ON 01910.Na Is Selectively Cytotoxic for Chronic Lymphocytic Leukemia Cells through a Dual Mechanism of Action Involving PI3K/AKT Inhibition and Induction of Oxidative Stress

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Abstract

Purpose: Chronic lymphocytic leukemia (CLL), a malignancy of mature B cells, is incurable with chemotherapy. Signals from the microenvironment support leukemic cell survival and proliferation and may confer chemotherapy resistance. ON 01910.Na (Rigosertib), a multikinase phosphoinositide 3-kinase (PI3K) inhibitor, is entering phase III trials for myelodysplastic syndrome. Our aim was to analyze the efficacy of ON 01910.Na against CLL cells in vitro and investigate the molecular effects of this drug on tumor biology.

Experimental Design: Cytotoxicity of ON 01910.Na against CLL cells from 34 patients was determined in vitro with flow cytometry of cells stained with Annexin V and CD19. Global gene expression profiling on Affymetrix microarrays, flow cytometry, Western blotting, and cocultures with stroma cells were used to delineate ON 01910.Na mechanism of action.

Results: ON 01910.Na induced apoptosis in CLL B cells without significant toxicity against T cells or normal B cells. ON 01910.Na was equally active against leukemic cells associated with a more aggressive disease course [immunoglobulin heavy-chain variable region unmutated, adverse cytogenetics] than against cells without these features. Gene expression profiling revealed two main mechanisms of action: PI3K/AKT inhibition and induction of ROS that resulted in an oxidative stress response through activating protein 1 (AP-1), c-jun-NH2-terminal kinase, and ATF3 culminating in the upregulation of NOXA. ROS scavengers and shRNA mediated knockdown of ATF3- and NOXA-protected cells from drug-induced apoptosis. ON 01910.Na also abrogated the prosurvival effect of follicular dendritic cells on CLL cells and reduced SDF-1–induced migration of leukemic cells.

Conclusions: These data support the clinical development of ON 01910.Na in CLL.

Introduction

Chronic lymphocytic leukemia (CLL), the most common leukemia in the Western world, is characterized by the accumulation of monoclonal CD5+ mature B cells in the peripheral blood (PB), lymph nodes (LN), and bone marrow (BM). The majority of cases is diagnosed in asymptomatic patients with an incidental finding of lymphocytosis or lymphadenopathy. The standard of care for CLL is watchful waiting of asymptomatic patients and chemoinmunotherapy for patients with active disease (1). This clinical approach to the CLL patient is guided by the absence of a curative chemotherapy regimen, the results of clinical trials that have shown no benefit for early chemotherapy in asymptomatic patients, and the relatively long natural history of the disease with a median survival of 11 years (2). CLL is divided into 2 main subgroups based on the presence or absence of acquired somatic mutations in the
immunoglobulin heavy chain variable region (IGHV) expressed by the leukemic B cells. Patients with mutated IGHV have a more indolent disease and longer overall survival than patients whose tumors express an unmutated IGHV gene. High expression of ZAP70 and CD38 are additional markers indicating more rapid disease progression (3). Cytogenetic alterations are also strong predictors of outcome. In particular, deletion of TP53 locus on 17p and deletion of the ATM locus on 11q are associated with more rapidly progressive disease and inferior response to chemotherapy. Increasingly, risk stratified treatment approaches are pursued for patients with these adverse prognostic markers (4).

CLL has historically been considered an accumulative disease of quiescent cells arrested in the G0/G1 phase of the cell cycle and endowed with resistance to apoptosis. This notion has been based primarily on the study of leukemic cells from the PB. However, more recently it has been recognized that a proliferating subpopulation localized in lymphoid organs contributes to disease progression (5–7). Interactions with the tissue microenvironment are crucial for CLL cell proliferation and survival (6, 8). We recently showed activation of the B-cell receptor (BCR) and NFκB pathways in CLL cells isolated from the LN microenvironment. BCR signaling was stronger in the clinically more aggressive CLL subtype expressing unmutated IGHV genes and tumor proliferation in the LN correlated with clinical disease progression (7). Thus, targeting the tumor microenvironment interactions and BCR signaling in particular has become a priority in CLL. Inhibitors of SYK (9), BTK (10), and phosphoinositide 3-kinase (PI3K; refs. 11, 12) have shown promising preclinical and clinical activity in CLL. The PI3K/AKT pathway in particular is a key signaling node for several pathways implicated in CLL pathogenesis and interactions with the microenvironment including the BCR (13, 14), CXCR4 (15), CD40 (16), CD44 (17), and IL4 (13). Moreover it has been shown that the PI3K pathway is active in freshly isolated CLL cells (18). PI3K activates AKT, a serine threonine kinase critically involved in cell growth and survival.

ON 01910.Na, a styryl benzylsulfone is a non-ATP competitive multikinase inhibitor with marked antiapoptotic and anticancer activity. A phase 1 study in patients with solid tumors showed good tolerability of the drug with an objective durable response in a patient with ovarian cancer (19). ON 01910.Na is currently being tested in a randomized phase III trial in patients with refractory myelodysplastic syndrome (MDS; registered as NCT01241500). Initially it was thought that the antiapoptotic activity was due to inhibition of polo-like kinase 1 (20). Subsequent studies did not support a direct effect on polo-like kinases (21) and a recent study correlated hyperphosphorylation of Ran GTPase-activating protein 1 with ON 01910.Na-induced cell-cycle arrest (22). However, whether ON 01910.Na directly or indirectly contributes to mitotic arrest remains elusive. Focusing on mantle cell lymphoma (MCL), Prasad and colleagues showed direct inhibition of PI3K, preferentially of the p110α, p110β isoforms, resulting in decreased phosphorylation of AKT, inhibition of cyclin D1 translation, and induction of apoptosis in MCL cell lines (23). These results prompted us to explore the potential activity of ON 01910 against CLL cells. Here, we show that ON 01910.Na induces apoptosis in CLL cells irrespective of markers predicting resistance to classic chemotherapy while sparing normal lymphocytes. Using gene expression profiling (GEP), we discovered that ON 01910.Na cytotoxicity is mediated by 2 main mechanisms: inhibition of the PI3K/AKT pathway and induction of an oxidative stress response. Moreover, ON 01910.Na remained active against CLL cells cocultured with stroma cells and reduced SDF-1α–induced migration. These studies provide a rationale for the development of ON 01910.Na as a therapeutic agent in CLL.

Materials and Methods

Patients and clinical samples

Blood samples from 34 CLL patients (Table 1) were obtained with written informed consent. PB mononuclear cells (PBMC) were isolated by gradient centrifugation with Lymphocyte Separation Medium (MP Biomedicals) and used fresh or cryopreserved in liquid nitrogen in 10% dimethyl sulfoxide, 90% fetal calf serum. Analysis of IGHV gene status was conducted as described (24). CLL samples were cultured in RPMI 1640 (Gibco-Invitrogen) supplemented with 10% fetal bovine serum (HyClone), 2 mmol/L L-glutamine, and penicillin/streptomycin (subsequently referred to as R10).

### Translational Relevance

Chronic lymphocytic leukemia (CLL) cells depend on signals from the microenvironment for proliferation and survival. Defective DNA damage response and stroma contact can protect CLL cells from chemotherapy-induced cytotoxicity. Therapies efficacious against tumor cells located in a protective microenvironment are needed. ON 01910.Na is a non-ATP competitive multikinase inhibitor in advanced clinical development for myelodysplastic syndrome. Here, we report that ON 01910.Na inhibits the phosphoinositide 3-kinase (PI3K)/AKT pathway, generates ROS, and selectively induces apoptosis in tumor cells, while sparing normal lymphocytes. ON 01910.Na was effective against tumor cells with p53 deletion and cells cocultured with follicular dendritic cells, an in vitro model of the tumor microenvironment. Quenching of ROS production with an antioxidant and knockdown of NOXA protected tumor cells from drug-induced apoptosis. These data support PI3K inhibition concomitant with ROS induction as a therapeutic principle with good activity and tumor selectivity. A clinical trial investigating ON 01910.Na in lymphoid malignancies has been initiated.
Stromal cell coculture
The follicular dendritic cell line (FDC) HK kindly provided by Dr. Yong Sung Choi (25), was cultured in IMDM (Gibco-Invitrogen) supplemented with 20% fetal bovine serum (HyClone), 2 mmol/L glutamine, and penicillin/streptomycin. HK cells were seeded in 12-well plates on day 0. The next day, IMDM was removed and CLL cells (2 × 10^6 cells/mL in R10) were added onto confluent stroma layers and cultured for the times indicated in the presence or absence of ON 01910.Na. CLL cells were recovered by gentle agitation, and the percentage of apoptotic cells was determined by flow cytometry after staining with CD19-FITC (FITC, fluorescein isothiocyanate) and Annexin V–PE.

BCR stimulation by immunoglobulin M cross-linking
CLL cells were reacted at 4°C for 30 minutes with 25 µg/mL Goat F(ab′)2 anti-human immunoglobulin M (IgM)-biotin (Southern Biotech) followed by the addition of 10 µg/mL streptavidin (Jackson ImmunoResearch Laboratories) for 20 minutes. Cells were transferred to 37°C for 30 minutes and stimulation was stopped with cold PBS. Where indicated cells were preincubated with ON 01910.Na for 1 hour.

Migration assays
SDF-1α/CXCL12-induced migration was evaluated in 24-well chemotaxis chambers containing polycarbonate

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*IGHV sequence is considered mutated when its homology to the germ line IGHV sequence is below 98%.

* Determined by interphase fluorescence in situ hybridization.

* Calculated by nonlinear regression with Prism 4 software.
inserts of 5 μmol/L pore size (Corning Life Science). A total of 100 μL cell suspensions at 5 × 10⁶ cells/mL pretreated or not with ON-01910.Na for 2 hours were added to the top chamber and SFD-1t (Peprotech) to the lower chamber. Media without SFD-1t was used as baseline. After 3 hours, cells in both chambers were counted separately in an Attune flow cytometer at high flow rate for 1 minute. The migration index was calculated as the ratio between cells that migrated in response to SFD-1t divided by those that passively migrated without the chemokine. The migration index (MI) of ON-01910.Na-treated cells was normalized to vehicle (dimethyl sulfoxide, DMSO) treated cells.

ATF3 and NOXA silencing by retroviral transfection of short hairpin RNA

The MCL cell line Jeko was transduced with retrovirus to generate shATF3 and shNoxa clones. Parental cells and short hairpin RNAs (shRNA) clones were cultured in RPMI 1640 (non-targeting control), shATF3, and shNOXA constructs were generated inserting the following sequences (shATF3: GGGTGGACTTCCTCCTCCTCA and TTTGGACTTCCTCCTCCTCA; shNOXA: GGGTGGACTTCCTCCTCCTCA and CAAATGTGAAACGTGCACC) into the pRetroSuper plasmid containing also GFP and puromycin-resistant elements (kind gift of Dr. Louis M Staudt, Metabolism Branch, National Cancer Institute, National Institutes of Health, Bethesda, MD). Viral particles were produced in Human Embryonic Kidney 293T cells (HEK-293T), and used to infect target cells by centrifugation (2500 rpm for 90 minutes) in the presence of DOTAP (Roche) followed by puromycin selection (2 μg/mL) for 7 days. The percentage of knockdown was assessed by quantitative RT-PCR using Sybr green-specific oligos designed for ATF3 and NOXA genes, using β2 microglobulin as housekeeping control (QIAGEN).

Treatments and cytofluorimetric assessment of apoptosis

ON 01910.Na (gift from Onconova Therapeutics, Newtown, PA) was dissolved in DMSO and stored at −80°C. To assess the lethal dose (LD₅₀, PBMCs were exposed to increasing concentrations of ON 01910.Na ranging from 0.25 μmol/L to 8 μmol/L for 48 hours. Cytotoxicity against B-CLL cells was evaluated by flow cytometry after staining with Annexin V–PE and CD19–FITC (BD Pharmingen). In CLL PBMCs less than 1% of CD19⁺ cells are typically normal B cells. To evaluate toxicity against T cells, CD3–APC was added. LD₅₀ was calculated by nonlinear regression with Prism 4.0 (GraphPad Software). Changes in the mitochondrial transmembrane potential (ΔΨm) were measured either by MitoTracker Green FM and MitoTracker Red CMX ROS (100 nmol/L each) or by 20 nmol/L 3,3-dihexyloxacarbocyanine iodide (DiOC₆[3]; Invitrogen). The generation of reactive oxygen species (ROS) was determined by staining cells with 2 μmol/L dihydroethidium (DHE; Invitrogen). Cells were incubated with each probe for 30 minutes at 37°C, and 10,000 cells per sample were analyzed in a FACs CANTO flow cytometer with FACs DIVA software (BD Biosciences). DiOC₆ and DHE staining was done simultaneously.

To detect activation of BAX, BAK, and caspase-3, 0.5 × 10⁶ cells were fixed with 4% paraformaldehyde, permeabilized with saponin 0.1% (Sigma), stained for 30 minutes with 1 μg/mL of antibodies against the active form of caspase-3 (BD Pharmingen), BAX (clone 6A7; BD Pharmingen), or BAK (clone Ab-1; Oncogene Research) for 30 minutes at room temperature, followed by goat anti-rabbit FITC or goat anti-mouse FITC (Jackson Immunoresearch) and then analyzed in FACs CANTO flow cytometer.

MTT assay

A total of 5 × 10⁶ cells per well were exposed to serial doubling concentrations of ON 01910.Na for 48 hours in flat bottom 96-well plates. For the last 4 hours, MTT reagent (Chemicon) at 0.5 mg/mL was added and analyzed as described (26).

Gene expression profiling and gene set enrichment analysis

Total RNA (2.5 μg) was subjected to GEP on Human Genome U133 plus 2.0 chips (Affymetrix). Processing, data extraction, and normalization was done as described (27). Significant gene signatures were identified using gene set enrichment analysis (GSEA) version 2.0 (Broad Institute at MIT; http://www.broadinstitute.org/gsea/) using the C3 (motif gene sets) from the Molecular Signature Database v2.5, and experimentally derived custom gene sets (28). An increasing profile analysis with 1,000 permutations of gene sets and a weighted metric was used. Bonferroni correction for multiple testing was applied and only gene sets with a false discovery rate (FDR) of 0.2 or less and a normalized enrichment score of more than 1.4 or less than −1.4 was considered significant. The leading edge genes, that is, the subset of genes that contributed most significantly to the identification of a gene set, were displayed with Cluster (version 2.11) and TreeView (version 1.6) softwares (Eisen Laboratory, Stanford University, CA).

Protein isolation and Western blot

CLL cells were lysed in 1% Triton buffer containing PhosphoSTOP and the protease inhibitors Complete EDTA-free (Roche). Nuclear and cytosol fractions were obtained with a commercial kit (Bovision). Western blot analysis was conducted with the NuPAGE Bis-Tris electrophoresis system on polyvinylidene difluoride (PVDF) membranes (Invitrogen) blocked in 5% nonfat milk or bovine serum albumin (BSA) with 0.05% Tween-20. Membranes were incubated with the primary antibodies (1:500 to 1:2,000 dilutions) in 5% milk or 0.05% Tween-20 overnight at 4°C or 1 hour at room temperature. Membranes were developed with enhanced chemiluminescence substrate (SuperSignal, Thermo Fisher Scientific) and visualized on a LAS4000 Fujifilm device. Protein quantification was done with Image Gauge Fujifilm software (Fujifilm Corporation). The following antibodies were used: pAKT (Ser 473) and pSAPK/c-Jun-NH₂-kinase (JNK; Tyr183/
Thr185) from Cell Signaling Technology; anti-c-jun (H-79), anti-MCL-1 (S19), anti-ATF3 (C-19), and anti-TBP (58C9) from Santa Cruz Biotechnology; anti-NOXA from Calbiochem (San Diego): anti-α-tubulin and anti-β-actin from Sigma.

Statistical analysis
Unpaired and paired t tests were used to assess differences between 2 groups. P < 0.05 was considered significant.

Results
ON 01910.Na activates the mitochondrial cell death pathway and is selectively toxic against CLL cells independent of IGHV mutational status and cytogenetic profile
To assess the cytotoxic effect of ON 01910.Na against CLL cells, we treated PBMCs from CLL patients and healthy donors ex vivo with increasing concentrations of drug. We measured drug-induced apoptosis in B- and T-cell populations separately with Annexin V staining in combination with antibodies against the lineage specific markers CD19 and CD3. ON 01910.Na-induced time- and dose-dependent cytotoxicity against B cells from CLL patients (Fig. 1A). In contrast, there was no cytotoxic effect on T cells from CLL patients or B and T cells from healthy donors at 24 hours. At 48 hours, normal B cells showed somewhat reduced viability albeit only at concentrations higher than those required to kill CLL cells. T cells from both normal donors and CLL patients remained largely unaffected even at concentrations of more than 8-fold the LD50 of CLL cells. Thus, ON 01910.Na is selectively toxic against CLL B cells.

We next determined the activity of ON 01910.Na against a panel of CLL samples from 34 patients stratified for disease markers that are associated with more aggressive disease and inferior survival (Table 1). We determined the percentage of CD19-positive cells staining for Annexin V and calculated the LD50 for each sample at 48 hours. As shown in Fig. 1B, most of the patients had LD50 values between 1 to 2 μmol/L, a concentration that is achieved in vivo (19). CLL cells expressing mutated or unmutated IGHV genes were equally sensitive to ON 01910.Na and most cases with 17p or 11q deletions had LD50 values comparable with samples without these adverse cytogenetic findings (Table 1, Fig. 1B).

To investigate whether ON 01910.Na activates the mitochondrial apoptotic pathway, CLL cells were treated with 2 μmol/L ON 01910.Na for 24 hours. We used the mitochondrion cell viability assay that measures mitochondrial depolarization to quantify drug-induced apoptosis (Fig. 1C). In parallel, we assessed activation of the proapoptotic proteins BAK and BAX with confirmation-specific antibodies. Oligomerization of BAX and BAK enables the formation of the mitochondrial pore leading to mitochondrial depolarization, followed by the release of apoptogenic factors and finally activation of effector caspases (29). Both mitochondrion staining and conformational changes in BAX and BAK indicate that ON 1910.Na activated the mitochondrial apoptotic pathway.

ON 01910.Na inhibits the PI3K/AKT pathway
ON 01910.Na has been shown to inhibit PI3K activity in vitro in cell free assays and in MCL cell lines (23). In contrast to CAL-101 (11), another clinical grade PI3K inhibitor, we noted a pronounced proapoptotic effect of ON 1910.Na against CLL cells already at 24 hours even in the low μmol/L range. Thus, we sought to better characterize the cellular stress response triggered by ON 01910.Na. We first carried out GEP of CLL cells treated with 1 and 2 μmol/L ON 01910.Na for 4 and 10 hours on Affymetrix whole genome arrays. To investigate the functional correlates of drug-induced gene expression changes, we used GSEA (28). This observer-independent statistical method can dissect gene expression data into defined signatures that correlate with cellular functions or the activity of defined transcription factors. Among the genes differentially expressed in ON 01910.Na-treated CLL cells, GSEA identified several significantly enriched gene sets (FDR < 0.2; Supplementary Table S1). ON 01910.Na-treated CLL cells showed gene expression changes significantly related to gene signatures derived from HL60 cells treated with the PI3K inhibitor LY-294002 (Fig. 2A). Thus, our gene expression data indicates inhibition of the PI3K pathway and is in agreement with findings by Prasad and colleagues in MCL cell lines (23). PI3K activation leads to activation of AKT which in turn phosphorylates and thereby inactivates forkhead transcription factors. Conversely, when PI3K is inhibited, these factors can translocate to the nucleus and initiate the transcription of proapoptotic genes (30). As shown in Fig. 2A, target genes of the transcription factors forkhead box O3 (FOXO3) and forkhead box O4 (FOXO4) were upregulated in ON 01910.Na-treated cells. In addition, we also observed a decrease in genes induced by BCR activation, consistent with a role of PI3K in BCR signaling (Supplementary Table S1; Fig. 2A).

To validate the inhibition of PI3K/AKT pathway at protein level, we treated cells from CLL patients with 2 μmol/L. ON 01910.Na for 6 hours and assessed the phosphorylation status of AKT by Western blot. pAKT was significantly reduced in most of the drug-treated samples (Fig 2B and C). Furthermore, expression of the antiapoptotic protein MCL-1, which is in part regulated through increased translation downstream of PI3K/AKT signaling, also decreased in cells treated with ON 01910.Na (Fig. 2B and C). Although about half of the samples showed more than 50% down-regulation of pAKT in response to ON 01910.Na, another half showed only minimal changes. This could be due to heterogeneity in cellular response to drug treatment or due to a relatively low baseline pAKT level in circulating CLL cells that makes it difficult to detect any further decrease. CLL cells in the PB are resting cells and, as we have recently shown, activation through signaling pathways, in particular the BCR occurs primarily in the LN microenvironment (7). Thus, having seen that treatment with ON 01910.Na down-regulates BCR target genes, we next tested whether ON 01910.Na is able to block BCR activation in vitro. Indeed,
ON 01910.Na was able to inhibit phosphorylation of AKT in response to BCR engagement by IgM cross-linking (Fig. 2D).

**ON 01910.Na induces an oxidative stress response through JNK and activating protein 1 leading to the upregulation of the proapoptotic BH3-only protein Noxa**

Although inhibition of the PI3K/AKT pathway may contribute to ON 01910.Na cytotoxic activity, this is unlikely to be the sole mechanism because cytotoxicity did not correlate well with the extent of PI3K/AKT blockade, and the degree of apoptosis observed with ON 01910.Na was higher than what has been reported for other PI3K inhibitors (11, 15). Further indicating a second mechanism of cytotoxic activity, GSEA identified a significant upregulation of genes controlled by the activating protein (AP-1) transcription factor (Supplementary Table S1; Fig. 3A). AP-1 protein dimmers most commonly composed of JUN, FOS, or members of the ATF family, upon activation by JNK kinase, act as transcriptional transducers of stress pathways. The JNK/AP-1 pathway is an integral part of the cellular response...
ON 01910.Na inhibits PI3K/AKT and activates FOXOs transcriptional activity. A, cells from patients CLL10 and CLL12 treated with 1 and 2 μmol/L ON 01910.Na for 4 and 10 hours and untreated cells cultured for the same time periods were analyzed on Affymetrix arrays. The leading edge genes of gene expression signatures that were significantly regulated by ON 01910.Na (FDR < 0.2; Supplementary Table S1) are shown in a heat map representation scaled as indicated. B, the effect of ON 01910.Na (2 μmol/L for 6 hours) on AKT phosphorylation and MCL-1 expression in CLL cells from 4 representative patients is shown by Western blotting. C, pAKT and MCL-1 expression normalized to γ-tubulin in samples treated as in B (n = 14) was quantified by densitometry. The log2 of the ratio between treated and untreated cells is depicted. D, CLL patients were stimulated with IgM for 30 minutes in the presence or absence of 2 μmol/L ON 01910.Na. Protein lysates were analyzed by Western blot for pAKT expression using γ-tubulin as loading control.
In keeping with the gene expression data, we detected a dose- and time-dependent increase in ROS in response to ON 01910.Na that paralleled mitochondrial depolarization (Fig. 3B and C). Thus, ON 01910.Na induces an oxidative stress response that activates the JNK pathway leading to the induction of AP-1 target genes.

To further determine the role of ROS in ON 01910.Na-induced apoptosis, we conducted cytotoxicity assays in presence of the antioxidant N-acetylcysteine (NAC). NAC reduced ROS generation by ON 01910.Na more than 50% (P = 0.002) and protected cells from the cytotoxic effect of the drug; while ON1910.Na on average killed more than 70% of cells at 48 hours, NAC potently antagonized the cytotoxic effect and all samples showed more than 50% viability (P = 0.004, Fig. 3D).

Next, we assessed the role of the JNK pathway in drug-induced apoptosis by Western blotting and shRNA knockdown experiments. ON 01910.Na-induced phosphorylation of JNK within 6 hours with concomitant upregulation of ATF3 and the nuclear accumulation of c-jun (Fig. 4A and B). In keeping with the known link between ROS and JNK activation (31), the ROS scavenger NAC reduced ON1910.Na-induced phosphorylation of JNK (Fig. 4C). We also found upregulation of the proapoptotic BH3-only protein NOXA in response to ON 01910.Na consistent with reports of a role for NOXA in the oxidative stress response (32, 33). Finally, we used shRNAs to knockdown NOXA and ATF3 in Jeko-1 cells (Fig. 4D). As there are no bona fide CLL cell lines, we chose the MCL cell line Jeko because the IC50 of ON1910.Na is comparable between the cell line and primary CLL cells. Efficient reduction of ATF3 and NOXA expression by shRNA was verified by RT-PCR (Supplementary Fig. S1). As shown in Fig. 4D, knockdown of ATF3 or NOXA expression significantly diminished the cytotoxic effect of ON 01910.Na, further supporting the important contribution of the oxidative stress response and the JNK pathway to ON 01910.Na’ mechanism of action.
ON 01910.Na overcomes stroma-mediated prosurvival effects on CLL cells

It is well documented that the host microenvironment contributes to CLL cell proliferation, survival, and drug resistance (7, 8). Thus, a critical need in CLL is the development of therapeutic approaches that remain highly active against tumor cells in the microenvironment. Given the prominent role of the LN microenvironment for CLL cells, we assessed the efficacy of ON 01910.Na in an in vitro model mimicking the LN environment by culturing CLL cells on the FDC HK (25). As expected, HK cells significantly increased the viability of cocultured CLL cells compared with media alone (Fig. 5A) in agreement with previous work from other groups (34, 35). Next, we tested the cytotoxic effect of ON 01910.Na on CLL cells cultured in this stroma model system.

Previous work has shown that the prosurvival effect of HK cells on CLL cells is mediated at least in part through upregulation of MCL-1 (35). We therefore tested if ON 01910.Na was able to counteract HK-induced upregulation of MCL-1. Indeed, ON 01910.Na greatly reduced MCL-1 expression in CLL cells cultured with HK cells (Fig. 5C). Moreover, ON 01910.Na also reduced AKT phosphorylation equally in CLL cells cultured with or without HK cells. Finally, activation of SAP/JNK by ON 01910.Na was not affected by HK cells, consistent with the preserved cytotoxic activity of the drug against CLL cells cultured in this stroma model system.

Finally, we addressed whether ON 01910.Na affects migration of CLL cells toward the chemokine SDF-1α/CXCL12. SDF-1α is a chemokine secreted by different types of stroma cells that may guide migration of CLL cells to the stroma microenvironment (36). It has been described recently that PI3K inhibitors targeting p110α or p110γ isoforms interfere with SDF-1α–induced migration of CLL cells (15). As shown in Fig. 5D, ON 01910.Na was very potent in blocking the migration of CLL cells in an SDF-1α chemokine gradient.

Discussion

We chose to investigate ON 01910.Na because of its reported activity against MCL cell lines through inhibition of the PI3K pathway (23) and its promising clinical activity in MDS, for which it has now entered a pivotal phase III study (NCT01241500). In initial experiments, we observed rapid induction of apoptosis selectively in CLL cells, while T
cells and normal B cells were not or only minimally affected. We were surprised by the strong proapoptotic effect of ON 01910.Na given reports of only minimal apoptosis in response to other clinical grade PI3K inhibitors (11). On the basis of its unique chemical structure and mechanism of kinase inhibition (20), we considered that ON 01910.Na may have additional effects. We chose to investigate the molecular effects and resultant cellular stress response using global gene expression profiling because this approach does not require prior assumptions. The transcriptional profile in ON 01910.Na-treated CLL cells was consistent with inhibition of PI3K signaling. In addition, we observed upregulation of gene signatures indicating a ROS-induced oxidative stress response. We went on to confirm a key role of ROS in ON 01910.Na-induced apoptosis in CLL cells: first, we showed a significant accumulation of ROS in response to drug that paralleled onset of apoptosis. Second, the antioxidant NAC effectively antagonized ON 01910.Na-mediated apoptosis. Third, we found that ON 01910.Na treatment induced a classic ROS triggered stress response pathway involving activation of pSAP/JNK, nuclear accumulation of c-jun, and induction of ATF3 and NOXA in all CLL samples tested. It is notable that this stress response differs in some aspects from the NRF2-dominated response, we described in MCL cells treated with the proteasome inhibitor bortezomib (33). NRF2, a key regulator of the protective antioxidant response, is in part regulated through PI3K. The combination of a PI3K inhibitor with a ROS-inducing agent has been shown to inhibit the translocation of NRF2 to the nucleus and block upregulation of NRF2 target genes (37, 38). In keeping with this, ON 01910.Na-treated cells mounted no significant NRF2 response (FDR > 0.2; data not shown).

Pharmacologic induction of ROS is increasingly recognized as a therapeutic principle to selectively kill transformed cells (39, 40). Cancer cells are particularly sensitive to disruption of redox homeostasis; a potential vulnerability thought to be due to increased ROS levels originating from oncogene activation (39). One of the first agents shown to have selective antitumor activity through upregulation of ROS is β-phenylethyl isothiocyanate (PEITC; ref. 41). This compound was subsequently shown to be effective against fludarabine-resistant CLL cells (42). Similarly, apoptosis induced in CLL cells by adaphostin, a tyrosine kinase inhibitor of the tyrphostin class (43), has been shown to depend on the induction of ROS.

In vitro, these agents remain active against chemotherapy resistant CLL cells with dysfunctional TP53; an effect that seems to be primarily due to the induction of NOXA in response to ROS (32). Despite their encouraging in vitro activity, these agents have no established clinical benefit in CLL; adaphostin has not entered clinical testing, and a phase I study of PEITC is only about to begin (NCT00968461). In contrast, ON 01910.Na has recently entered a pivotal phase III clinical trials in MDS and is in phase I/II studies for other indications. Interestingly, ON 01910.Na has been reported to be selectively cytotoxic for the myeloid tumor cells without affecting normal hematopoiesis (44). Although induction of oxidative stress has not been described as a therapeutic principle in MDS, ROS plays an important role in the pathogenesis and is increased in MDS cells compared...
with normal BM cells (reviewed in ref. 45). Given our findings, it thus seems plausible that generation of ROS contributes to the selective antitumor activity of ON 01910.Na in MDS.

Consistent with a report that ON 01910.Na inhibits PI3K signaling, we detected a transcriptional response in drug-treated CLL cells indicative of substantial PI3K inhibition. To do this, we selected 2 representative patients for gene expression analysis and exposed PBMCs to ON 01910.Na in a dose- and time-dependent fashion. In this way, we generated 4 replicates of drug-treated cells in each patient that also incorporate dose and time effects. Gene expression in treated cells was compared with untreated cells obtained at the same time points. We then tested whether ON 01910.Na induces the gene expression fingerprint of a PI3K inhibitor using GSEA to estimate the connection of ON 01910.Na-induced gene expression changes with previously established signatures of the pan-PI3K inhibitor LY 294002. This approach termed “connectivity mapping” has been developed and used successfully to identify compounds affecting the same cellular pathways (46). Indeed, the connection between the gene signatures of LY 0294002 and ON 01910.Na was highly significant (FDR 0.01 for downregulated genes; FDR 0.17 for upregulated genes; Supplementary Table S1, Fig. 2A). Further supporting “on target” effects are the upregulation of genes controlled by FOXO. Transcription factors of the FOXO family are retained in the cytoplasm by PI3K and/or ERK-dependent phosphorylation. Upon downregulation of PI3K activity, unphosphorylated FOXO translocates to the nucleus and upregulates numerous genes involved in cell-cycle arrest, apoptosis, and stress response (47). Finally, we also observed downregulation of a subset of genes that we have previously identified as target genes of BCR signaling in CLL cells (7). Possible reasons why we observed only a partial downregulation of the BCR signature include PI3K-independent regulation of some BCR target genes and incomplete inhibition of the pathway by ON 01910.Na, which has been reported to primarily inhibit PI3Kα and PI3Kβ (23), while BCR signaling activates PI3Kδ (11). As reported by Prasad and colleagues, we did observe a decrease in pAKT in most samples (Fig. 2C), while effects on p-mTOR, pS6K, and p4EBP1 were apparent in some but not other CLL samples tested (data not shown). These differences may be due to the former study testing only 2 cell lines or could reflect differences between cell lines and primary samples.

The tumor microenvironment is increasingly recognized as a significant factor contributing to treatment resistance in CLL (8). Strategies to target cells in the protective environment are therefore a priority. ON 01910.Na combines 2 complementary activities that could endow it with significant clinical activity. ROS activation can induce apoptosis, particularly in cells with high baseline ROS levels. CLL cells are reported to have high baseline ROS levels (42) and some of the key signals activated in the secondary lymphoid struc-
References


ON 01910.Na Is Selectively Cytotoxic for Chronic Lymphocytic Leukemia Cells through a Dual Mechanism of Action Involving PI3K/AKT Inhibition and Induction of Oxidative Stress

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