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# Apoptosis in T Cell Acute Lymphoblastic Leukemia Cells after Cell Cycle Arrest Induced by Pharmacological Inhibition of Notch Signaling

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## SUMMARY

**In this report, inhibitors of the γ-secretase enzyme have been exploited to characterize the antiproliferative relationship between target inhibition and cellular responses in Notch-dependent human T cell acute lymphoblastic leukemia (T-ALL) cell lines. Inhibition of γ-secretase led to decreased Notch signaling, measured by endogenous NOTCH intracellular domain (NICD) formation, and was associated with decreased cell viability. Flow cytometry revealed that decreased cell viability resulted from a $G_0/G_1$ cell cycle block, which correlated strongly to the induction of apoptosis. These effects associated with inhibitor treatment were rescued by exogenous expression of NICD and were not mirrored when a markedly less active enantiomer was used, demonstrating the γ-secretase dependency and specificity of these responses. Together, these data strengthen the rationale for using γ-secretase inhibitors therapeutically and suggest that programmed cell death may contribute to reduction of tumor burden in the clinic.**

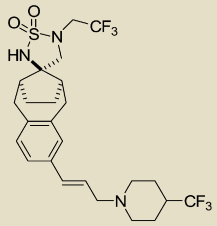
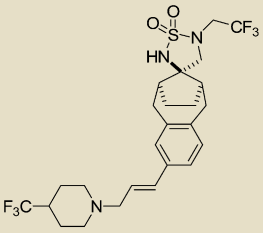
## INTRODUCTION

In multicellular organisms, the Notch pathway [1–3] participates in diverse fundamental processes, including cell-fate decisions during development and tissue renewal and repair in the adult. Both positive and negative Notch pathway modulators act on a core signaling cascade [4], in which extracellular ligand binding activates the receptor, which, in turn, stimulates its sequential cleavage by the proteases TNF-α converting enzyme (TACE) and γ-secretase to finally release the NOTCH intracellular domain (NICD). Once released from the membrane-bound receptor, the cytosolic NICD polypeptide then translocates to the nucleus to modulate transcription of downstream genes and, subsequently, cell growth [5, 6].

Increased Notch signaling has been associated with tumorigenesis [7–9], and NOTCH1 was first discovered via a chromosomal translocation found in a small subset of T cell acute lymphoblastic leukemias (T-ALL) [10]. Subsequently, enforced NOTCH1 signaling has been shown both to act as a potent inducer of T-ALL in mice and to sustain the growth of a human T-ALL cell line [11, 12]. In addition, constitutively active mutants of the NOTCH family have been associated with a range of cancers, including some mammary and mucoepidermoid carcinomas [13–16]. In other cancers, increased expression of NOTCH wild-type family members leads to greater flux through the Notch signaling pathway. For example, some lymphoproliferative cancers show increased expression of normal NOTCH pathway members without evidence of associated mutations [17, 18]. An analogous increase in pathway flux has been associated with ~50% of human breast cancers, on account of reduced expression of the NOTCH inhibitor Numb [19]. Also, a novel animal model suggests that increased Notch signaling may also be associated with medulloblastomas [20].

Consequently, inhibitors of γ-secretase, originally developed as a potential treatment for Alzheimer's disease, may prevent NOTCH receptor cleavage [21–23] and

**Table 1. Structures and Calculated Potencies for MRK-003 and MRK-006**

| | MRK-003 | MRK-006 |
|---|---|---|
| Structure |  |  |
| SH-SY5Y SPA4CT cells | 0.4 nM (0.08) | 110.5 nM (2.56) |
| HEK NOTCHΔE/APP cells | 0.52 nM versus Aβ (40) | – |
| | 0.72 nM versus NICD | – |

Data in parentheses represent standard errors of the mean.

provide an effective cancer therapy [24]. Although translocations originally associated with T-ALL are extremely rare [25], affecting less than 1% of the T-ALL population, two recent reports strengthened the rationale for γ-secretase inhibition as a viable intervention in this disease. First, it has been confirmed that some truncations that remove a large portion of the NOTCH receptor ectodomain and result in ligand-independent signaling can remain membrane-associated and therefore dependent on γ-secretase for release of the NICD signaling fragment [26]. Second, extensive sequencing has indicated that 56% of human T-ALL cases encode novel activating mutations that enhance either NICD production or stability [27]. The therapeutic potential of γ-secretase inhibitors for treating Notch-related T-ALL cases is therefore greater than originally suggested by the rarity of the original chromosomal translocations.

Despite these findings, important questions relating to the downstream results of γ-secretase inhibition remained unanswered. In this study, we used a novel and extremely potent γ-secretase inhibitor from the recently disclosed cyclic sulfamide structural series [28] as a chemical tool. This has enabled us to decipher the consequences of cell cycle arrest in representative human T-ALL cell lines (HPB-ALL and DND-41) and to investigate its relationship to the induction of apoptosis.

## RESULTS

### γ-Secretase Inhibitors Used in This Study

In order to explore further the role of Notch signaling in T-ALL, two novel γ-secretase inhibitors were employed (Table 1). MRK-003, initially developed from a screening hit [28], is a cyclic sulfamide γ-secretase inhibitor structurally distinct from molecules such as DAPT (*N*-[*N*-(3,5-difluorophenacetyl)-L-alanyl]-(*S*)-phenylglycine *t*-butyl ester) [29] and the benzodiazepine Compound E [30]. MRK-003 is a potent γ-secretase inhibitor with a subnanomolar in vitro potency for the inhibition of Aβ secretion from human neuroblastoma cells. It has been shown to reduce brain Aβ production in vivo after oral dosing of mice [28]. In contrast, MRK-006 is its less active enantiomer and was selected as a negative control in order to differentiate specific mechanism-based activity from idiosyncratic effects. MRK-003 was characterized along with a selection of over 50 inhibitors for its in vitro effects on the cleavage of APP and the Notch receptor in HEK293 cells [31]. In this system, MRK-003 inhibited the γ-secretase-mediated cleavage of the Notch receptor with essentially the same potency as that of APP (Table 1).

### MRK-003, a Potent γ-Secretase Inhibitor, Decreases T-ALL Cell Viability

Three T-ALL cell lines (DND-41, HPB-ALL, and TALL-1) previously identified as being dependent on γ-secretase for proliferation [27] were chosen to evaluate the effects of the γ-secretase inhibitor MRK-003 on cell viability. The HPB-ALL cell line demonstrated a time-dependent decrease in cell viability between day 3 and day 8 after incubation with inhibitor, with a highly potent effective dose ($ED_{50}$ 9 nM) observed at day 8. MRK-006 was shown to be far less effective at inhibiting viability in the same experiment (Table 2). Inhibition of proliferation by MRK-003 was also recorded in the two additional T cell lines, with DND-41 being less responsive than either HPB-ALL or TALL-1 cells (Table 3). Clearly, exposure to MRK-003 leads to a marked decrease in measurable metabolic activity of multiple T-ALL cell lines, indicative of a decrease in cell viability/proliferation.

### Dosage- and Time-Dependent Cell Cycle Arrest with MRK-003

In order to investigate further the cause of the decreased viability, treated cells were fixed and their DNA was labeled with propidium iodide, followed by FACS analysis. This method allows the assignment of cell cycle populations according to their DNA content. HPB-ALL cells, which are derived from a patient with T-ALL, were chosen

**Table 2. Calculated Potencies for Inhibition of Cellular Proliferation by MRK-003 and MRK-006 in HPB-ALL Cells**

| | Cell Viability $ED_{50}$ (μM) | | |
|---|---|---|---|
| Day | MRK-003 | MRK-006 | MRK-003/-006 |
| 3 | 3.300 | 11.7 | 4 |
| 6 | 0.060 | 4.7 | 78 |
| 8 | 0.009 | 5.2 | 578 |

Cells were incubated with compound as described in Experimental Procedures. Data indicate the potencies for inhibition of proliferation in HPB-ALL cells at three time points. Individual potencies for each compound, in addition to a ratiometric comparison, are given.

**Table 3. Comparative Potencies for Inhibition of Cellular Proliferation by MRK-003 and MRK-006 in Different T-ALL Cell Lines**

| | Cell Viability $ED_{50}$ (μM) | | |
|---|---|---|---|
| Cell Line | MRK-003 | MRK-006 | MRK-003/-006 |
| HPB-ALL | 0.009 | 5.2 | 578 |
| TALL-1 | <0.005 | 2.3 | >460 |
| DND-41 | 0.320 | 13.0 | 41 |

Cells were incubated with compound as described in Experimental Procedures. Data indicate the potencies for inhibition of proliferation at day 8 in three cell lines. Individual potencies for each compound, in addition to a ratiometric comparison, are given.

as a representative cellular model for the remainder of this work. As shown in Figure 1A, 10 μM MRK-003 led to a marked arrest in cell cycle progression after 7 days, as characterized by loss of cells in $G_2$ and S phases and an accumulation of cells in $G_1$. This is in agreement with the mechanism of $G_0/G_1$ cell cycle arrest seen with an alternative γ-secretase inhibitor (Compound E) reported previously [27]. It is noteworthy that the inhibitor treatment also appears to increase the abundance of the sub-G1 population, which suggests DNA fragmentation as a result of apoptosis.

The time dependence of the $G_0/G_1$ cell cycle arrest is shown by analysis of cultures incubated with increasing concentrations of MRK-003 for 2, 3, 4, 5, and 6 days. As shown by the representative histograms in Figure 1B and by the plotted $G_2$-S populations in Figure 1C, the time required for arrest to be manifested depends to some extent on the concentration of the inhibitor. However, the top doses begin to cause arrest after about 3 days, and a maximal effect is seen after 5–6 days. For this reason, 7 days was selected as the incubation time for subsequent studies.

A comprehensive titration of the effect of MRK-003 on cell cycle arrest after 7 days is shown in Figure 2A. Analysis of MRK-003 between 10 μM and 10 nM shows a gradual loss of cell cycle arrest as levels of inhibitor are reduced. Nonlinear regression analysis allows the $ED_{50}$ for this inhibition to be estimated as 400 nM (Figure 2B).

## Apoptosis Documented by FACS Analysis

Since a transient arrest of the cell cycle in tumor cells would result in only a temporary cytostatic delay in growth, it is vitally important to understand the fate of the arrested cells. In particular, for any future therapeutic application we wanted to investigate whether programmed cell death is a potential outcome of this phenotype. To this end, cells were incubated with inhibitor as described above for 7 days and then collected for labeling with FITC-labeled Annexin V. FACS analysis of vehicle-treated cultures indicated that all cells showed only low (or no) surface staining with this protein since its binding partner, phosphatidylserine, is normally an asymmetric resident of the inner membrane. However, in cells that had been treated with MRK-003, but not MRK-006, this asymmetric distribution is lost, and a second population with increased Annexin V staining can be detected (Figure 3). This population is likely to be an early indicator of apoptosis rather than of late-stage necrosis since it is not also associated with a general increase in cell permeability to DNA staining (data not shown). Supporting the observation of apoptosis, an increase in the sub-$G_1$ populations (e.g., Figure 1A) is also seen in parallel with the Annexin V staining effects, although this effect was slightly less robust between experiments.

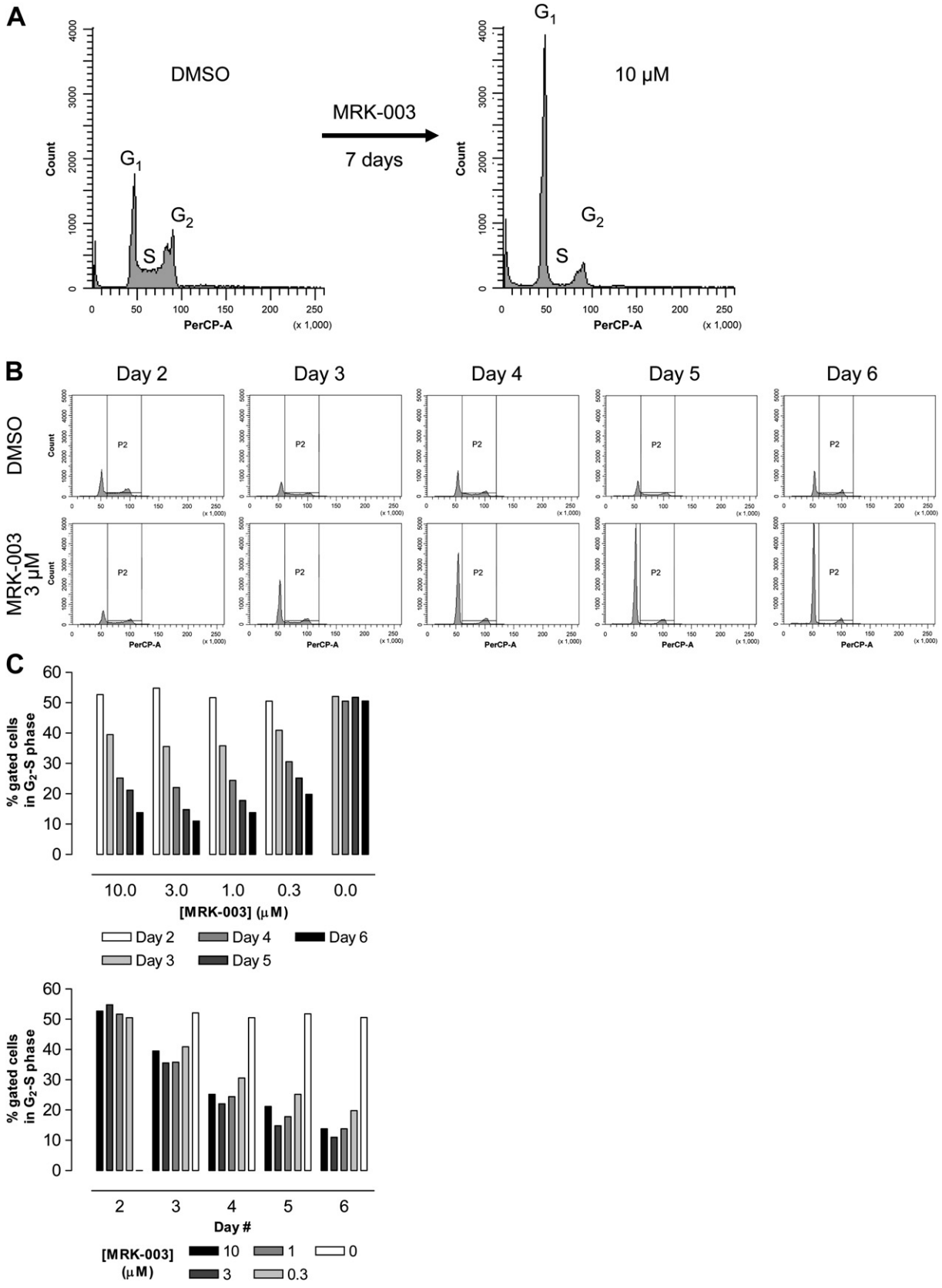
We were able to titrate out this effect with lower doses of inhibitor and estimate $ED_{50}$s for the changes to the high- and low-staining Annexin V peaks (and the ratio of the two). In contrast, no apoptosis was seen in cells that had insufficiently efficacious doses of MRK-003, in cells not incubated for enough time, or in cell lines reported not to depend on γ-secretase for proliferation (data not shown).

## Correlations between Cell Cycle Arrest and Induction of Apoptosis

Because the apoptosis analysis (Figure 3) was conducted on cells incubated in parallel with those that underwent analysis of cell cycle arrest (Figure 2), we were able to compare the potencies and correlate the two processes. As shown in Figure 3C, excellent correlations between interference with cell cycle progression and the induction of apoptosis after γ-secretase inhibition are generated, whether the changes in the low- or high-staining Annexin V peak (or a ratio of the two) are compared. The $ED_{50}$s for the observed changes in cell cycle (400 nM; Figure 2B) and apoptotic populations (440 nM) also show good agreement (Figure 3D).

## γ-Secretase Inhibition Leads to Notch Receptor Signaling

To verify that γ-secretase inhibition led to loss of Notch signaling in T cells, we sought to detect endogenous levels of the NICD cleavage product in HPB-ALL cells. As shown in Figure 4, lysates from HPB-ALL cells showed specific labeling of a NICD band with a MW of ~85 kDa, in good agreement with the expected mass for full-length



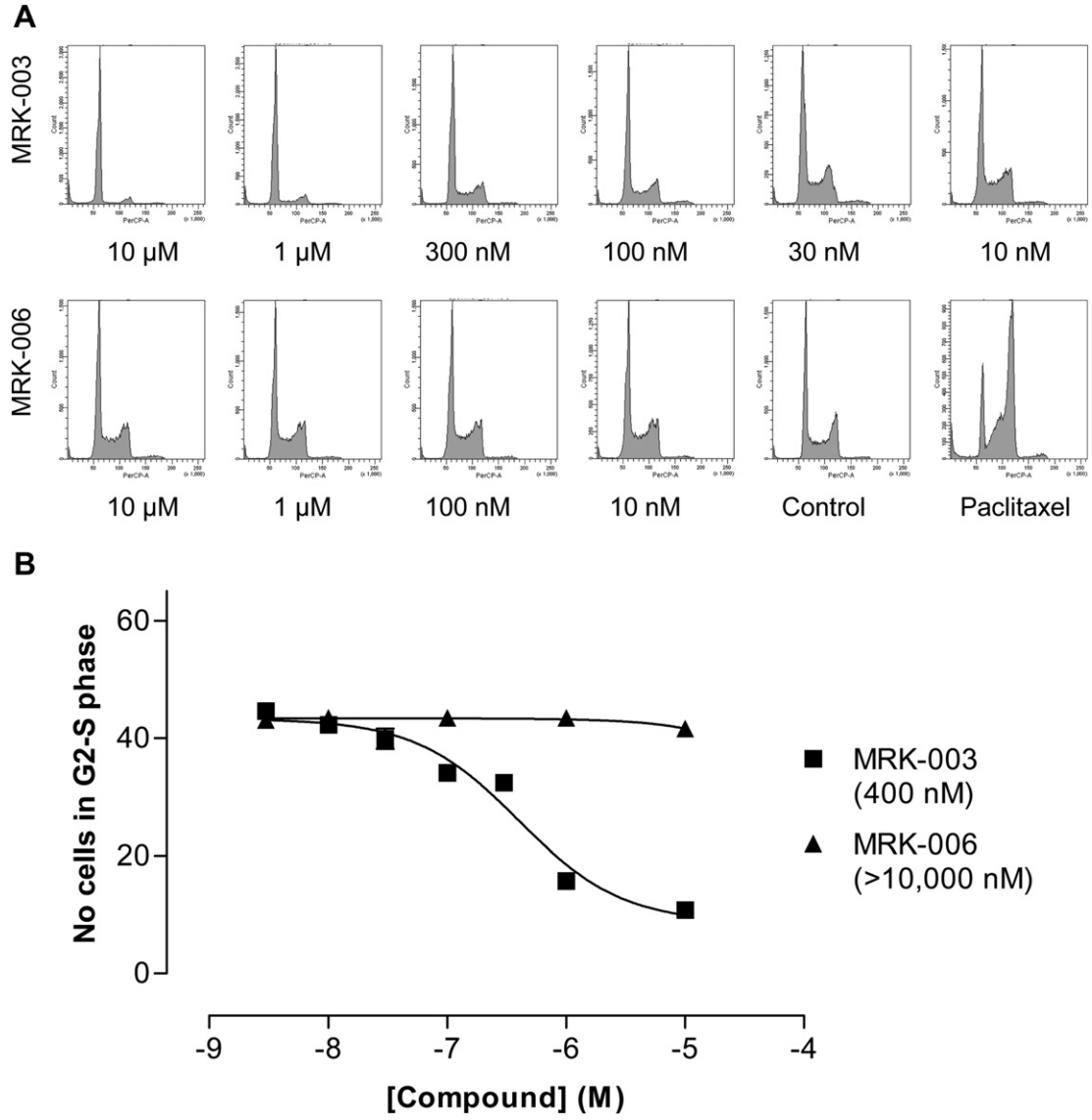



**Figure 2. Dose Responsiveness of Cell Cycle Arrest**

(A) Representative FACS traces indicate titration of cell cycle arrest seen at 7 days with MRK-003, but not MRK-006.

(B) The data are also plotted to estimate relative potencies. Note that error bars indicating standard error of the mean (SEM) are smaller than the symbols on the graph.

NICD, which disappeared upon incubation with MRK-003 at 10 μM. A gradual reappearance of this NICD band was seen with decreasing doses of inhibitor. Lysates from cells overexpressing the NOTCHΔE construct (included as a positive control) led to the detection of a band with a slightly lower molecular weight, consistent with the reported deletion of the C-terminal PEST sequence from this construct in order to enhance NICD stability [32]. Incubation with a comprehensive range of concentrations allows an $ED_{50}$ for NICD generation to be estimated to be 30 nM by densitometry.

### Rescue of Antiproliferative and Proapoptotic Effects of γ-Secretase Inhibition by Expression of NOTCH Intracellular Domain

In order to confirm that the observed effects of the γ-secretase inhibitor MRK-003 on cell cycle arrest and induction of apoptosis are solely mediated through inhibition

**Figure 1. Demonstration of Cell Cycle Arrest after γ-Secretase Inhibition**

(A and B) Representative FACS histogram traces indicate the (A) extent and (B) timing of cell cycle arrest.

(C) The time course data (single point) are tabulated more fully.

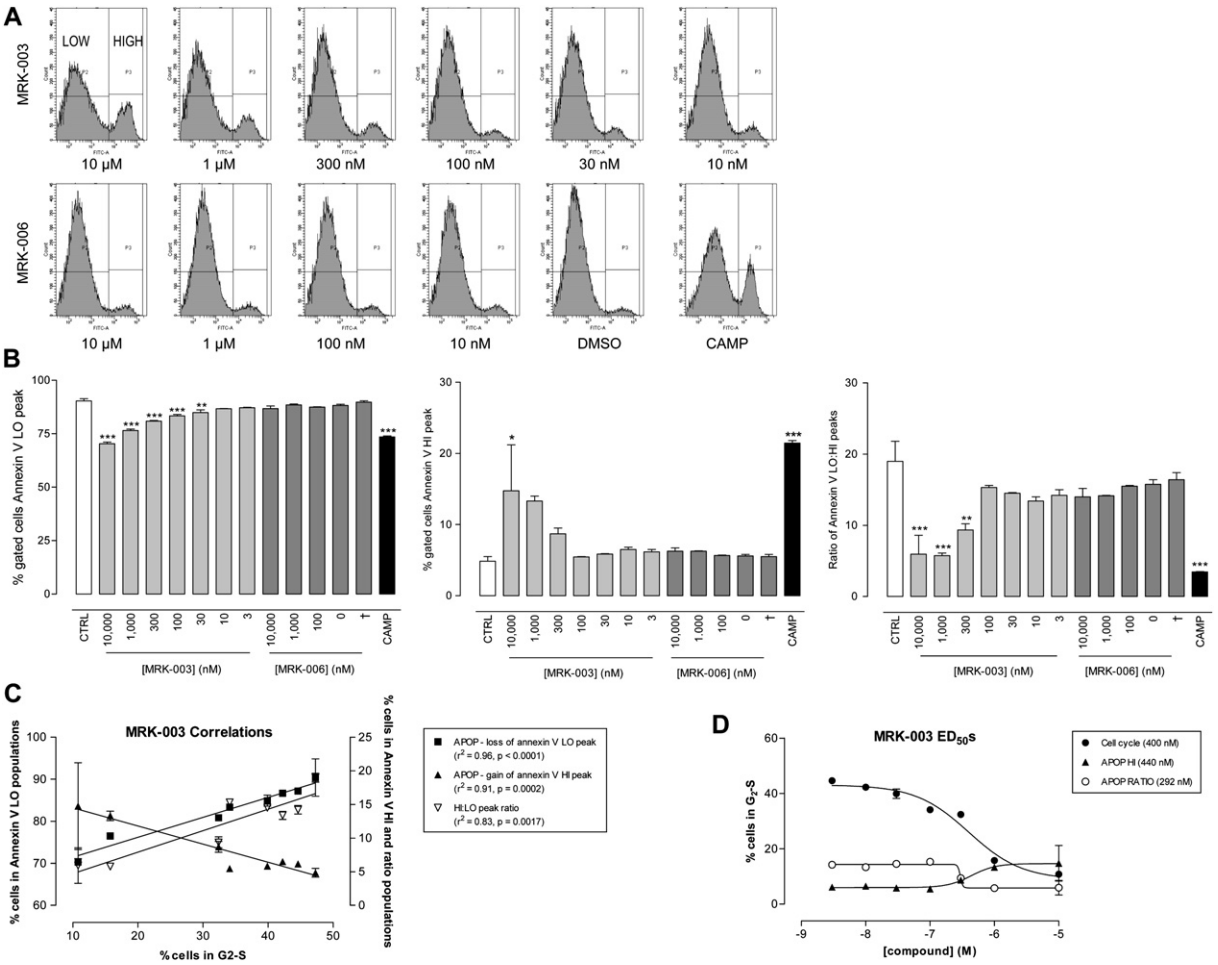



**Figure 3. Dose Responsiveness of Apoptosis**

(A) Representative FACS traces indicate titration of cell cycle arrest seen at 7 days with MRK-003, but not MRK-006.

(B) The data are also shown in bar charts in order to quantify the changes in the low- and high-staining Annexin V peaks, as well as a ratio of the two. Induction of apoptosis generally leads to the appearance of a population (labeled "HIGH") that stains well for Annexin V in parallel with a loss of cells from the unstained ("LOW") group. Camptothecin (6 μM, 24 hr) is included as a positive control. Error bars represent SEM.

(C and D) Comparative correlations and potencies for effects of MRK-003 on cell cycle arrest and apoptosis are shown. Error bars represent SEM.

of Notch signaling, and not via the cleavage of other substrates [33, 34], the ability of NICD expression to overcome these phenomena was examined. As shown in Figure 5, DND-41 cells (previously shown to be responsive to γ-secretase inhibition in Table 1) lose the ability to undergo both $G_0/G_1$ arrest and apoptosis in response to MRK-003 (1 μM) if infected with a retrovirus encoding NICD, in contrast to cells containing the empty vector control construct (MigR1).

## DISCUSSION

The ability of γ-secretase inhibitors to induce cell cycle arrest and apoptosis in human T-ALL cell lines strengthens the rationale for their use in the treatment of cancer. Cell cycle block, induction of apoptosis, and specificity observed through the NICD rescue experiment, as well as cycle arrest observed with human T-ALL cell lines, agree with studies conducted with an alternative γ-secretase inhibitor (Compound E) in the engineered murine T6E cell line [35] and demonstrate that the cyclic sulfamide structural series, specifically MRK-003, is a useful tool for studying γ-secretase inhibition and Notch pathway modulation. In T-ALL cells, MRK-003 blocked cell cycle progression and decreased cellular metabolism in a process that became apparent after a few days. Closer examination of the affected populations indicates that they exhibit a marked loss of cells in $G_2$ and S phases of the cell cycle and an accumulation of cells in $G_0/G_1$. The efficacy of MRK-003 has been well studied in a number of cell lines in vitro, but, to our knowledge, this is the first published report to demonstrate, by western blotting, an ability to inhibit the processing of endogenous Notch receptor in a T cell line. The degree of γ-secretase inhibition, directly monitored by inhibition of Notch receptor processing, correlated well with cell cycle arrest and apoptosis. Evidence

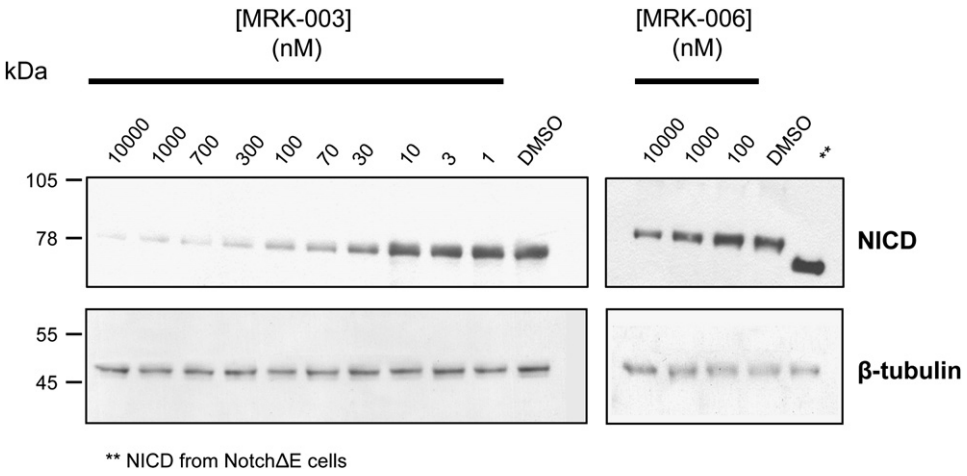



**Figure 4. Demonstration of Endogenous NICD in HPB-ALL and Inhibition by MRK-003**

HPB-ALL cells were incubated with MRK-003 for 48 hr, and the cells were collected, lysed, and solubilized as described. Levels of NICD were detected by western blot relative to β-tubulin as a loading control.

of apoptosis, monitored by flow cytometry analysis with FITC-conjugated Annexin V, was supported further by evidence of DNA fragmentation, represented by sub-$G_0/G_1$ populations. This correlation suggests that cell death is an irrevocable result of the cell cycle disruption triggered by γ-secretase inhibition in T-ALL cells. Finally, exogenous expression of active NICD in a T-ALL cell line and the concomitant loss of responsiveness to MRK-003 clearly demonstrate that both cell cycle arrest and apoptosis are absolutely dependent on Notch signaling, which, in turn, can be pharmacologically modulated with γ-secretase inhibitors.

Our studies help explain a number of recent reports that link γ-secretase inhibition to antiproliferative effects in T cell lines [27, 35], cell and animal models of melanoma [36], and lung cancer cell lines overexpressing NOTCH3 [37]. Similarly, reports consistent with the model of γ-secretase inhibitor-mediated induction of apoptosis are also beginning to emerge. For example, apoptosis is reported to follow γ-secretase inhibition in T cells [35], in lung cell lines [37], in Kaposi's sarcoma tumor cells [39], and in medulloblastoma xenografts [20]. Activated Notch signaling has been linked to inhibition of apoptosis in Hodgkin's and anaplastic large-cell lymphoma [18], in

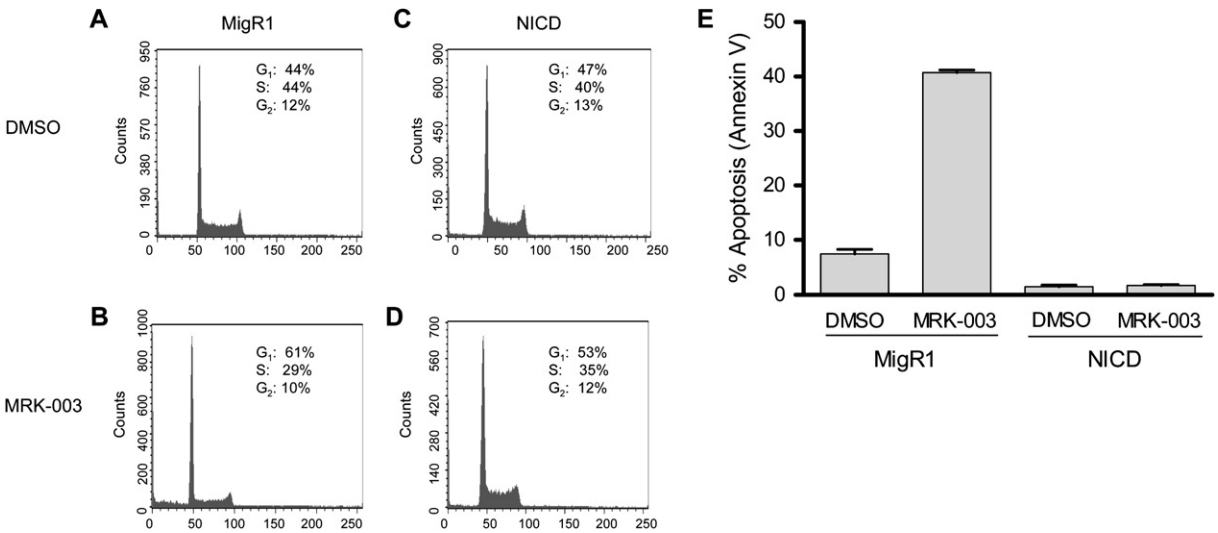



**Figure 5. Overexpression of the Intracellular Domain of NOTCH1, NICD, Rescues the Cell Cycle Block and Apoptosis Induction of Treating T-ALL Cells with MRK-003**

(A–E) DND-41 cells infected with either MigR1 (empty vector) or MigR1 NICD were treated with 1 μM MRK-003 for 7 days. The effects on the cell cycle and apoptosis induction were assessed by flow cytometry. Error bars indicate SEM.

papillomavirus-induced cervical cancer [38], and in myeloma [40]. Also consistent with this model are data suggesting that the NOTCH1 and lactation-dependent mammary tumors regress initially at weaning due to apoptosis, but that this response is substantially reduced as the tumors progress toward invasive adenocarcinomas [41]. An interesting report of Notch signaling conversely inducing apoptosis in neural progenitor cells may be indicative of contrary networks of Notch signaling and/or apoptosis at this distinct developmental stage [42].

Although these reports document antiproliferative and proapoptotic effects of γ-secretase inhibition, these phenomena have seldom been addressed simultaneously prior to this report. These studies confirm induction of cell cycle arrest in γ-secretase-sensitive human T-ALL cell lines [27] and expand previous findings by demonstrating parallel induction of apoptosis in the selected cell lines. The correlation between arrest and apoptosis demonstrated here by using HPB-ALL cells (Figure 3C) is both novel, to our knowledge, and extremely strong and has also been reproduced in another T-ALL cell line (DND-41) after exposure to γ-secretase inhibitors (data not shown). Our work benefits from the ability to assess both read-outs from parallel treatments in the same experiments. Additionally, the dose-response curves employed allow for accurate estimation of the potencies for the incidence of both phenomena. Of note, some published apoptosis studies in Kaposi's sarcoma cells [39] and B cell chronic lymphocytic leukemia [43] may be partially confounded by the use of compounds like Z-Leu-Leu-Nle-CHO that have broad specificity against other proteases such as the proteasome. The work in this report is also strengthened by exploiting the potent and specific inhibitor MRK-003, plus its far less active enantiomer, and extends its use as a sound research tool.

The finding that NICD expression is able to rescue DND-41 from undergoing cell cycle arrest and apoptosis strongly suggests that loss of Notch signaling is the most critical result of γ-secretase inhibition, despite the growing list of proteins that are proposed to be additional substrates of this enzyme complex [33, 34]. Taken together, these biological data complement and fully support the conclusions from the γ-secretase inhibitor studies. To our knowledge, the ability to quantify endogenous NOTCH receptor cleavage in cultured T cell lines also provides a novel means for investigating the effects of γ-secretase inhibition. Previous studies have usually relied on increased levels from overexpressed polypeptides in order to demonstrate genetic or pharmacological efficacy against Notch processing. Although our assay was routinely incubated for 2 days to maximize the magnitude of the effect, NICD levels were also substantially reduced after treatment with γ-secretase inhibitors for 24 hr, a time frame consistent with cleavage effects on other Notch assays [31]. That incubations of 4–7 days are routinely required for antiproliferative effects to become manifest implies that increased time might be required for the action of γ-secretase inhibitors to be transmitted from Notch to cooperating cell cycle interactors.

The growing list of such partners proposed to interact with Notch to stimulate oncogenesis includes regulators such as C-MYC [44], E2A-PBX1 [45], and IKAROS [46], plus many common oncoproteins, including adenovirus E1A [47], HPV E6 and E7 [48, 49], RAS [50], and SV40T [51]; it will be interesting to discover the downstream nuances of how Notch directly impacts proliferation pathways. Additionally, the disconnect between the potencies for inhibition of Notch cleavage and the potency for effects on cell cycle/apoptosis is similarly indicative of additional inputs into the proliferation cycle that would benefit from further study. Mechanistic studies in myeloma systems suggest that Notch signaling influences apoptosis via effects on p53 in some [38, 42], but not all [52], cases, and mediators like p21 [40, 52] and phosphorylated AKT [37, 52] may also be involved in Notch-mediated oncogenesis. It is highly likely, however, that these associations, like most Notch pathway effects, will prove to be subject to complex temporal and spatial regulation dependent on the cellular context in question.

In conclusion, we demonstrate that a potent, specific γ-secretase inhibitor is able to cause both cell cycle arrest and, in parallel, induce apoptosis through a Notch-dependent mechanism in cells originated from T-ALL patients. These processes correlate extremely well, suggesting that γ-secretase inhibitor-induced apoptosis might contribute to the reduction of Notch-related tumor burden in vivo. Given that Notch signaling has been linked to cancers of the breast [19], colon [53], lung [54], pancreas [55], and prostate [56], as well as to gliomas [57], medulloblastomas [20], and mesotheliomas [51], it will be interesting to ascertain whether this general model will have similar therapeutic benefits for other tumor types.

## SIGNIFICANCE

**Growing evidence supports the use of inhibitors of Notch signaling for therapeutic intervention against a number of cancers linked with increased Notch activity. This work strengthens the rationale for therapeutic intervention by further characterizing the antiproliferative mechanisms using newly disclosed, potent inhibitors as chemical tools in immortalized T-ALL lines. Clear cessation of cell growth and blockage of cell cycle progression (at $G_0/G_1$) are reproducibly observed in a time-, dose-, and Notch signaling-dependent manner. This finding highlights a strong connection between cell cycle arrest and induction of apoptosis when both phenomena are measured in parallel after inhibition with a γ-secretase inhibitor. The excellent correlation between these processes supports the possibility that inhibition of γ-secretase might contribute substantially to the reduction of tumor burden, as well to arrested proliferation, in the clinic. The loss of endogenous NOTCH cleavage product in T cells with increasing doses of γ-secretase inhibitor and the lack of inhibitor responsiveness observed with exogenous overexpression of NICD also support the Notch-dependent phenotype of these**

**compounds. Future work is needed to confirm whether apoptosis correlates with cell cycle arrest in other cancers associated with increased Notch signaling.**

## EXPERIMENTAL PROCEDURES

### Compounds

MRK-003 and MRK-006, described previously [28], were synthesized according to standard medicinal chemistry procedures. Stocks at 10 mM in DMSO were used for the dilutions described in this study. Both positive control treatments, paclitaxel and camptothecin, were sourced from Sigma Aldrich Fluka.

### Proliferation Assays

HPB-ALL, DND-41, and TALL-1 cell lines were seeded to 96-well plates ($1.0 \times 10^4$ cells in 90 μl/well) in media specified by the cell line supplier (DSMZ, German National Resource Centre for Biological Material) that contained 10% FBS. After overnight incubation of 90 μl at 37°C in 5% $CO_2$, 10 μl media containing 10× γ-secretase inhibitor stock was added, yielding a final concentration of 0.1% DMSO, and cells were resuspended by gentle pipetting. Media containing inhibitor (75 μl) were replaced after a brief centrifugation every 2 days, and the cells were completely resuspended. Cell viability was measured after 8 days of treatment by using ATPlite (PerkinElmer, Wellesley, MA) according to the manufacturer's instructions.

### Quantification of Cell Cycle Arrest by FACS

HPB-ALL cells were seeded at a density of $0.6 \times 10^6$ in 1 ml in 24-well plates with RPMI media containing 10% FBS and were cultured for 7 days with γ-secretase inhibitor (10 μM and lower). Compound levels were replenished, and the cells were split (usually 2-fold), so as to remain in the supplier's recommended linear growth range, on days 2 and 4. At the end of the incubation, the cells were pelleted by brief centrifugation, resuspended in PBS, and fixed in 70% ethanol on ice for up to 2 hr. The fixed cells were then stained with 20 μg/ml propidium iodide in a PBS buffer that also contained 0.1% Triton X-100 and 0.2 mg/ml RNase. Labeled cell cycle populations were quantified by using a Fluorescence Activated Cell Sorting (FACS) Canto instrument from Beckton Dickenson (Franklin Lakes, NJ). Wells previously incubated with 4–6 μM paclitaxel (which caused $G_2$/M block due to stabilization of microtubules) for 24 hr were included as positive controls.

### Quantification of Apoptosis by FACS

Cells were incubated with inhibitors and were split exactly as described in the preceding section. At the end of the incubation, the cells were collected, and any increase in apoptosis was assessed by using the Annexin V staining kit from BD Biosciences (#556547) according to manufacturer's instructions. Phosphatidylserine, which binds Annexin V, is usually located only on the inner leaflet of the plasma membrane in cells. Loss of this asymmetry and increased staining for Annexin V on the outer membrane are early indicators of apoptosis. Briefly, cells are labeled with FITC-labeled Annexin V, and changes in cell populations stained by this antibody are recorded by FACS. Lack of accessible nucleic acid staining by propidium iodide under the kit buffer conditions is used in parallel to rule out any whole-scale loss of membrane integrity reported in necrosis. Wells incubated with 6 μM camptothecin for 24 hr were included as positive controls for induction of apoptosis.

### Western Blot Analysis of Notch Receptor Processing/NICD Generation

HPB-ALL cells were cultured at $4 \times 10^6$ cells in 3 ml media in 3 cm diameter dishes with the specified γ-secretase inhibitors from DMSO stocks (at 1000× concentrations) and were incubated for 48 hr at 37°C in 5% $CO_2$. The cells were harvested, washed with PBS, and lysed in 50 μl lysis buffer (50 mM Tris [pH 7.4] containing 150 mM sodium chloride, 1% Triton X-100, 0.5% NP-40, and 0.2% SDS). After gentle agitation for 25 min at 4°C, lysates were centrifuged for 10 min at ~14,000 × g, and the protein concentration of the supernatant was determined by using BCA reagent (Pierce, Rockford, IL) with bovine serum albumin as a standard. Equal amounts of protein (30 μg) were loaded and separated on 10%–20% Tricine gels (Novex) prior to transfer onto nitrocellulose membranes. The membranes were blocked for 2 hr in phosphate-buffered saline (PBS) containing 10% skim milk powder (Merck, Darmstadt, Germany). The blots were then probed with anti-cleaved NOTCH1 Val-1744 antibody (Cat #2421, Cell Signaling Technologies, Danvers, MA) for 2 hr at 1:400 in PBS, followed by PBS washes (five washes for 5 min/wash) and incubation with the secondary antibody, HRP-conjugated donkey anti-rabbit (GE Healthcare, Bucks, UK), for 1 hr at room temperature at 1:5,000 in PBS. Proteins from washed membranes were visualized by using ECL reagent from Amersham. Parallel detection with β-tubulin antibody (Sigma, #T5293) was used to provide a loading control.

### Virus Production and T-ALL Cell Line Infection

HEK293T cells were transfected with either the empty vector (MigR1) or with a vector encoding the intracellular form of NOTCH1 (MigR1 NICD) along with pKatAmpho and pCMV-VSV-G by using Lipofectamine PLUS (Invitrogen). Viral supernatants were removed 60 hr later, were filtered, and were used to infect DND-41 cells in the presence of polybrene. Infected cells were collected by flow sorting of GFP+ cells.

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