD4/CD8 immunophenotypes of the HSB-2, JURKAT and LOUCY cells (*data not shown*).

Combinations of γ-secretase inhibitors and kinase inhibitors can offer a therapeutic advantage

Results presented in previous reports and our results described above indicate that a GSI as a single agent may only have modest effects on T-ALL growth and survival, except when long-term treatment is possible.[8,15,19-21] We, therefore, investigated whether combining GSI with other anti-cancer agents used for T-ALL treatment could offer a therapeutic advantage over single agent therapy.

ABL1 fusions are present in 8% of T-ALL cases and are currently the most frequent known mutations providing the cells with proliferation and survival advantages.[1] We sequenced NOTCH1 in one T-ALL patient with an *EML1-ABL1* fusion gene, and in five T-ALL patients with the *NUP214-ABL1* episomal fusion.[24,25] *NOTCH1* was mutated in all six patients (two patients with a truncating PEST domain mutation, four patients with a hetero-dimerization mutation, *Online Supplementary Table S1*), indicating that *ABL1* fusions are preferentially associated with *NOTCH1* mutations. This observation raised the question of whether the combined inhibition of the *ABL1* fusion protein with imatinib, and activated *NOTCH1* with a GSI could offer a therapeutic advantage over single agent therapy.

We previously showed that the ALL-SIL cell line expresses, apart from mutant *NOTCH1*, the NUP214-ABL1 fusion tyrosine kinase and that it is sensitive to imatinib.[25] This cell line, therefore, represented an experimental model to study the effect of combinations of imatinib and GSI. When imatinib and Compound E were added at the same time to ALL-SIL cell cultures, the inhibitory effect of imatinib on cell proliferation was antagonized by Compound E (Figure 4A). This antagonism was also observed for the combination of imatinib with L-685458, a transition state analog inhibitor of γ-secretase with a different structure from that of Compound E (*data not shown*). These data indicate that this antagonism is not restricted to a combination of imatinib and Compound E, but that it is also observed with a structurally different GSI. It was shown that γ-secretase can modulate the activity and trafficking of transporter molecules.[26,27] We hypothesized that the antagonism between imatinib and the GSI could be due to a decreased influx of imatinib into the cells. However, imatinib potently inhibited phosphorylation of NUP214-ABL1 in the presence of Compound E, demon-

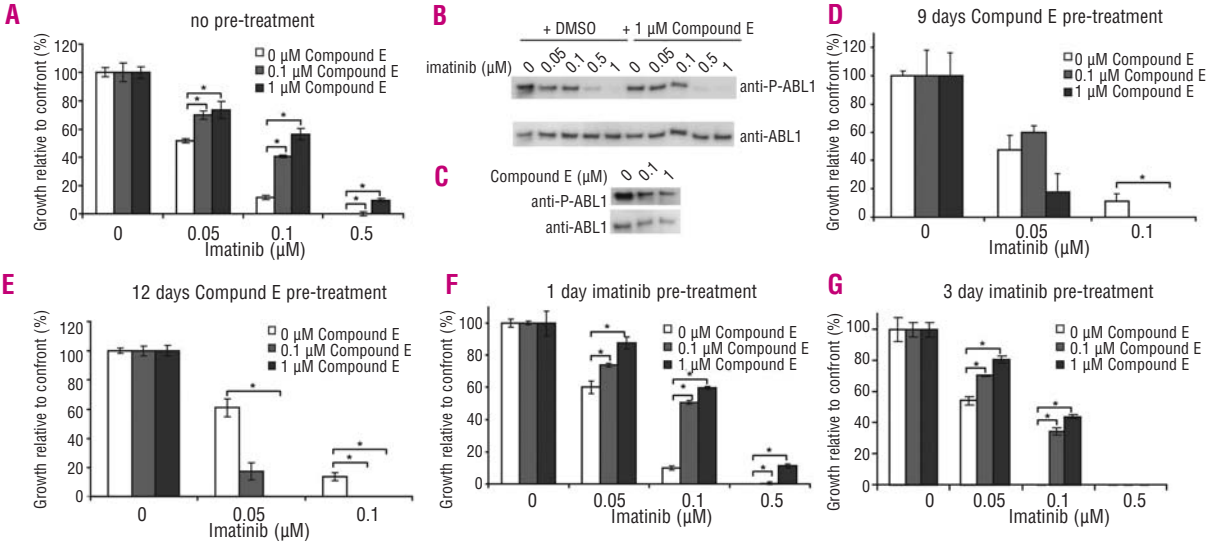

Figure 4. Kinase inhibitors and γ-secretase inhibition can act synergistically or antagonistically, depending on the sequence in which they are administered. All experiments were performed on the ALL-SIL cell line. The concentrationsof the GSI are indicated by the different colors of the bars. Kinase inhibitor concentrations are indicated on the X-axis. The Y-axis represents the growth relative to DMSO-treated cells. After the pre-treatment conditions indicated above the graphs, cells were incubated for 3 additional days with indicated combinations of imatinib and Compound E. After these 3 days, the amount of viable cells was determined on a Vi-cell XR cell viability analyzer and proliferation of treated cells relative to untreated cells was calculated. An asterisk indicates a statistically significant difference between the groups connected by the line underneath the asterisk. (A) ALL-SIL cells were treated for 3 days with imatinib and Compound E, without pre-treament. (B) Western blot showing the effect of addition of Compound E on the inhibition of NUP214-ABL1 phosphorylation by imatinib in the ALL-SIL cell line. The blot was stripped and reprobed with anti-ABL1. (C) The effect of Compound E treatment on NUP214-ABL1 activity was assessed by detecting NUP214-ABL1 phosphorylation on western blot with anti-phospho-ABL1 antibody after treatment with 0, 0.1µM or 1 µM Compound E for 2 days. The blot was stripped and reprobed with anti-ABL1. (D, E) ALL-SIL cells were pre-treated for 9 or 12 days with 0, 0.1µM or 1 µM Compound E. After the pre-treatment, cells were treated for 3 more days with the indicated combinations of imatinib and Compound E. (F, G) ALL-SIL cells were pre-treated for 1 or 3 days with 0.025 µM imatinib. After this pre-treatment, cells were treated for 3 more days with the indicated combinations of imatinib and Compound E.

strating that intracellular imatinib levels were not reduced (Figure 4B). The observed antagonism between Compound E and imatinib in ALL-SIL cells was also not due to a direct increase of NUP214-ABL1 activation by Compound E, as we could not detect an increase in NUP214-ABL1 phosphorylation upon Compound E treatment (Figure 4C).

We next tested whether pre-treatment of the cell lines for 9 or 12 days with a GSI could sensitize the cells to imatinib. After 9 days of pre-treatment with Compound E, imatinib was added to the Compound E treatment and cells were incubated with this combination of drugs for 3 more days. No significant interaction was observed at a concentration of 0.1 μM Compound E, but 1 μM Compound E sensitized the cells to the effect of imatinib (Figure 4D). After 12 days of pre-treatment, this sensitization was also observed for 0.1 μM Compound E (Figure 4E). This can probably be explained by the fact that inhibition of cell proliferation by 0.1 μM Compound E treatment occurs with a slight delay compared to treatment with the 1 μM concentration. We also tested pre-treatment with the kinase inhibitor instead of with the GSI. ALL-SIL was pre-treated for 1 day or 3 days with a low dose of imatinib. As can be seen in Figure 4 F and G, kinase inhibitor pre-treatment did not abolish the antagonism between the kinase inhibitor and the GSI.

Combining γ-secretase inhibitors with vincristine does not offer a common therapeutic advantage

We next tested combinations of the chemotherapeutic agent vincristine and a GSI. Treatment of T-ALL cell lines with vincristine alone resulted in potent inhibition of proliferation at concentrations below 10 nM. In the ALL-SIL cell line, we tested concomitant treatment with Compound E and vincristine without pre-treatment of the cells (Figure 5A), after pre-treatment with the GSI (Figure 5B) and after pre-treatment with vincristine (Figure 5C). No significant interaction or antagonism occurred in ALL-SIL cells, independently of the administration scheme of the compounds. Combinations of vincristine and Compound E after pre-treatment with the GSI were also tested in DND-41, RPMI-8402, HSB-2 and KARPAS-45, the four other cell lines that showed a proliferation defect upon GSI treatment. For RPMI-8402, significant synergy was observed when combining vincristine with 1 μM Compound E, but not when combining it with the lower dose of 0.1 μM. No significant therapeutic advantage of combining the GSI and vincristine could be documented in the other cell lines (Figures 5 D-G).

γ-secretase inhibitor treatment can sensitize T-ALL cells to the effect of dexamethasone

Glucocorticoids have been used for a long time for the

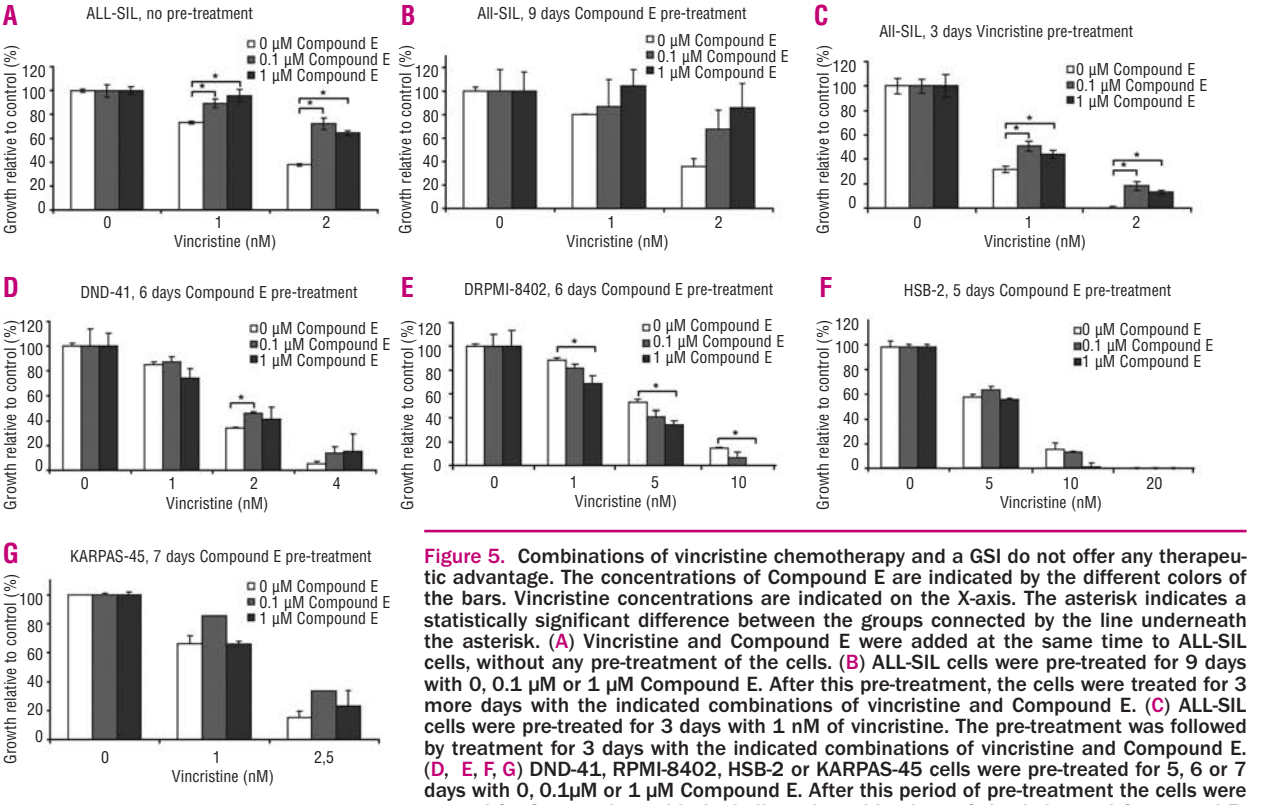

Figure 5. Combinations of vincristine chemotherapy and a GSI do not offer any therapeutic advantage. The concentrations of Compound E are indicated by the different colors of the bars. Vincristine concentrations are indicated on the X-axis. The asterisk indicates a statistically significant difference between the groups connected by the line underneath the asterisk. (A) Vincristine and Compound E were added at the same time to ALL-SIL cells, without any pre-treatment of the cells. (B) ALL-SIL cells were pre-treated for 9 days with 0, 0.1 μM or 1 μM Compound E. After this pre-treatment, the cells were treated for 3 more days with the indicated combinations of vincristine and Compound E. (C) ALL-SIL cells were pre-treated for 3 days with 1 nM of vincristine. The pre-treatment was followed by treatment for 3 days with the indicated combinations of vincristine and Compound E. (D, E, F, G) DND-41, RPMI-8402, HSB-2 or KARPAS-45 cells were pre-treated for 5, 6 or 7 days with 0, 0.1μM or 1 μM Compound E. After this period of pre-treatment the cells were treated for 2 more days with the indicated combinations of vincristine and Compound E.

treatment of ALL.[28] We, therefore, tested the interaction between a GSI and the glucocorticoids dexamethasone and corticosterone. In ALL-SIL and DND-41, dexamethasone treatment showed a weak, but significant inhibitory effect on the proliferation of the cells, which was significantly enhanced when cells were pre-treated for 7 days or 14 days with 1 μM Compound E (Figure 6). The RPMI-8402 and LOUCY cell lines showed the weakest sensitivity to dexamethasone as a single agent, but GSI pre-treatment also made these cell lines more sensitive to the effect of dexamethasone (Figure 6). Maximal inhibition of proliferation of ALL-SIL, DND-41, RPMI-8402 and LOUCY cells was already obtained with 1 μM of dexamethasone alone or in combination with 1 μM Compound E (*data not shown*). No clear interactions could be documented for corticosterone combinations (*data not shown*).

Discussion

The identification and validation of novel molecular targets for cancer therapy are important objectives of cancer research. The aims are to reduce the toxicity of current treatments and to further improve treatment outcomes. Mutant *NOTCH1* could represent an important new target for therapy of T-ALL patients, since *NOTCH1* is frequently mutated in T-ALL and the generation of activated NOTCH1 can be inhibited by γ-secretase inhibitors.[2,8] Treatment of T-ALL cell lines with GSI was shown to induce a G_0/G_1 cell cycle block, but the exact therapeutic consequences of γ-secretase inhibition in the context of T-ALL treatment have not been fully established.[8,15,19-21] In this study, we have further evaluated the consequences of GSI treatment of T-ALL cell lines, and investigated possible synergism between GSI and other anti-cancer agents.

In agreement with previous reports, we observed that the generation of activated NOTCH1 was completely inhibited after 2 days of GSI treatment in all cell lines, but that the effect on proliferation only became evident 5 to 7 days later, and correlated with a G_0/G_1 block in the cell cycle. Our study demonstrates that these effects are reversible and that treatment periods of 14 days or longer are required to induce apoptosis. We also observed decreased telomerase activity in cell lines with GSI induced proliferation and cell cycle defects. As telomerase activity is known to vary depending on the stage in the cell cycle and is minimal during the G_0/G_1 phase,[29] this observation could be explained by the G_0/G_1 cell cycle block induced by GSI treatment in these cell lines. In addition, we observed GSI-induced differentiation of some T-ALL cell lines with *NOTCH1* mutations, indicating that mutant *NOTCH1* could be partially responsible for the differentiation defect observed in T-ALL. Indeed, expression of activated *NOTCH1* has been shown to block differentiation of $CD4^+CD8^+$ T cells.[23] Differentiation in T-ALL is also believed to be affected by the abnormal expression of transcription factors such as TLX1, TLX3, HOXA10, TAL1 and MYB.[1,4,30] It is, therefore, unlikely that GSI treatment alone can completely release the block of differentiation, but this aspect may also contribute to the therapeutic potential of GSI treatment. As these GSI-induced differentiation changes also occurred in MOLT-4, a cell line expressing mutant *NOTCH1*, but not showing any inhibition of proliferation upon GSI treatment, there seems to be no clear correlation between differentiation and proliferation effects of GSI treatment.

Similar to previous findings, we observed that not all cell lines with *NOTCH1* mutations were sensitive to GSI treatment.[8] We observed that JURKAT and MOLT-

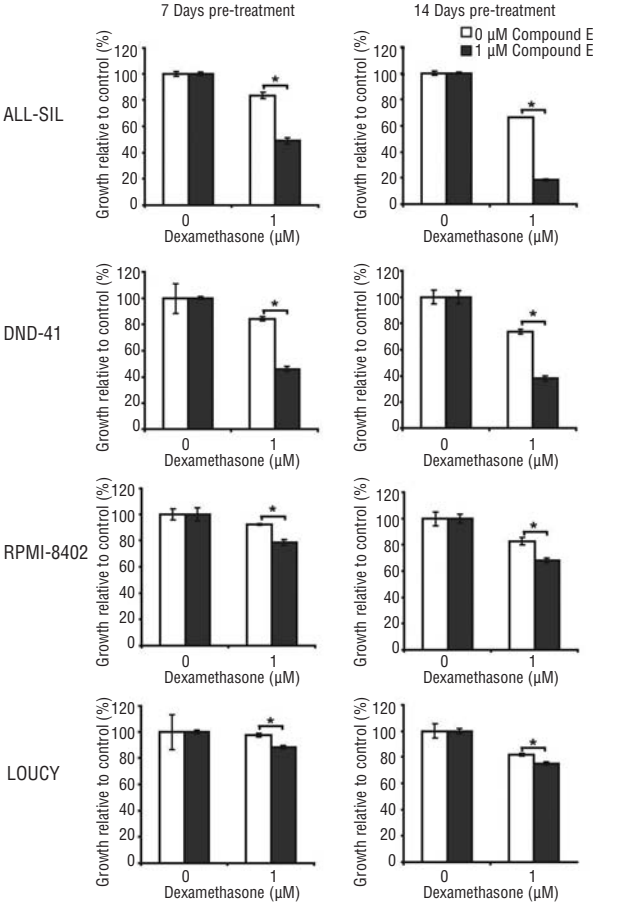

Figure 6. Pre-treatment with a GSI sensitizes dexamethasone-responding cells. ALL-SIL, DND-41, RPMI-8402 and LOUCY cells were treated with the glucocorticoid dexamethasone after DMSO pre-treatment (white bars), or with a combination of 1 μM Compound E and dexamethasone, after pre-treatment with 1 μM Compound E (dark bars). The pre-treatment period was either 7 days (left part of figure) or 14 days (right part of figure). After the pre-treatment period, cells were treated for 2 days (DND-41, RPMI-8402 and LOUCY) or 3 days (ALL-SIL) and cell growth relative to DMSO-treated cells was calculated. The asterisk indicates a statistically significant difference between the groups connected by the line underneath the asterisk.

4, two cell lines displaying detectable levels of activated intracellular NOCH1 on western blot, were resistant to the GSI-induced proliferation and cell cycle defects observed in the GSI-sensitive cell lines. It was recently reported that loss of the tumor suppressor gene *PTEN* is associated with resistance to GSI in cell lines with *NOTCH1* mutations.[5] From the collection of T-ALL cell lines we tested, MOLT-4 and JURKAT lack *PTEN* expression,[31] which likely explains why these cell lines are resistant to GSI treatment. Our results also confirm that *NOTCH1* mutation-negative cell lines can be sensitive to GSI treatment. The proliferation of HSB-2 cells was found to be weakly inhibited by GSI treatment in the absence of detectable NOTCH1 activation. Weng *et al.* observed that the TALL-1 cell line displayed cell cycle block upon GSI treatment although this cell line also lacked *NOTCH1* mutations.[8] In addition, murine GSI-sensitive cell lines without mutant *NOTCH1* have been described.[19] Possible mechanisms for the observed GSI sensitivity in these cell lines could be the presence of activating mutations in *NOTCH2*, *NOTCH3* or *NOTCH4* or aberrant expression of NOTCH ligands.[32] Alternatively, these observed effects could be due to aspecific effects of the GSI on another target. Our findings differ in some points from the results reported by Weng *et al.*, as we found that RPMI-8402 and KARPAS-45 cells are sensitive to GSI treatment. For RPMI-8402, the difference is likely to be caused by the different assays, and different time-points that we used for the read-outs, so that we were able to observe that RPMI-8402 is in fact sensitive to GSI treatment. This is also in agreement with the fact that RPMI-8402 does not have a complete loss of *PTEN*.[5] For KARPAS-45, the *NOTCH1* DNA sequencing and western blot analysis revealed that the difference is due to the fact that our KARPAS-45 is different from the cell line reported by Weng *et al.* Such differences between cell lines have been reported before, and warrant a full analysis by sequencing and western blot of each cell line used in functional studies.

The fact that GSI only induce apoptosis after long period of treatment, and the fact that the effects of gamma-secretase inhibition are reversible, suggests that the use of a GSI as a single agent for the treatment of T-ALL may be limited. In addition, it was previously shown that GSI treatment for 15 days is associated with toxic effects on the gastrointestinal tract and on normal T-cell development.[18] To investigate possible synergistic effects with other currently used therapeutic agents, we tested whether the addition of GSI could enhance the inhibitory effect of tyrosine kinase inhibitors or other cytotoxic drugs. We recently identified *NUP214-ABL1* and *EML1-ABL1* fusions in T-ALL, and showed that these are sensitive to the small molecule kinase inhibitor imatinib.[24,25] Here we show that these *ABL1* fusions are frequently associated with the presence of *NOTCH1* mutations (six out of six cases), supporting the possible application of combined NOTCH1- and ABL-targeted therapy for the treatment of these cases. Our results indicate, however, that GSI and imatinib cannot unconditionally be combined with each other, and that the exact timing and order of administration could determine whether the combinations have synergistic/additive or antagonistic effects. In addition, the finding that *ABL1* fusions occur together with *NOTCH1* mutations provides genetic evidence that these mutations are co-operating events in the pathogenesis of T-ALL.

In contrast to imatinib, pre-treatment of T-ALL cell lines with a GSI did not make the cells more sensitive to the cytotoxic chemotherapeutic vincristine. This may be explained by the fact that GSI induces a cell cycle block, and that cytotoxic chemotherapy is dependent on cell division to induce its effect. We also tested whether combinations of a GSI and glucocorticoids (dexamethasone or corticosterone) offer any therapeutic advantage over glucocorticoids as single agent therapy. For dexamethasone, pre-treatment with the GSI enhanced the weak inhibitory effects of dexamethasone as a single agent; we found the strongest synergy between the GSI and dexamethasone in the cell lines that were most sensitive to dexamethasone. It remains to be determined to what extent the observed *in vitro* interactions could translate into responses of T-ALL patients to these drug combinations. Our data indicate that GSI pre-treatment of T-ALL cells can make the cells more susceptible to other inhibitors, and this could provide important therapeutic advantages.

The clinical application of small molecule tyrosine kinase inhibitors for the treatment of chronic myeloid leukemia has demonstrated the efficacy and low toxicity of targeted therapies.[33] The introduction of GSI in the treatment protocols for NOTCH1 mutation-positive T-ALL could further reduce the toxicity of current treatments, and improve long-term outcomes. To date, however, exclusive targeting of mutant NOTCH1 is not possible, and the currently used GSI generally inhibit γ-secretase with associated effects on wild type *NOTCH1*, the three other NOTCH members, as well as a variety of other proteins that are cleaved by the γ-secretase complex. Our data show that combining GSI with kinase inhibitors or dexamethasone could potentially enhance the anti-proliferative effects of GSI on leukemic cells, which may improve their application as agents for the treatment of T-ALL.

Authorship and Disclosures

KDK and IL designed and performed research, analyzed data and wrote the paper; NM and CF performed research; LVN and SB provided analytical tools and performed research; PV, MDO, PM and JC designed the study, analyzed data and wrote the paper.

The authors reported no potential conflicts of interest.

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