'From the cradle to the grave': novel therapeutic approaches to attack the microenvironment in chronic lymphocytic leukemia

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γ-secretase inhibitor (GSI)-1 induces apoptosis in CLL cells via proteasome inhibition and Noxa upregulation

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ABSTRACT

Deregulation of the Notch pathway has been suggested to contribute to the pathogenesis of B cell chronic lymphocytic leukemia (CLL). γ-Secretase inhibitors (GSIs) can block intracellular processing of all four different Notch receptors. We found that GSI-1 (z-Leu-Leu-Nle-CHO) was a potent inducer of apoptosis in CLL, but this appeared not to be due to inhibition of Notch signaling. Instead, efficient blocking of proteasomal activity by GSI-1 was observed, equivalent to established proteasome inhibitors bortezomib and MG-132. In contrast with GSI-1, another γ-secretase inhibitor GSI-9/DAPT neither affected proteasome activity nor induced apoptosis in CLL. GSI-1-induced apoptosis was associated with a transcription-independent accumulation of the BH3-only protein Noxa. The pivotal role of Noxa in GSI-1 mediated apoptosis was demonstrated via RNAi in model systems. Our data offer an explanation for certain conflicting notions on Notch signaling in B cell malignancies, and suggest that GSI-1 or related compounds may hold promise for therapeutic application in CLL.
INTRODUCTION

B cell chronic lymphocytic leukemia (CLL) is characterized by the relentless accumulation of monoclonal B cells that coexpress CD5, CD19 and CD23. The malignant B cells have a low proliferative activity, but prolonged cell survival. Although over the last few years new (immuno)chemotherapy regimens have resulted in strongly improved quality of remissions and progression free survival, at present there still is no curative treatment⁴.

A prominent feature of CLL cells is the overexpression of the transmembrane glycoprotein CD23². It was reported that Notch signaling is involved in the up-regulation of the CD23a isoform in CLL cells, and that this may be linked with the aberrant apoptosis regulation in CLL³;⁴. The Notch gene family consists of four evolutionarily conserved transmembrane receptors that play a fundamental role in cell fate decisions including cell proliferation, differentiation and apoptosis. Notch signaling is initiated by receptor-ligand interaction resulting in two successive proteolytic cleavages of the Notch receptor by TACE (TNF-α-converting enzyme) and the γ-secretase/presenilin complex, which liberates the cytoplasmic domain of the Notch receptor (Notch intracellular domain; NIC). The NIC enters the nucleus leading to transcriptional activation of downstream target genes⁵. Recent reports suggest that deregulated Notch signaling is associated with various malignancies, in which Notch may function either as an oncogene or as a tumor suppressor⁶. Variable mRNA expression levels of Notch1 and Notch2 (receptors); Delta-like 1 (ligand); Deltex (a regulator molecule) and Hairy/Enhancer of Split-1 (Hes-1, the most well-known target gene of Notch⁵) were found in CLL⁷. In general, studies into the role of Notch signaling in B cell malignancies have led to disparate reports, with suggestions that Notch activity induces apoptosis⁸, while others have indicated that in fact inhibition of Notch signaling induces apoptosis⁹.

Various studies implicate the ubiquitin-proteasome pathway in the control of Notch activity⁴;¹⁰;¹¹, and proteasome inhibitors such as bortezomib induce apoptosis. Following the successful application of proteasome inhibitors in multiple myeloma (MM)¹²;¹³, this topic has attracted broad interest as novel treatment strategy for cancer. Exposure to proteasome inhibitors results among others in induction of the pro-apoptotic BH3-only protein Noxa, which was preceded in melanoma and myeloma cells by enhanced transcription of Noxa mRNA¹⁴. We recently showed that Bortezomib-mediated apoptosis in CLL also involves Noxa protein accumulation¹⁵.

In the present study we investigated the possible role of Notch signaling in CLL by examining the effect of γ-secretase inhibitors. These chemical inhibitors can block processing of all Notch receptors¹⁶;¹⁷. Unexpectedly, we observed that in CLL GSI-1
is a potent inducer of apoptosis without discernable involvement of Notch signaling. Instead, the compound efficiently inhibits proteasomal activity, leading to accumulation of the pro-apoptotic BH3-only protein Noxa, which has a pivotal role in GSI-1-induced cell death.

MATERIALS & METHODS

Isolation of leukemic, normal B and normal T lymphocytes
Peripheral blood samples were obtained from CLL patients from the outpatient clinic of the department of Hematology of the Academic Medical Center, Amsterdam; the department of Internal Medicine, Meander Medical Center, Amersfoort and the department of Internal Medicine, The Netherlands Cancer Institute, Amsterdam. Informed consent was obtained according to the guidelines of the local Ethical Review Board. Clinical characteristics of patients are presented in Table 1. This study was conducted in accordance with the ethical standards in our institutional medical committee on human experimentation, as well as in agreement with the Helsinki Declaration of 1975, revised in 1983. Peripheral blood mononuclear cells (PBMC) from CLL patients and healthy donors, obtained via density-gradient centrifugation were frozen in 15% fetal calf serum (FCS) containing 10% dimethyl sulphoxide (DMSO; Sigma Chemical Co., St Louis, MO, USA) and stored in liquid nitrogen before use or directly used for experiments. Expression of CD3, CD5, CD19 (all antibodies from Becton Dickinson (BD) Biosciences, San Jose, CA, USA) and CD23 (clone MHM6 from DAKO, Glostrup, Denmark) on leukemic cells were assessed by flow cytometry (FACScalibur, BD Biosciences) and CellQuest software (BD Biosciences)15.

Cell lines
Cell lines were cultured in Iscove’s modified Dulbecco’s medium (IMDM; Gibco Life Technologies, Paisley, Scotland), supplemented with 10% (v/v) heat-inactivated fetal FCS (ICN Biomedicals GmbH, Meckenheim, Germany), 100 U/ml penicillin, 100 μg/ml streptomycin and 5 mM L-glutamine (Gibco). The RPMI myeloma cell line was kindly provided by Dr. Spaargaren (Department of Pathology, AMC, Amsterdam, The Netherlands). The CLL cell line Mec-1 was a kind gift of Dr. Caligaris-Cappio (Milano, Italy).
Mock and Noxa shRNA sequence (N7, N8)18 were retrovirally transduced into Ramos FSA (with enhanced sensitivity to CD95)19, Mec-1 and Jurkat (J16) cell lines. To improve knockdown of Noxa, a limiting dilution of the Ramos FSA N8 cell population was performed according to standard procedures. Resulting clones were selected
for increased resistance to bortezomib and further characterized by Western blot to assess reduced Noxa expression. Clone N8G10 was used for experiments in figure 7.

Reagents and antibodies
Roscovitine, fludarabine and propidium iodide (PI) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). The pan-caspase inhibitor zVAD-fmk was obtained from Alexis Biochemicals (Läufelringen, Switzerland). APC-labeled Annexin V was from IQ Products (Groningen, The Netherlands) and MitoTracker Orange was from Molecular Probes (Leiden, The Netherlands). Proteasome inhibitors MG-132 and bortezomib, were obtained from Calbiochem, (Amsterdam, The Netherlands) and Janssen-Cilag, (Tilburg, The Netherlands), respectively. γ-secretase inhibitors, GSI-120 (Z-LLNle-CHO – Cat.nr. 565750) and GSI-921 (DAPT (Difluorophenacetyl-L-alanyl)-S-phenylglycine t-Butyl Ester) - Cat.nr. 565770) were purchased from Calbiochem. Anti-human Fas10 (agonistic antibody to the CD95 receptor) was a kind gift from Prof. Dr. L. Aarden (Sanquin, Amsterdam, The Netherlands). Monoclonal antibody to CD123 (IL-3Rα) conjugated to PE was purchased from Becton Dickinson. Anti-BDCA2-APC was obtained from Miltenyi Biotec (Bergisch Gladbach, Germany).

Analysis of apoptosis & Western Blot
For the analysis of apoptosis of CLL cells (>90% CD19+CD5+ PBMC suspensions) and PBMCs from healthy donors, 5×10^6 cells/ml were incubated for indicated time points with GSIs or other drugs. Apoptosis of CD19+ CLL and normal B cells and CD3+ T cells was measured by Annexin-V or MitoTracker staining as described before.

Western blotting was performed as described previously. Blots were probed with monoclonal anti-Noxa from Imgenex (San Diego, CA, USA), monoclonal anti-Bim Chemicon, Temecula, CA, USA), monoclonal anti-Mcl-1 (BD Biosciences) and antiserum to β-actin from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Anti-ubiquitin was a kind gift from Prof. Rapoport (Department of Cell Biology, Harvard Medical School, Boston, MA, USA). p53 functionality of CLL samples was screened by the effect of radiation on the expression of p53 and p21 by Western Blot analysis.
Notch-dependent differentiation assay
Isolation of CD34⁺ cells from postnatal thymus tissue was described previously by Dontje et al.⁶. The development of pDCs was assessed by coculturing $5 \times 10^4$ CD34⁺CD1a⁻ progenitor cells with $5 \times 10^4$ OP9 cells in MEMα (Invitrogen Life Technologies, Carlsbad, CA, USA) with 20% FCS (Hyclone, Logan, UT, USA), 5 ng/mL IL-7 and 5 ng/mL Flt3L. Differentiation assays for pDCs were analyzed after 1 week of coculture. Flow cytometric analyses were performed on an LSRII FACS analyzer (Becton Dickinson).

Reverse transcription (RT) PCR
Total RNA was isolated using the Nucleospin RNA isolation kit (Macherey-nagel, Düren, Germany). For RT-PCR (Reverse Transcriptase Polymerase Chain Reaction), total RNA was extracted as described above, and cDNA was synthesized by means of oligodeoxythymidine (oligo(dT)) and Superscript II RNase H reverse transcriptase (Invitrogen Life Technologies). Noxa, Notch1 and Notch2 transcripts were amplified by PCR as described before⁶;¹⁸. Products were electrophoresed on 1% agarose gel. PCR for Hes-1 was performed on an iCycler PCR (Bio-Rad, Hercules, CA) as described before⁶.

Proteasome activity assay
Cytoplasmic extracts (Assay buffer: 250 mM HEPES (pH7.5), 5 mM EDTA, 0.5% NP-40 and 0.01% SDS) from freshly isolated PBMCs from CLL patients were used to measure proteasome activity using a 20S proteasome activity assay kit (Chemicon, part of Millipore; Billerica, USA) following the manufacturer’s instructions. The assay is based on detection of the fluorophore 7-amino-4-methylcoumarin (AMC) after cleavage from the labeled substrate LVVY-AMC. The free AMC fluorescence was quantified using a 380/460-nm filter set in a VICTOR² D fluorometer (Wallac-PerkinElmer) PerkinElmer, Massachusetts, USA). Proteasomal activity was calculated from the changes in fluorescence over time and expressed per μg of protein in the extract.
RESULTS

GSI-1 is a potent, p53-independent inducer of apoptosis of primary CLL cells

Notch expression has been characterized previously in CLL\textsuperscript{3,7}, and we could confirm expression of Notch1 and Notch2 in CLL, while Notch3 and Notch4 were not present in the samples tested (data not shown). γ-Secretase inhibitors (GSIs) are pharmacological agents able to block Notch signaling\textsuperscript{16,17}. GSI-1 (0.25 – 50 μM) induced a significant dose-dependent increase in apoptosis of CLL cells after 24 hours (Fig. 1A). Similar experiments were performed with GSI-9/DAPT, another class of small molecule inhibitors of γ-secretase activity. DAPT, however, had no effect on apoptosis in CLL (1.0 – 50 μM) (Fig. 1A). To determine the effect on normal B and T lymphocytes, PBMCs from 3 healthy individuals were treated for 24 hrs with GSI-1 at concentrations ranging from 0.5 to 50 μM. The cytotoxicity of GSI-1 towards normal CD19+ B cells was comparable to that observed for CLL cells, while normal CD3+ T cells were less sensitive to GSI-1 (Fig. 1B), and showed a maximum of approximately 50% apoptotic cells. Next, CLL samples of patients lacking functional p53 were analyzed following incubation with GSI-1, in comparison with induction of apoptosis by γ-irradiation (γ-IR). Both p53 deficient (n=2) CLL samples showed prominent cell death following treatment with 5 μM GSI-1, comparable to WT samples (see also Fig. 1A), whereas they resisted radiation-induced apoptosis (Fig. 2). Taken together, these results indicate that Notch1 and Notch2 are expressed in primary CLL cells and treatment with GSI-1 but not DAPT results in rapid, p53-independent cell death.

Figure 1: Apoptosis of CLL cells, normal B and T lymphocytes upon GSI-1 treatment.
\textbf{(A)} Apoptosis response as analyzed by MitoTracker staining of CLL cells upon 24 hours incubation with GSI-1 (▲; n=13; 0.25 - 50 μM) or DAPT (Δ; n=3; 1 - 50 μM). Data represent mean ± SD.
\textbf{(B)} Apoptosis response as analyzed by MitoTracker staining of PBMCs from healthy donors (HD) (n=3). CD19+ normal B cells (●) and CD3+ normal T cells (○) were treated with 1 – 50 μM GSI-1. Data represent mean ± SD from 3 independent experiments.
Notch signaling does not contribute to CLL cell survival in vitro, and GSI-1 cannot block Notch signaling

To test whether triggering via Notch influenced in vitro survival of CLL cells, freshly isolated CLL cells were evaluated in two co-culture systems; murine fibroblast L cells or stromal OP-9 cells transfected with the Notch ligands Jag1 or DL1. CLL survival was evaluated every day up to day 7. No significant or reproducible difference in survival compared to co-culture with untransfected control cells or in medium could be observed (11 CLL samples tested at various cell densities, data not shown).

To address whether Notch signaling can be specifically blocked by GSI-1, we applied an established cell culture system where lineage decision between T cells and CD123^+BDCA2^+ plasmacytoid dendritic cells (pDCs) depends on Notch signals. The OP-9 cell line expressing human DL1 inhibits the development of pDCs from CD34^+CD1a^- thymic progenitor cells, but as mentioned above it has no effect on CLL survival. Addition of DAPT could overcome the inhibition of pDC development, whereas GSI-1 could not (Fig. 3A). In fact, as can be seen from the FACS plots, GSI-1 caused massive apoptosis at high concentration (5 μM). The effect of GSI-1 and DAPT on the expression of the Notch specific target gene Hes-1 was evaluated by quantitative RT-PCR (Fig. 3B). The mRNA levels were normalized to expression in untreated CLL cells (relative value of 1) using β-actin as internal control. Expression of Hes-1 shows large variation among untreated CLL cases and the median expression of Hes-1 was lower than in normal B cells (0.1-17.5 % of the average of normal B cells). However, incubation of CLL cells (n=2) with DAPT (1 or 10 μM) blocked the basal Hes-1 expression, whereas GSI-1 (1 or 10 μM) showed no clear inhibitory effect. Thus, triggering of Notch receptors on CLL did not affect their survival and in contrast to DAPT, GSI-1 was unable to prevent Notch signaling in an established system of lineage decision.

Figure 2: Apoptotic response to GSI-1 does not require functional p53.

Apoptosis response of 3 CLL patients: CLL17 (black bar; p53 wt); CLL27 and CLL28 (gray bar and white bar; p53 dysfunctional), to GSI-1 (5 μM) and γ-IR (5Gy) assessed by MitoTracker staining at 24 hours. (med = medium)
GSI-1-induced apoptosis is accompanied by Noxa protein accumulation

It was reported that in melanoma cells GSI-1 induced pro-apoptotic Bim and Noxa. Similarly, in CLL cells addition of GSI-1 resulted in a rapid and massive increase in Noxa protein levels, surpassing the Noxa-inducing capacity of the proteasome inhibitor bortezomib (Fig. 4A, upper panel). We recently showed that in CLL, Mcl-1 interacts with Noxa and Bim, so we also evaluated protein expression of Mcl-1 and Bim. No change in Bim EL and Bim L protein levels occurred after 4 hours GSI-1 or bortezomib exposure. After 24 hours, Bim protein levels declined (Fig. 4A, second panel), which probably reflects the transition of Bim to the insoluble fraction when cells undergo apoptosis. Mcl-1 levels increased after 4 hr treatment with 10 μM GSI-1. The decrease in Mcl-1 protein in GSI-1 and bortezomib treated cells at 24 hours (Fig. 4A, third panel) is most probably the result of caspase-mediated degradation, as it could be inhibited by zVAD (data not shown).

To investigate whether Noxa upregulation was through increased transcription, we performed RT-PCR of CLL cells incubated with GSI-1 or bortezomib. No induction of Noxa mRNA was observed after 4 hours GSI-1 and bortezomib (Fig. 4B).
After 24 hours of treatment with these agents, mRNA could hardly be extracted because of the high proportion of dead cells (data not shown). In addition, Figure 4 B illustrates that in the myeloma cell line RPMI 8226, bortezomib does however induce upregulation of Noxa mRNA at intermediate concentration, in agreement with a previous report30. In conclusion, addition of GSI-1 to CLL cells causes a rapid accumulation of Noxa protein levels, independent of transcriptional regulation.

**Figure 4: Effect of GSI-1 and bortezomib on Noxa mRNA and protein levels.**

(A) CLL cells were treated for 4 and 24 hrs with GSI-1 (1 or 10 μM) or bortezomib (B) (10 or 30 nM). Cell lysates were probed for expression of Noxa, Bim, Mcl1 and β-actin protein by Western Blot. Apoptosis of cells was determinated by Annexin-VAPC/PI staining and is indicated above the lanes. Results are representative for 2 separate experiments.

(B) CLL cells or the myeloma cell line RPMI 8226 were incubated for 4 hours with GSI-1 or bortezomib. RNA was isolated and RT-PCR for Noxa and GAPDH was performed. Results are representative for 2 separate experiments.

**GSI-1 inhibits proteasome activity and induces accumulation of polyubiquitinated proteins**

Given the aforementioned results, demonstrating Noxa protein accumulation upon treatment with GSI-1 and bortezomib, we determined the effect of GSI-1 on the total level of ubiquitinated proteins in HeLa cells and the proteasomal activity in primary CLL samples. First, HeLa cells were incubated for 16 hours with GSI-1 or acknowledged proteasome inhibitors bortezomib and MG-132. Figure 5A (upper panel) illustrates that addition of GSI-1, bortezomib or MG-132 led to accumulation of polyubiquitinated proteins. Concomitant with increased levels of ubiquitinated proteins, Noxa protein levels accumulated over time (Fig. 5A; middle panel). Second, incubation of lysates from freshly isolated CLL cells with GSI-1, bortezomib or MG-132 resulted in clear inhibition of proteasome activity (Fig. 5B). In contrast, roscovitine and fludarabine, both chemotherapeutic agents capable of inducing apoptosis in CLL cells, or the gamma secretase inhibitor DAPT had no measurable effect on proteasomal activity (Fig. 5B).
In addition, proteasomal activity was measured in lysates obtained from CLL cells after 4 and 24 hours incubation with various drugs. Again, GSI-1, bortezomib and MG-132 showed blocking of the proteasome, whereas roscovitine did not (Fig. 5C). Note that upon 24 hours after triggering with roscovitine proteasomal activity was also undetectable, probably as a result of massive apoptosis, and subsequent block in proteasomal activity. Apoptosis responses under these various conditions are represented in figure 5D. In conclusion, GSI-1 blocked proteasomal activity, leading to accumulation of polyubiquitinated proteins, similar to the effects of well known proteasome inhibitors such as MG-132 and bortezomib.

**Figure 5: Effect of GSI-1 on ubiquitin / proteasome system.**

(A) Accumulation of polyubiquitinated proteins after MG-132, bortezomib or GSI-1 treatment. HeLa cells were treated with 10 μM MG-132, 10 nM bortezomib or 5 μM GSI-1 for 16 hours. Lysates were probed for expression of ubiquitinated proteins, Noxa and β-actin by Western blot.

(B) The enzymatic activity of the 20S proteasome was measured after adding bortezomib (20 nM), MG-132 (0.5 μM), GSI-1 (5 μM), DAPT (5 μM), roscovitine (25 μM) or fludarabine (100 μM) to cytoplasmic extracts from freshly isolated cells from 3 CLL patients. After 15 minutes incubation, the fluorogenic proteasome substrate LVVY-AMC was added. Results are expressed as change in AMC fluorescence per minute per μg protein.

(C) CLL cells were treated for 4 (black bar) and 24 (white bar) hrs with GSI-1 (1 or 5 μM), bortezomib (20 nM), MG-132 (0.5 μM) and roscovitine (25 μM). 20 S proteasomal activity was measured as described above (4B). Data represent mean ± SD from four different CLL patients.

(D) Apoptosis response of CLL cells from the experiments described in C were measured by MitoTracker staining after 4 (black bar) and 24 (white bar) hours.
Surface expression of CD23 is reduced upon proteasomal inhibition

Exposure of CLL cells to the proteasome inhibitors bortezomib and MG-132 induced apoptosis and decreased expression of CD23, which was proposed to be a consequence of Notch inhibition. To test the alternative possibility that CD23 down-regulation is a direct consequence of proteasomal inhibition, we incubated CLL cells (n=3) with GSI-1, proteasome inhibitors bortezomib and MG-132, or the Notch inhibitor DAPT. As can be seen in figure 6, GSI-1 led to a reduction of CD23 surface expression similar to that seen with bortezomib or MG-132, while CD23 expression remained essentially unchanged during 24 hours incubation with DAPT.

![Figure 6: CD23 expression on CLL cells upon incubation with GSI-1.](image)

ClL cells were cultured for 24 hours with 30 nM bortezomib (n=2), 0.5 μM MG-132 (n=2), 5 μM GSI-1 (n=4) and 5 μM DAPT (n=3). The percentage CD23-positive cells was measured by FACS analysis: within the viable population, CD5 and CD19 double positive cells were gated. Data represent mean from ± SD.

Noxa knock-down results in decreased sensitivity to GSI-1

The previous experiments showed that Noxa protein accumulates upon GSI-1 treatment, but do not establish whether this is required to induce apoptosis. To investigate a direct role for Noxa in GSI-1 mediated apoptosis, we studied various cell lines transduced with retroviral vectors encoding two different Noxa shRNA sequences (N7 and N8). Figure 7A illustrates significant but not complete knock-down of Noxa protein in the different cell lines. Incubation with GSI-1 (+/- pre-treatment with zVAD-fmk) clearly raised Noxa protein levels, and this in fact still occurred in the knock-down lines. Ramos FSA cells expressing two types of shRNA, exhibited a decreased sensitivity to GSI-1-induced apoptosis compared to the mock transduced cells, similar to results obtained with bortezomib (Fig. 7B). The effects of Noxa shRNA on GSI-1-mediated cell death were reproducible in other cell lines, Mec-1 and Jurkat (Fig. 7C&D). Consistent with previous results, no effect of Noxa protein reduction was observed on apoptosis triggered via other pathways, such as fludarabine treatment or triggering of the CD95 receptor (Fig. 7E). In all cases resistance to GSI-1-mediated
apoptosis was not complete, most probably due to the fact that remaining levels of Noxa protein in the knock-down lines were still augmented by GSI-1 (Fig. 7A). In summary, these data demonstrate that decreased expression of Noxa has a direct and specific effect on the susceptibility to apoptosis induced by GSI-1.

Figure 7: Knockdown of Noxa with RNAi specially prevents apoptosis induction by GSI-1.

(A) Ramos FSA-M (=Mock, lane 1-4), Ramos FSA-N7 (lane 5-8) and Ramos FSA-N8G10 cells (lane 9-12) were treated with the pan-caspase inhibitor zVAD-fmk (100 μM) and GSI-1 (1μM) as indicated. Noxa protein levels were monitored by Western blotting. Noxa knockdown in Mec-1 cells and Jurkat T (J16) cells has been described previously 18;23. Equal protein loading is shown by reprobing for β-actin.

(B) Mock (●), N7 (▼) and N8,G10 (◇) Ramos FSA cells were incubated for 24 hours in the presence of indicated concentrations of GSI-1 and bortezomib. Viability was assessed by MitoTracker staining and FACS analysis. Data represent mean ± SD from 3 independent experiments, and one experiment with N7 and bortezomib.

(C) Mock (●) and N8 (◇) transduced Mec-1 cells were incubated and analyzed as in panel B. Data represent mean ± SD from 6 independent experiments for GSI-1 and 1 experiment for bortezomib treatment.

(D) Mock (●) and N8 (◇) transduced J16 cells were incubated for 24 hours in the presence of indicated concentrations of GSI-1 and analyzed as in panel B. Data represent mean ± SD from 3 independent experiments.

(E) Mock (●) and N8,G10 (◇) transduced Ramos FSA cells were incubated for 24 hours in the presence of 100 μM fludarabine (F) and 0.25 μM α-CD95, and analyzed as in panel B. Medium = M. Data represent mean ± SD from 3 independent experiments.
DISCUSSION

The major findings of our study are that GSI-1 is a potent inducer of apoptosis in CLL and that this response does not involve Notch signaling. Rather, efficient inhibition of proteasomal activity and subsequent Noxa accumulation seems to play a key role. Dysregulation of the Notch pathway has been suggested in CLL, mainly because of the observation that overexpression of CD23 in CLL cells is regulated by Notch2. In agreement with other reports we found that Notch1 and Notch2 receptors are present in CLL cells. To explore a possible functional role of the Notch pathway in CLL we performed two kinds of experiments. First we co-cultured primary CLL cells with either L cells or OP9 cells expressing the Notch ligands DL1 and Jagged1. This did not affect cell survival in CLL cells. To exclude that this observation is due to already optimal autocrine or paracrine Notch stimulation in CLL we performed a set of experiments aimed at blocking Notch signaling. To this end we used the inhibitors DAPT and GSI-1, both assumed to specifically block γ-secretases. Here we encountered a surprising dichotomy: whereas DAPT was unable to induce apoptosis in CLL, it did inhibit pDC development in the presence of the Notch ligand DL1, as described previously. In contrast, this was not the case for GSI-1, which was capable of potent induction of apoptosis in CLL. A similar dichotomy has been described with respect to Notch2 dependent CD23 upregulation. Treatment of CLL cells with proteasome inhibitors led to inhibition of nuclear Notch2 intracellular domain (Notch2IC) activity and downregulation of CD23. However, CD23 down-regulation could not be achieved with DAPT, as we have also observed. With respect to CD23 down-regulation we could confirm that this is the result of proteasome- rather than Notch inhibition (Fig. 6). CD23 is a known transcriptional target of NF-κB, and NFκB-p52 constitutively binds to the CD23 promoter region in murine cells. Thus, a possible explanation for the down-regulation of CD23 by proteasome inhibitors could be their well-known negative effect on NF-κB signaling. However, in experiments using specific inhibitors of NF-κB signaling we observed no influence on the expression of CD23 (data not shown).

One of the best characterized Notch targets is the HES family of transcription factors. The majority of the CLL patients display substantially lower HES-1 transcripts compared to normal B cells. In agreement, we could detect low HES-1 in two CLL samples, which could further be decreased by DAPT but not significantly by GSI-1 (Fig. 3B). Our data agree with previous studies showing Noxa upregulation and apoptosis upon GSI in melanoma cell lines, melanoma xenografts and myeloma cells. To our knowledge, no data are available that would directly demonstrate repression of Noxa by HES-1.
In CLL, GSI-1-induced apoptosis was accompanied by rapid and extensive accumulation of Noxa protein, due to blocking of proteasomal function, similar to earlier findings in CLL and other cell types that proteasome inhibitors like bortezomib and MG-132 effectively block the proteasome and upregulate Noxa protein\textsuperscript{15}. Effects of GSI-1 on BH3-only proteins Noxa and Bim in melanoma cells\textsuperscript{14}, were apparently also regulated at transcriptional level\textsuperscript{14,36}. The latter is clearly not the case in CLL, since Noxa mRNA levels do not increase upon treatment with bortezomib or GSI-1. In support of a direct role for Noxa accumulation, Noxa shRNA experiments demonstrated a clear reduction in apoptosis mediated by GSI-1, whereas other apoptosis pathways were unaffected.

The demonstration that GSI-1 acts as a proteasome inhibitor is perhaps not surprising since the structure of GSI-1 (Z-LLNle-CHO) resembles that of other chymotrypsin inhibitors and also that of MG-132 (z-LLL-CHO). Others have also remarked that the aldehyde group on the GSI-1 peptide is probably able to covalently inhibit certain serine protease\textsuperscript{20}. Our data agree with that view and we propose that many, if not all, of the apoptosis-inducing activities ascribed to GSI-1, and related compounds such as GSI-12 (Z-IL-CHO)\textsuperscript{9} are probably due to proteasome rather than Notch inhibition. An intriguing remaining question concerns the mechanism behind the rapid and massive upregulation of Noxa protein by proteasome inhibition. Accumulation of higher molecular weight species of Noxa, indicative of (poly)ubiquitination, were never observed by us. Also, efforts to detect ubiquitination of Noxa in cells transiently transfected with differentially tagged Noxa and ubiquitin, and treated with proteasome inhibitors, were unsuccessful (data not shown). A possible explanation for this might be that Noxa accumulation occurs because its binding partner Mcl-1 is ubiquitinated and degraded\textsuperscript{37,38}, whereby Noxa dissociates and passively accumulates. Support for such a chaperone-like role for Noxa in controlling the degradation of Mcl-1 has indeed been obtained\textsuperscript{39}.

In CLL, new therapies that act independently of p53 are urgently required. We observed that GSI-1 is effective in CLL cells from p53 dysfunctional patients, and a variety of compounds related to GSI are already in the clinic for the treatment of Alzheimer’s disease\textsuperscript{40}. In that setting, side effects are limited\textsuperscript{41,42}. Bortezomib has proven efficacy as single agent in multiple myeloma and some forms of non-Hodgkin’s lymphoma\textsuperscript{43-45}, although a number of toxicities are described\textsuperscript{46,47}. For unknown reasons, clinical trials with bortezomib in patients with fludarabine-refractory CLL showed only limited responses although biological activity was observed\textsuperscript{48}. We propose that our studies provide a basis to continued studies into alternative proteasome inhibitors such as GSI-1 or related compounds as potential treatment for chemoresistant CLL.
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