The Aurora A and B kinases are up-regulated in bone marrow-derived chronic lymphocytic leukemia cells and represent potential therapeutic targets

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ABSTRACT

Background
The malignant B cells in chronic lymphocytic leukemia receive signals from the bone marrow and lymph node microenvironments which regulate their survival and proliferation. Characterization of these signals and the pathways that propagate them to the interior of the cell is important for the identification of novel potential targets for therapeutic intervention.

Design and Methods
We compared the gene expression profiles of chronic lymphocytic leukemia B cells purified from bone marrow and peripheral blood to identify genes that are induced by the bone marrow microenvironment. Two of the differentially expressed genes were further studied in cell culture experiments and in an animal model to determine whether they could represent appropriate therapeutic targets in chronic lymphocytic leukemia.

Results
Functional classification analysis revealed that the majority of differentially expressed genes belong to gene ontology categories related to cell cycle and mitosis. Significantly up-regulated genes in bone marrow-derived tumor cells included important cell cycle regulators, such as Aurora A and B, survivin and CDK6. Down-regulation of Aurora A and B by RNA interference inhibited proliferation of chronic lymphocytic leukemia-derived cell lines and induced low levels of apoptosis. A similar effect was observed with the Aurora kinase inhibitor VX-680 in primary chronic lymphocytic leukemia cells that were induced to proliferate by CpG-oligonucleotides and interleukin-2. Moreover, VX-680 significantly blocked leukemia growth in a mouse model of chronic lymphocytic leukemia.

Conclusions
Aurora A and B are up-regulated in proliferating chronic lymphocytic leukemia cells and represent potential therapeutic targets in this disease.

Key words: chronic lymphocytic leukemia, microenvironment, Aurora kinase, novel therapeutic targets.

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The online version of this article has a Supplementary Appendix.

Introduction

Chronic lymphocytic leukemia (CLL) is characterized by the progressive accumulation of mature, monoclonal, CD5-positive B cells in the peripheral blood (PB), bone marrow (BM) and secondary lymphoid organs, such as lymph nodes and spleen.[1,2] Traditionally, the disease has been considered a disorder of cells with defective apoptosis that accumulate because of extended survival. Consistent with this view, the majority of CLL cells in the PB are arrested in the G0/G1 phase of the cell cycle and show a gene expression profile of resting B cells.[3] However, more recent studies using deuterated water have shown that a notable proliferative compartment exists, which continuously replenishes the bulk population of non-cycling CLL cells.[4] This proliferative compartment contributes to disease progression not only by increasing the leukemic cell burden but also by facilitating the accumulation of new mutations that increase the aggressiveness and chemoresistance of the malignant clone.

The CLL proliferating compartment is located in imperfectly defined structures in the lymph nodes and BM, named proliferation centers or pseudofollicles. These structures are composed of focal aggregates of neoplastic prolymphocytes and paraimmunoblasts that are interspersed with auxiliary cells, such as $CD4^+$ T cells, follicular dendritic cells, stromal cells and nurse-like cells.[2,5] In these structures, CLL cells are believed to receive various signals that stimulate their proliferation and survival. Examples of such microenvironmental signals include interleukin (IL)-4,[6] CD40 ligand,[7,8] CXCL12,[9] BAFF,[10] CD31,[11] VCAM-1,[12] CpG-DNA,[13,14] antigen,[15,16] and contact with stromal,[17] nurse-like,[10,12] and follicular dendritic cells.[18] *In vitro*, most of these signals only increase the resistance of CLL cells to spontaneous and chemotherapy-induce apoptosis. However, certain combinations can induce relatively efficient proliferation, such as unmethylated CpG oligonucleotides (CpG-ODN) with IL-2,[13,14] or CD40L/IL-2/IL-10 in co-culture with stromal cells.[8]

Despite the progress in identifying potential microenvironmental signals that can induce the proliferation or increase the survival of CLL cells *in vitro*, the microenvironmental signals that sustain the growth of the malignant clone *in vivo* remain poorly defined. In addition, the intracellular pathways that transduce the proliferation signal in the malignant B cells have not been fully characterized. Identification of the signals and pathways that operate *in vivo* is important for the development of strategies to block the microenvironmental interactions that regulate the proliferation of the malignant clone. Such strategies could be particularly important in patients with CLL with high proliferation rates, in whom they could prevent the development of dangerous clonal variants or eliminate them as they occur.

To further define the pathways that transduce the microenvironmental signals in CLL cells *in vivo* and to identify novel potential targets for therapeutic intervention, we investigated the gene expression profiles of purified CLL cells from paired BM and PB samples by microarray and real-time reverse transcriptase polymerase chain reaction (RT-PCR) analysis. A number of genes involved in cell cycle and mitosis were found to be significantly up-regulated in BM-CLL cells. Among these, the Aurora A (AURKA) and Aurora B (AURKB) kinases appeared as particularly interesting molecules for further study, considering that selective small molecule inhibitors of these kinases have been developed and have already shown promising activity against a wide range of other malignant diseases.[19,20] To determine whether these kinases could also represent potential therapeutic targets in CLL, we investigated the effects of down-regulation or inhibition of Aurora A and Aurora B in CLL cell lines, primary CLL cells induced to proliferate by CpG-ODN/IL-2, and murine leukemias that develop in the Eμ-TCL1 transgenic model of CLL.

Design and Methods

Chronic lymphocytic leukemia and normal B-cell samples

Blood samples satisfying standard morphological and immunophenotypic criteria for B-cell CLL were collected from patients. Informed consent was obtained from all patients according to the Declaration of Helsinki and approval for the study was obtained from the Ethical Committees at the "A. Gemelli" Catholic University Hospital, Rome (Italy) and the University Clinical Hospital of the Faculty of Medicine, Ribeirão Preto (Brazil).

CLL B cells from PB and BM that were used for the microarray and real-time RT-PCR experiments were purified by negative selection using the RosetteSep human B cell-enrichment cocktail (StemCell Technologies, Vancouver, Canada). For all other experiments, CLL cells were first isolated by Ficoll gradient centrifugation and then further purified by negative selection with anti-CD3, anti-CD14 and anti-CD16 mouse monoclonal antibodies and Dynabeads coated with pan-anti-mouse IgG antibody (Dynal Biotech, Oslo, Norway). The purity of the selected B-cell populations was >95%, as determined by staining with anti-CD5 and anti-CD19 antibodies and analysis on a FACSCalibur flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA).

Gene expression profiling and real-time reverse transcriptase polymerase chain reaction analysis

Microarray profiling of total RNA extracted from purified BM and PB CLL cells was performed using Whole Human Genome Oligo microarrays (Agilent, Palo Alto, CA, USA) containing 41,000 distinct probes. Complete microarray data sets have been deposited in NCBI Gene Expression Omnibus and are accessible through GEO Series accession number GSE30896. Differentially expressed transcripts were identified by a two-tailed paired t-test, considering *P* values of less than 0.05 as statistically significant. Functional classification analysis was performed using the online Database for Annotation, Visualization and Integrated Discovery (DAVID), version 6.7. Expression of *AURKA*, *AURKB*, *BIRC5* and *CDK6* was further quantified by RT-PCR analysis with pre-designed TaqMan probes in a 7300 Real-Time PCR System (Applied Biosystems). Details on the gene expression profiling and quantitative RT-PCR analysis are provided in the *Online Supplementary Design and Methods*.

Cell culture, RNA interference and immunoblotting experiments

Freshly isolated CLL B cells were cultured in the presence of CpG oligonucleotide 2006 (Microsynth, Balgach, Switzerland), IL-2 (R&D Systems, Minneapolis, MN, USA), goat anti-human IgM (Southern Biotechnology Associates, Birmingham, AL, USA) coated on Dynabeads M-450 Epoxy (Dynal Biotech) or 3T40L cells as described elsewhere.[14,15] The pan-Aurora kinase inhibitor VX-680 (Cayman Chemical, Ann Arbor, Michigan) was used as indicated.

Stealth Select small interfering (si) RNA (Invitrogen) were used to silence the expression of Aurora A and B in MEC1 and EHEB cells (DSMZ, Braunschweig, Germany), as previously described.[14] Briefly, $0.6x10^7$cells were resuspended in 100 μL of Cell Line Nucleofector Solution L (Amaxa Biosystems GmbH, Cologne,

Germany) and mixed with 2.5 μg of siRNA. Nucleofections were performed on an Amaxa Nucleofector II device (Amaxa Biosystems GmbH) using the C-005 program. Cells were collected and processed for subsequent analysis at the indicated times.

Immunoblotting analysis was performed as described elsewhere.[14] The following antibodies were used: Aurora A (Invitrogen), Aurora B, phospho-AURKA^{T288}, phospho-histone H3^{S10}, rabbit IgG-horseradish peroxidase (HRP), mouse IgG HRP-linked (Cell Signaling Technology, Danvers, MA, USA), and β-actin (Sigma-Aldrich).

Cell cycle, apoptosis and proliferation assays

The cell cycle was analyzed by propidium iodide staining and flow cytometry. Annexin-A5-fluorescein isothiocyanate (FITC) (Nexins Research, Kattendijke, The Netherlands) was used to evaluate the percentage of viable and apoptotic cells. For analysis of proliferation, 5-bromo-2-deoxyuridine (BrdU) 10 μM was added to synchronized or unsynchronized MEC1 or EHEB cells for 1 h prior to harvesting. In experiments with primary CLL cells, BrdU was added after 48 h of stimulation with CpG-ODN and IL-2. Cells were cultured for another 18 h prior to harvesting. Incorporated BrdU was detected with anti-BrdU-FITC antibody (BD Biosciences). Further details are provided in the *Online Supplementary Design and Methods*.

Treatment of mice with adoptively transferred Eμ-TCL1 leukemias

For adoptive transfer, 1.2x10^7 TCL1 leukemia cells were thawed, resuspended in 500 μL of phosphate-buffered saline and injected intraperitoneally into B6/C3H F1 female mice (6-8 weeks old; Harlan Laboratories B.V., Horst, The Netherlands). The VX-680 solution was first dissolved in PEG400 (Sigma-Aldrich) and then diluted 2-fold with 50 mM sodium phosphate pH 4.0 (the pH was adjusted to 7.1 before injection). Five days after adoptive transfer, treatment was started in groups of seven to ten mice with VX-680 40mg/kg bid or vehicle control, intraperitoneally. Leukemia development was monitored by measuring white blood cell counts on a Hemavet HV950FS hematology analyzer (Drew Scientific, Inc., Dallas, TX, USA) and flow-cytometry analysis of CD5$^+$/B220$^+$ cells. All animal procedures were performed in accordance with Italian national law (Italian legislative decree 116/92 and European directive 8/609) and ICGEB institutional guidelines.

Statistical analysis

Student's *t* tests and Mann-Whitney rank sum tests were performed to determine the significance of the differences between mean and median values as appropriate. Fisher's exact test was used to investigate the correlation between response to CpG-ODN/IL-2 stimulation and *IGHV* mutation status or ZAP-70 expression. The survival curves and medians were calculated within subgroups with the Kaplan-Meier method. The log-rank test was used to compare differences between estimated survival curves. All statistical analyses were performed using the SigmaStat 3.1 program (Systat Software, Richmond, CA, USA).

Results

Genes involved in cell cycle and mitosis are significantly up-regulated in chronic lymphocytic leukemia cells from bone marrow

To identify the pathways that are activated by microenvironmental stimuli in CLL cells *in vivo*, we performed gene expression profiling of purified BM and PB CLL cells. Six paired BM and PB samples were used for this analysis. A total of 272 genes were identified as differentially expressed between BM and PB CLL cells, with expression levels that were at least two-fold different (*Online Supplementary Table S1*). Of these, 56 were up-regulated and 216 were down-regulated in BM-CLL cells. Among the significantly up-regulated genes in BM-CLL cells several important regulators of the cell cycle were identified, such as *survivin* (*BIRC5*), *AURKA*, *E2F2*, *CDK6*, *CDC45L*, and *CDCA2* (Figure 1). In addition, functional annotation analysis with the DAVID tool revealed that many of the other up-regulated genes also belong to gene ontology categories that are related to cellular proliferation, including the categories cell cycle (GO:0007049), mitosis (GO:0007067), nuclear division (GO:0000280) and M phase of mitotic cell cycle (GO:0000087)(*Online Supplementary Table S2*).

In order to validate the gene expression data, we reanalyzed the expression of *AURKA*, *CDK6* and *survivin* by real-time RT-PCR in the six samples used for the microarray analysis and in an additional set of nine paired BM and PB CLL samples. *AURKB*, an *AURKA* homologue that was not identified as differentially expressed by the microarray profiling, was also included in this analysis because it is induced in other cell types during cell cycle progression and is also involved in mitosis regulation.[21] As shown in Figure 2, all four genes, including *AURKB*, were found to be expressed at significantly higher levels in BM-CLL cells, with mean values exceeding the levels in PB-CLL cells by 10-fold. Collectively, these data show that genes involved in cell cycle control and proliferation are up-regulated in BM-CLL cells.

Down-regulation of AURKA and AURKB induces growth arrest and apoptosis in chronic lymphocytic leukemia cell lines in vitro

Among the various proliferation/cell cycle regulatory genes that were up-regulated in BM-CLL cells, the Aurora kinases appeared as particularly interesting for further study, as they have recently emerged as promising therapeutic targets in a broad range of malignant diseases. These kinases play critical roles in chromosome segregation and cytokinesis during mitosis and are frequently deregulated in cancer, which has led to the development of several pharmacologically active small-molecule inhibitors that selectively inhibit *AURKA*, *AURKB* or both. Preclinical studies with several of these agents have shown promising activity, resulting in inhibition of tumor growth in a variety of *in vivo* xenograft models at well-tolerated doses.[22-24]

To determine whether the Aurora kinases could be appropriate therapeutic targets in CLL, we first evaluated the functional consequences of *AURKA* and *AURKB* knockdown in the CLL-derived cell lines MEC1 and EHEB. As shown in Figure 3A and 3B, both Aurora kinases were efficiently down-regulated by RNA interference, with an approximately 70-80% reduction in mRNA and protein levels with respect to cells transfected with control siRNA. To evaluate the effect of Aurora kinase knockdown on proliferation, the percentage of dividing cells was determined by BrdU incorporation analysis 48 h after siRNA transfection. In both cell lines, down-regulation of *AURKA* or *AURKB* significantly reduced the percentage of BrdU-positive cells (Figure 3C and 3D). Inhibition of proliferation appeared even greater when *AURKA* and *AURKB* were simultaneously silenced, which was particularly evident in MEC1 cells.

To further clarify the role of *AURKA* and *AURKB* in regu-

lating CLL proliferation, we synchronized MEC1 and EHEB cells and evaluated their cell cycle distribution 8 and 24 h after release from thymidine block. At both time points, down-regulation of *AURKA* or *AURKB* reduced the percentage of cells in G1 and increased the percentage of cells in G2/M, indicating a block at this phase of the cell cycle (Figure 3E). This effect was more pronounced in cells in which both kinases were silenced. In addition, silencing of *AURKA* and *AURKB* induced a modest but significant increase in the percentage of apoptotic subG1 cells, especially at the later time point. Induction of apoptosis following down-regulation of *AURKA* and *AURKB* was further confirmed by annexin V/propidium iodide staining, which showed an increase in the percentage of apoptotic, annexin V-positive cells (Figure 3F and 3G).

The Aurora kinase inhibitor VX-680 blocks proliferation and induces apoptosis in chronic lymphocytic leukemia cell lines and CpG-oligonucleotide/interleukin-2-stimulated primary chronic lymphocytic leukemia samples

The RNA interference experiments with MEC1 and EHEB cells revealed that simultaneous silencing of *AURKA* and *AURKB* was more effective in inducing mitotic arrest and apoptosis than individual silencing of each of the two Aurora kinases. We, therefore, decided to investigate the activity of the dual AURKA and AURKB inhibitor VX-680 (MK-0457)[22] against MEC1 and EHEB cells. Incubation of MEC1 and EHEB cells with nanomolar concentrations of VX-680 significantly inhibited BrdU incorporation and induced a block at the G2/M stage of the cell cycle (*Online Supplementary Figure S1A-C*). These effects correlated with a decrease in both Aurora A and Aurora B kinase activity, as evidenced by reduced autophosphorylation of Aurora A at Thr288 and reduced phosphorylation of Histone H3 at Ser10, which is a site directly phosphorylated by Aurora B (*Online Supplementary Figure S1D*).[25,26] In addition, VX-680 induced moderate levels of apoptosis, as evidenced by an increase in the subG1 population and a decrease in the percentage of annexin V-negative cells (*Online Supplementary Figure S1C,E*).

We next investigated whether VX-680 also affects the proliferation of primary CLL cells. To induce proliferation, PB-CLL cells were stimulated for 48 h with CpG-ODN and IL-2. As shown in Figure 4A and 4B, CpG-ODN/IL-2 induced an increase in the percentage of proliferating, BrdU-positive cells in some but not all CLL samples. Substantial proliferation (more than 6% BrdU-positive cells) was detected in ten samples, six of which expressed unmutated *IGHV* genes, two expressed mutated *IGHV* genes and in two the *IGHV* sequence was not determined (*Online Supplementary Table S3*). Of the five non-proliferating cases (less than 6% BrdU-positive cells), four expressed mutated *IGHV* genes and one expressed an unmutated *IGHV* gene. This heterogeneous response to CpG-ODN/IL-2 stimulation is consistent with our previous studies, which showed that CpG-ODN induces proliferation mainly in *IGHV*-unmutated CLL cells, whereas *IGHV*-mutated CLL cells usually do not respond or undergo apoptosis.[14,27] A similar trend was observed with respect to induction of *AURKA* and *AURKB*. As shown in Figure 4C, all six unmutated CLL cases showed an increase in AURKA and AURKB expression, whereas only two of the seven investigated mutated CLL cases responded (P=0.021 by Fisher's exact test). An association with ZAP-70 expression was also observed (5/5 ZAP-70-positive and 1/6 ZAP-70-negative cases showed an increase in *AURKA* and *AURKB* mRNA levels, P=0.015 by Fisher's exact test).

VX-680 inhibited BrdU incorporation in all samples that proliferated in response to CpG-ODN/IL-2 stimulation (Figure 4A and 4B). Somewhat higher concentrations were required to inhibit proliferation of CpG-ODN/IL-2-stimulated primary CLL cells in comparison with the CLL cell lines. In terms of survival, VX-680 had no effect on the viability of resting CLL cells, but prevented or reduced the viability gain in samples that showed increased survival in the presence of CpG-ODN and IL-2 (Figure 4D and *Online Supplementary Table S3*). This effect was particularly evident at the 2.5 μM concentration of VX-680 (P<0.001).

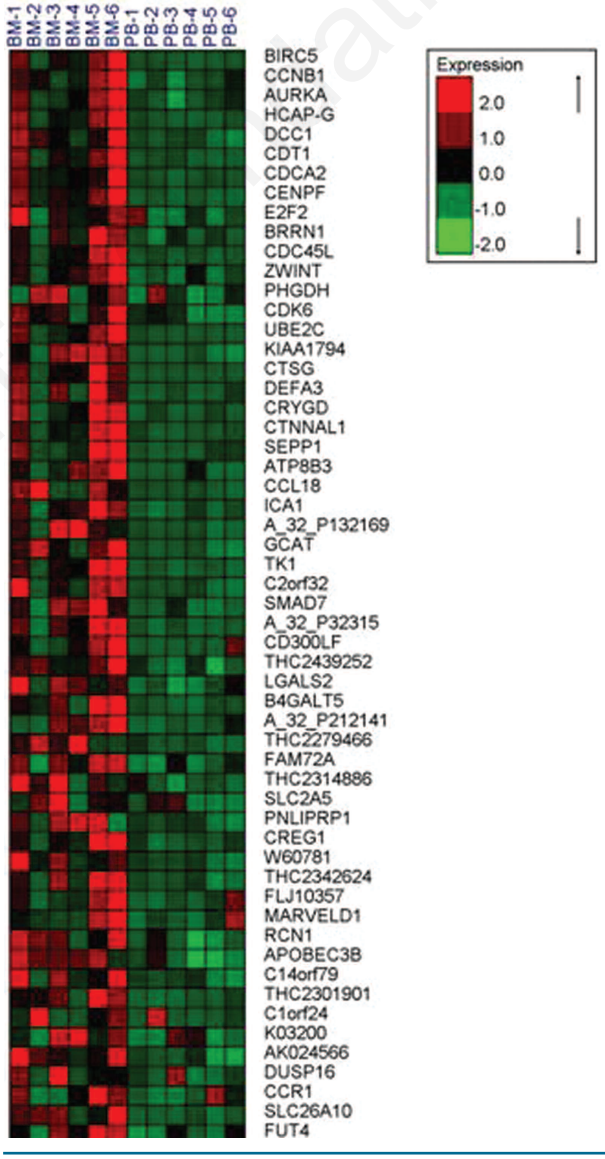

Figure 1. Gene expression profiling analysis of purified PB- and BM-derived CLL cells. The heatmap was generated using microarray row-normalized transcript levels of the 56 differentially expressed transcripts found at higher levels in BM than in PB. The relative level of gene expression is depicted according to the color scale shown in the upper right panel. Patients' samples are arranged in columns keeping the same order in the BM and PB groups.

VX-680 blocks the growth of murine Eμ-TCL1 leukemias in vivo

We next investigated the activity of VX-680 against leukemias that develop in the Eμ-TCL1 transgenic mouse model of CLL. These leukemias display many of the typical features of aggressive human CLL and are commonly used to test novel agents for this disease.[28-30] The Eμ-TCL1 leukemias do not grow *in vitro*, but can be effectively propagated *in vivo* by adoptive transfer in syngeneic recipients, suggesting that they rely on microenvironmental signals for proliferation and survival. Previously, we established and characterized several such lines,[30] of which two (TCL1-002 and TCL1-870) were used in the current study.

The two Eμ-TCL1 leukemias were adoptively transferred in cohorts of seven to ten syngeneic mice. Treatment with VX-680 or vehicle control was started 5 days later. The drug was administered intraperitoneally, 40 mg/kg bid, for 15 consecutive days. Leukemia growth was monitored every second week by automated white blood cell counts and by screening with flow cytometry for the appearance of CD5-positive B cells in the PB.

TCL1-002 leukemia cells became detectable in the PB of all animals from the control group (n=10) by day 35 following adoptive transfer (Figure 5A). At this time, leukemic cells could not be detected in any of the animals treated with VX-680 (n=7). However, several weeks later all VX-680-treated animals developed overt leukemia, suggesting that VX-680 inhibited leukemia growth but did not eradicate the disease. A similar response was seen in the experiment with the TCL1-870 leukemia, which grows more slowly than TCL1-002 (Figure 5A, right panel).

Treatment with VX-680 significantly prolonged the survival of the studied animals. The median survival of animals with TCL1-002 leukemia that were treated with vehicle control was 51 days, whereas the median survival of VX-680-treated animals was 99 days ($P<0.001$) (Figure 5B, left panel). Similarly, control group animals with TCL1-870 leukemia had a median survival of 136 days, whereas the median survival had not been reached at 170 days in the VX-680 group ($P=0.001$) (Figure 5B, right panel).

Discussion

This study provides further evidence that the BM is an important site of CLL cell activation and proliferation. A large proportion of the transcripts that were identified by gene expression profiling as significantly enriched in BM-CLL cells belonged to gene ontology categories that are involved in cell cycle, mitosis and nuclear division. Some of these genes, such as *BIRC5*, *AURKA*, *AURKB*, *CDT1* and *TK1* were also identified in a recent study by Herishanu *et al.*[31] as genes that are significantly up-regulated in lymph node- and to a lesser extent in BM-derived CLL cells. The less pronounced changes in BM- compared to lymph node-derived CLL cells could suggest that the lymph node microenvironment provides stronger stimuli for leukemic cell proliferation. Alternatively, some of the differences in the gene expression profiles could be related to technical issues, such as dilution of the BM aspirate with PB-CLL cells or underrepresentation of CLL cells in direct contact with the BM stroma. The latter possibilities may also account for some of the differences in the gene expression profiles observed in the study by Herishanu *et al.* and our study. Nonetheless, both studies provide evidence for significant enrichment of proliferation/cell cycle regulatory genes in BM-derived CLL cells, suggesting that the BM, in addition to the lymph nodes, is a site in which CLL cells receive microenvironmental signals that drive their proliferation.

The concept that proliferation of CLL cells is a result of interactions with the tumor microenvironment suggests that targeting the intracellular pathways that propagate these signals should be a rational therapeutic strategy in CLL. The study by Herishanu *et al.* revealed significant up-regulation of *BCR* and *TLR* target genes in lymph node CLL cells, suggesting that these pathways are activated *in vivo* and may possibly be involved in propagating the proliferation signal.[31] The lack of suitable inhibitors precludes tests to determine whether the TLR pathway could represent a potential therapeutic target in CLL, but several small molecule inhibitors of the BCR signaling pathway have recently been developed and have shown encouraging activity in animal models and early clinical trials.[30,32-34] Whether the activity of these compounds against CLL cells is because of inhibition of proliferation or survival is currently unclear. However, based on *in vitro* data, these compounds would be expected to target primarily the survival signal, considering that CLL cells display increased viability but do not proliferate upon stimulation with anti-IgM.[15,16]

An alternative approach to target the proliferating compartment in CLL would be to use inhibitors of regulatory molecules that are directly involved in leukemic cell prolif-

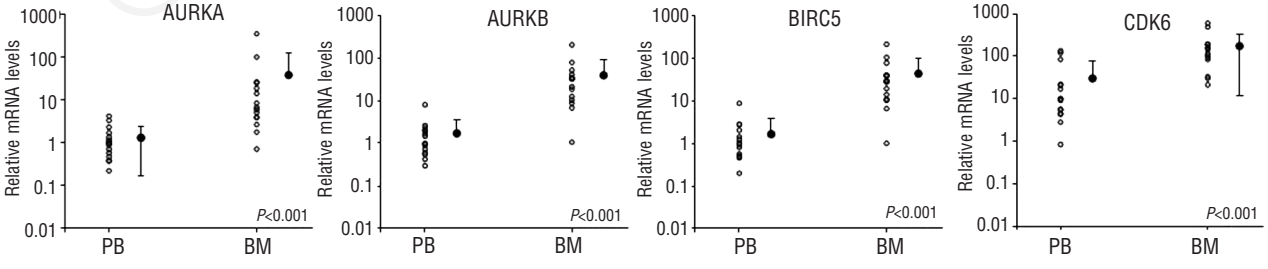

Figure 2. Expression of selected cell cycle-regulatory genes in PB- and BM-derived CLL cells. Quantification of *AURKA*, *AURKB*, *CDK6* and *BIRC5* mRNA levels was performed by real-time RT-PCR in 15 paired PB-CLL and BM-CLL samples. Open circles represent the relative mRNA levels of individual samples expressed as fold-difference relative to one PB-CLL sample that was used as a calibrator; mean values and SD are indicated by filled circles and error bars, respectively.

eration. Cell cycle inhibitors, such as the cyclin-dependent kinase inhibitors flavopiridol, roscovitine and SNS-032, have already been tested in CLL and have shown promising activity both *in vitro* and *in vivo*.[35-37] The Aurora A and B kinases, which were found to be significantly up-regulated in BM-CLL cells in this study, also appear as interesting targets for several reasons. First, both molecules are induced in PB-CLL cells by CpG-ODN/IL-2, suggesting that their expression is tightly linked to leukemic cell proliferation. Second, siRNA experiments with the CLL-derived cell lines MEC1 and EHEB showed that silencing of Aurora A and B induces G2/M arrest and apoptosis, suggesting that these kinases are essential for CLL cell cycle progression. Third, pharmacological inhibitors of Aurora kinases are available and are already in advanced stages of clinical development for other malignant diseases.[19,20]

To investigate the potential of Aurora kinase inhibitors as therapeutic agents in CLL, we performed *in vitro* and *in vivo* experiments with VX-680, an inhibitor of both Aurora kinases. This compound significantly inhibited proliferation

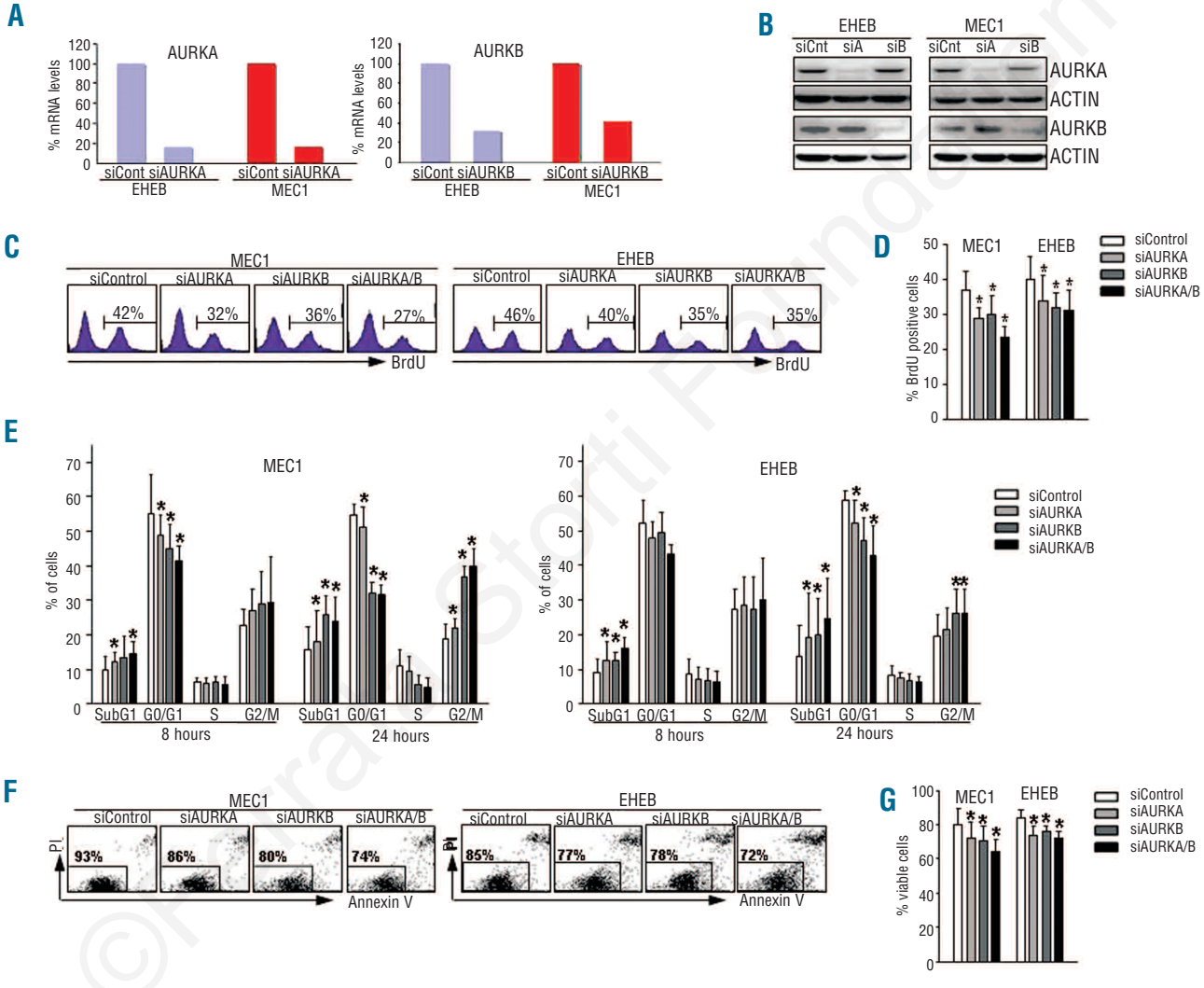

Figure 3. Down-regulation of *AURKA* and *AURKB* inhibits growth of EHEB and MEC1 cells and induces apoptosis. (A) Quantification of *AURKA* and *AURKB* mRNA levels by quantitative RT-PCR in EHEB and MEC1 cells collected 24 h after transfection with control or Aurora-specific siRNA. (B) Immunoblotting analysis of AURKA and AURKB protein levels in EHEB and MEC1 cells transfected with control or Aurora-specific siRNA. Actin was used as a loading control. One representative experiment out of three is shown. (C) Analysis of BrdU incorporation in EHEB and MEC1 cells transfected with control or Aurora-specific siRNA. BrdU was added 48 h after transfection. The cells were harvested 1 h later and the percentage of dividing $BrdU^+$ cells was determined by flow cytometry analysis. The percentage of $BrdU^+$ cells is indicated in the histogram plots. (D) Summary of five different BrdU incorporation experiments with MEC1 and five with EHEB cells. Mean values ± SD are shown. Significant differences ($P<0.05$) with respect to cells transfected with siControl are indicated by asterisks. (E) Analysis of cell cycle distribution in synchronized MEC1 and EHEB cells. Cells were transfected with control or Aurora-specific siRNA, synchronized with thymidine for 48 h and analyzed for cell-cycle distribution by propidium iodide staining and flow cytometry 8 and 24 h after release from thymidine block. Graphs represent the average of five experiments with MEC1 and five experiments with EHEB cells. Percentages of cells (mean values ± SD) in the subG1, G0/G1, S, and G2/M phases of the cell cycle are shown. Significant differences ($P<0.05$) with respect to cells transfected with siControl are indicated by asterisks. (F) Analysis of cellular viability 48 h after transfection with control or Aurora-specific siRNA. Unsynchronized MEC1 and EHEB cells were used in this experiment. The percentage of viable, annexin V/propidium iodide-double-negative cells (boxed) was determined by flow cytometry. (G) Summary of the results of five different experiments with MEC1 and five different experiments with EHEB cells. The mean percentage of viable cells ± SD is shown. Significant differences ($P<0.05$) between cells transfected with siControl and cells transfected with Aurora kinase-specific siRNA are indicated by asterisks.

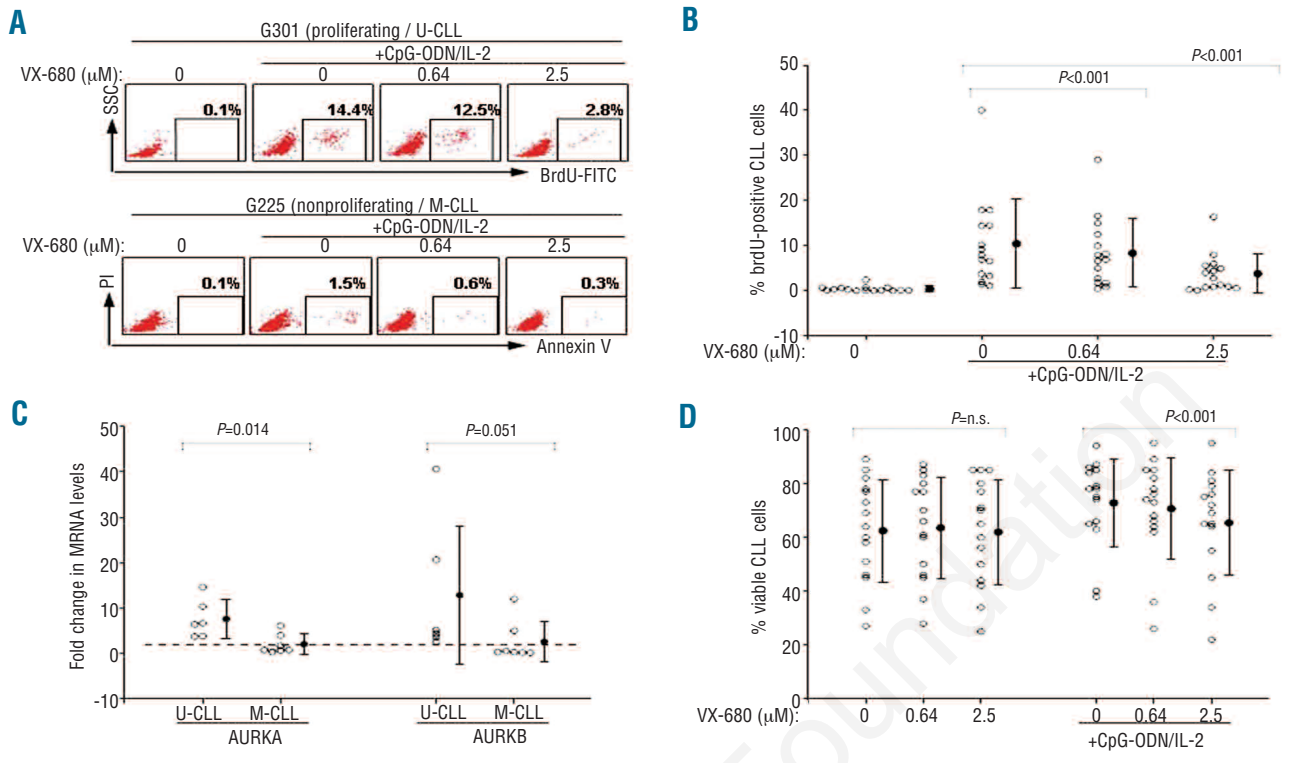

Figure 4. VX-680 inhibits proliferation and induces apoptosis in primary CLL cells stimulated with CpG-ODN and IL-2. (A) BrdU incorporation in primary PB-CLL cells stimulated with CpG-ODN/IL-2 in the presence of the indicated concentrations of VX-680. The results of a representative experiment with a proliferating (upper panel) and one representative experiment with a non-proliferating sample (lower panel) are shown. (B) Summary of BrdU incorporation experiments performed with CpG-ODN/IL-2-stimulated PB-CLL cells from 15 patients. Open circles represent the percentage of $BrdU^+$ cells in individual samples. Mean values and SD are indicated by filled circles and error bars, respectively. (C) Induction of *AURKA* and *AURKB* mRNA by CpG-ODN/IL-2 in *IGHV*-unmutated (U-CLL) and *IGHV*-mutated (M-CLL) samples. Open circles represent individual samples; mean values and SD are indicated by filled circles and error bars, respectively. The dashed line separates responding (≥2-fold change in mRNA levels) from non-responding (<2-fold change in mRNA levels) cases. (D) Effects of VX-680 on the viability of unstimulated and CpG-ODN/IL-2-stimulated PB-CLL cells. VX-680 was added to the culture medium 1 h before CpG-ODN/IL-2. The percentage of viable CLL cells was determined by annexin V/propidium iodide staining 48 h after addition of VX-680. Open circles represent the percentage of viable cells in individual samples. Mean values and SD are indicated by filled circles and error bars, respectively.

of MEC1 and EHEB cells and blocked proliferation of primary CLL cells stimulated with CpG-ODN and IL-2. In addition, a mild cytotoxic effect was observed against proliferating CLL cells, in agreement with the RNA interference experiments and consistent with similar observations in myeloma cells.[38,39]

VX-680 also displayed considerable activity against adoptively-transferred Eμ-TCL1 leukemias. These leukemias are a well-established animal model of CLL and a commonly used preclinical tool to test the activity of novel agents for this disease.[28-30] Treatment with VX-680 resulted in significant inhibition of leukemia growth and significantly prolonged the survival of VX-680-treated mice compared to mice treated with a vehicle control. It should be noted, however, that none of the animals was cured from the leukemia, suggesting that combinations with other agents will be required to improve the efficacy of this treatment.

In terms of clinical experience with VX-680, the safety and toxicity of this compound was recently investigated in two phase 1 clinical trials in patients with advanced solid tumors.[40,41] The drug was generally well tolerated, causing few non-hematologic toxicities. Dose-limiting toxicity was neutropenia, which has also been observed with most other Aurora kinase inhibitors.[19,20] This toxicity profile is consistent with results of previous *in vitro* studies with VX-680, which showed no effect against resting lymphohematopoietic cells, but inhibited the proliferation of phytohemagglutinin-stimulated PB lymphocytes and depressed the formation of erythroid, granuocyte-monocyte, granulocyte-erythroid-monocyte-megakaryocyte colony-forming units and burst-forming unit-erythroid from BM progenitor cells.[38]

The relatively good toxicity and tolerability profile of Aurora kinase inhibitors, together with the promising preclinical activity of VX-680 observed in our study, suggest that these compounds are worth further evaluation in clinical trials of CLL. Aurora kinase inhibitors may be particularly useful in combination with chemotherapy or chemoimmunotherapy, together with agents that target resting CLL cells. In this context, it is worth noting that inhibition or down-regulation of Aurora kinases has been shown to potentiate the activity of several standard chemotherapeutic agents in various solid tumors.[19,42]

In addition to inhibiting Aurora kinases, VX-680 has certain off-target effects that may be beneficial in CLL. In a recent screen against a large panel of human kinases, VX-680 was shown to inhibit ABL, FLT-3, PLK4, RET and TRKA at concentrations similar to those required to inhibit Aurora kinases.[43] The ability to inhibit ABL was exploited in a recent clinical trial of Philadelphia chromosome-positive chronic myeloid and acute lymphocytic leukemia, which

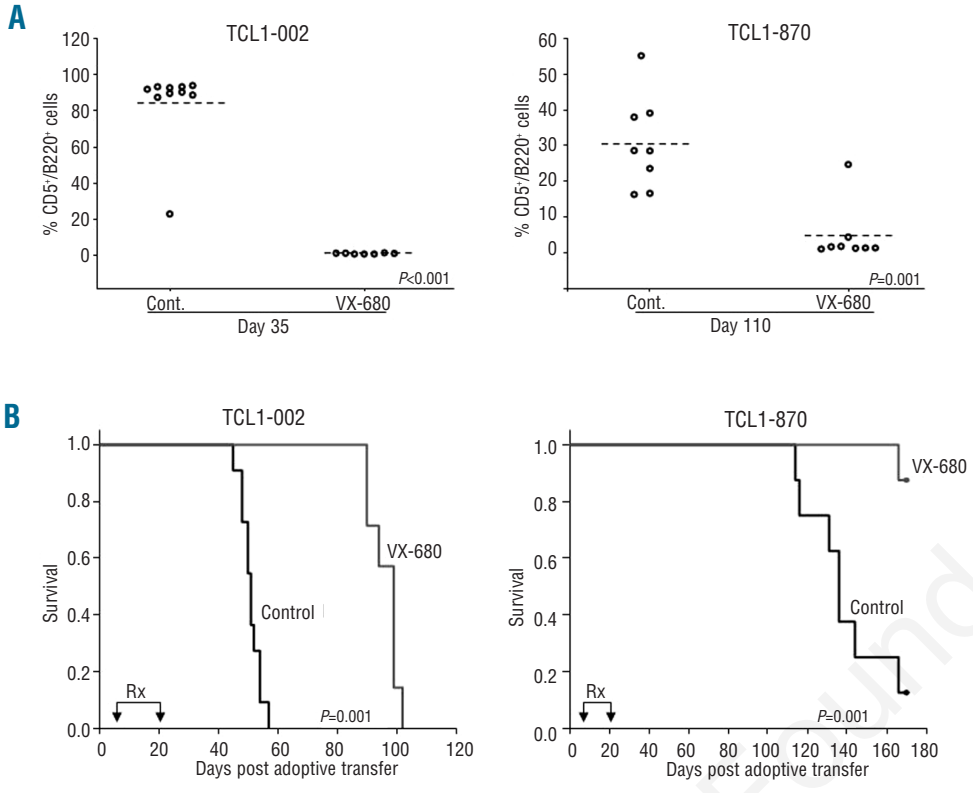

Figure 5. Treatment of adoptively transferred Eμ-TCL1 leukemias with VX-680. (A) B6/C3H F1 female mice (6-8 weeks old) received $1.2x10^7$ TCL1-002 or TCL1-870 leukemia cells by intraperitoneal injection. Five days later treatment was initiated with VX-680 40 mg/kg bid or vehicle control (Cont.). Treatment was administered by intraperitoneal injection for 15 days. Mice were monitored every 2 weeks for leukemia development by flow-cytometry analysis of peripheral blood samples. The percentages of leukemic $CD5^+/B220^+$ cells at day 35 for mice with TCL1-002 leukemia and at day 110 for mice with TCL1-870 leukemia are shown. (B) Kaplan-Meier survival curves of mice with adoptively transferred TCL1-002 or TCL1-870 leukemia treated with VX-680 or vehicle control. The period of treatment (Rx) is indicated by arrows.

demonstrated considerable clinical activity of VX-680 in this setting.[44] The ABL kinase may also represent a therapeutic target in CLL, as this kinase is highly expressed and constitutively active in CLL cells.[45] Moreover, inhibition or down-regulation of Abl has been reported to reduce the expression of the anti-apoptotic protein Mcl-1 and induce leukemic cell apoptosis.[45-46] Although we did not observe a direct cytotoxic effect of VX-680 against resting CLL cells, it remains possible that this compound could enhance the sensitivity of these cells to other cytotoxic agents by down-regulating Mcl-1.

In conclusion, this study provides preclinical evidence that Aurora kinase inhibitors could be a class of agents active in CLL. Although these compounds are not entirely selective for the malignant clone, their good tolerability profile and the absence of significant toxicities suggest that they may represent a useful addition to the current therapeutic armamentarium in CLL.

Authorship and Disclosures

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