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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Citation: de Paula Careta F, Gobessi S, Panepucci RA, Bojnik E, Morato de Oliveira F, Mazza Matos D, Falcão RP, Laurenti L, Zago MA, and Efremov DG. The Aurora A and B kinases are up-regulated in bone marrow-derived chronic lymphocytic leukemia cells and represent potential therapeutic targets. Haematologica 2012;97(8):1246-1254. doi:10.3324/haematol.2011.054668

Online Supplementary Design and Methods

Gene expression profiling and data analysis

Total RNA was extracted using TRIZOL-LS (Invitrogen), treated with DNase and purified using the RNAeasy kit (Qiagen, Valencia, CA, USA). RNA quality was assessed by agarose gel electrophoresis and quantified using a NanoDrop 1000 spectrophotometer (NanoDrop, Wilmington, DE, USA). Microarray profiling was performed using Whole Human Genome Oligo microarrays (Agilent, Palo Alto, CA, USA) containing 41,000 distinct probes. The Quick Amp Labeling Kit, one-color kit (Agilent) was used to generate Cy3-labeled cRNA, which was then fragmented and hybridized to the microarray slides. Slides were scanned using a GenePix 4000B scanner (at 535 nm, 5 μm/pixel resolution) and the "GenePix Pro 6.0" software (Molecular Devices, Sunnyvale, CA, USA). Images were analyzed with Agilent Feature Extraction software (version 9.5.3.1) and the 75th percentile processed signal of each array was used to normalize the data, allowing inter-array comparisons. Complete microarray data sets have been deposited in the NCBI Gene Expression Omnibus and are accessible through GEO Series accession number GSE30896 (*http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE30896*).

After averaging replicated probes in the arrays, transcripts differentially expressed between bone marrow and peripheral blood chronic lymphocytic leukemia (CLL) cells were identified by a two-tailed paired t-test, with *P* values less than 0.05 indicating statistically significant differences. Transcripts showing at least a two-fold difference between bone marrow and peripheral blood averaged expression values were submitted to a functional classification analysis, using the online Database for Annotation, Visualization and Integrated Discovery (DAVID) version 6.7 (http://david.abcc.ncifcrf.gov/). Briefly, differentially expressed transcripts that were present at higher levels in bone marrow CLL cells than in peripheral blood CLL cells were first classified into functionally-related groups based on annotated Gene Ontology terms; the enrichment of specific terms in the list of differentially expressed transcripts was then statistically evaluated by comparing their observed frequency in the whole human genome. To allow the visualization of selected differentially expressed genes, microarray transcript levels were used to generate a heatmap (row-normalized sorting), using the HeatMap Builder software, available at http://ashleylab.stanford.edu/tools_scripts.html.

Quantitative real-time reverse transcriptase polymerase chain reaction analysis

RNA (500 ng) was used to produce single-stranded cDNA with the High Capacity cDNA Archive Kit (Applied BioSystems, Foster City, CA, USA), following the manufacturer's instructions. Pre-designed TaqMan probes and MasterMix (Applied Biosystems) were used to quantify the expression of *AURKA*, *AURKB*, *BIRC5*, *CDK6*, and *GAPDH* (reference gene) in a 7300 Real-Time PCR System (Applied Biosystems). The fold-difference in gene expression was calculated using the formula 2-ΔΔCt, where the ΔCt value was obtained by subtracting the mean Ct of the reference gene from the mean Ct of the target gene for each sample; the ΔΔCt value was calculated by subtracting, in each sample, the ΔCt of one peripheral blood CLL sample that was used as a calibrator.

Analysis of cell cycle, proliferation and apoptosis

MEC1 and EHEB cells were synchronized for 18 h with 2 mM thymidine (Sigma-Aldrich), washed with phosphate-buffered saline, cultured for 9 h in complete RPMI, and treated a second time with 2 mM thymidine for an additional 12 h. After the second incubation in thymidine, cells were washed twice in phosphate-buffered saline to release the thymidine block and resuspended in complete RPMI. Cells were harvested after 8 and 24 h, resuspended in DNA staining buffer (0.1% sodium citrate; 0.1% Triton X-100 and 50 μg/mL propidium iodide) by vortexing, incubated for 30 min at 4°C and analyzed for DNA content on a FACSCalibur cytometer (BD Biosciences). Cell doublets and debris were excluded from further analysis by gating individual cells in peak area *versus* peak width plots.

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-oligonucleotide/interleukin-2 and the cells were cultured for another 18 h before harvesting. Cells were washed

with phosphate-buffered saline and fixed overnight at -20°C in 70% ethanol. Cells were permeabilized with 2N HCl and 0.5% Triton X-100 for 30 min at room temperature and neutralized with 0.1M Na2B4O7 x 10H2O, pH 8.5. Incorporated BrdU was detected with anti-BrdU-fluorescein isothiocyanate (FITC) antibody (BD Biosciences) and flow cytometry.

The percentage of viable cells was determined by staining with annexin-A5-FITC conjugate (Nexins Research, Kattendijke, The Netherlands) and propidium iodide. Data were analyzed on a FACSCalibur flow cytometer with the CellQuest software (BD Biosciences). Viable cells are annexin V/propidium iodide double-negative.

Online Supplementary Table S1. (SEE PDF FILE)

Online Supplementary Table S2. Enriched Gene Ontology (GO) categories among up-regulated genes in BM-CLL cells.

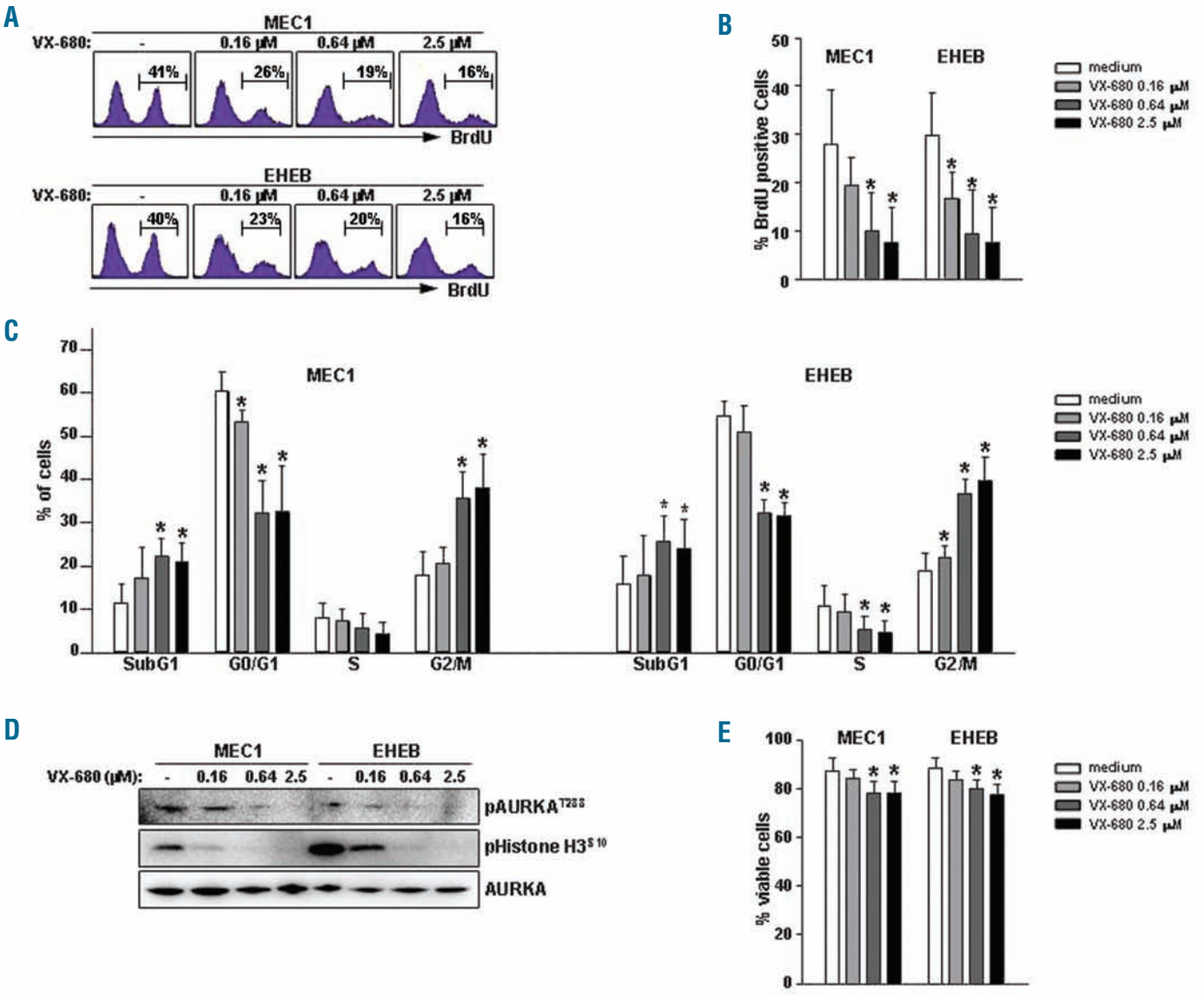
GO category ID	Function name	Number of genes/category*	*P* value
GO:0007049	cell cycle	16	1.25E-07
GO:0007067	mitosis	10	1.81E-06
GO:0000280	nuclear division	10	1.81E-06
GO:0000087	M phase of mitotic cell cycle	10	2.12E-06
GO:0048285	organelle fission	10	2.57E-06
GO:0000278	mitotic cell cycle	11	1.13E-05
GO:0022403	cell cycle phase	11	3.26E-05
GO:0022402	cell cycle process	12	5.55E-05
GO:0000279	M phase	10	5.9E-05
GO:0051301	cell division	9	0.000361
GO:0051726	regulation of cell cycle	9	0.00086
GO:0007059	chromosome segregation	6	0.00108
GO:0000075	cell cycle checkpoint	5	0.047612

**Out of the 37 genes identified in the DAVID database, from the 56 up-regulated in BM-CLL cells.*

Online Supplementary Table S3. Clinical and laboratory features of CLL patients and responses to CpG-ODN/IL2 and VX-680

Patient #	Age/ gender	Stage	VH gene (% homology)	ZAP-70	CD38*	FISH	% BrdU-positive cells				% Annexin V-negative cells			
							Unst	CpG/IL2 0.64 μM	CpG/IL2 VX-680 2.5 μM	CpG/IL2 VX-680	Unst	CpG/IL2 0.64 μM	CpG/IL2 VX-680 2.5 μM	CpG/IL2 VX-680
G6	M/66	A/0	V3-23 (92.6%)	pos.	7%	neg.	0.6	3.3	1.7	0.8	45	40	26	22
G42	M/50	A/0	V3-23 (91.9%)	pos.	1%	neg.	0.0	10.2	8.2	4.9	82	74	73	72
G68	M/48	A/II	V4-39 (93.3%)	neg.	7%	neg.	0.8	2.0	1.8	0.6	33	38	36	34
G148	M/75	A/0	V4-39 (96.7%)	pos.	1%	neg.	n.d.	n.d.	n.d.	n.d.	69	86	82	76
G155	M/68	A/0	V3-23 (91.5%)	pos.	1%	neg.	0.1	7.9	6.9	5.6	85	87	89	81
G160	M/64	A/0	V3-11 (100%)	pos.	20%	neg.	0.3	17.9	15.	6.0	58	66	62	45
G225	M/68	B/II	V4-34 (94.8%)	neg.	1%	del13q14	0.1	1.4	0.5	0.3	64	75	74	65
G248	M/62	B/II	V3-30 (100%)	pos.	63%	del11q22, del13q14	0.0	17.9	16.6	16.4	27	84	85	75
G267	M/74	B/II	n.d.	pos.	69%	neg.	0.0	6.9	5.0	0.1	77	63	64	65
G269	M/56	A/0	V3-74 (100%)	pos.	6%	del 13q14	0.1	6.6	6.6	4.4	46	65	68	55
G273	F/59	B/II	V1-08 (98.9%)	n.d.	1%	neg.	0.3	9.2	8.0	4.0	60	78	76	69
G275	M/65	A/0	V3-23 (95.0%)	neg.	12%	neg.	0.7	1.1	0.9	1.0	73	78	66	64
G290	F/70	A/0	V3-35 (100%)	neg.	12%	del13q14	0.7	14.5	10.0	0.9	51	79	78	79
G301	F/72	A/0	V3-48 (99%)	n.d.	1%	del11q22, del13q14	0.1	14.4	12.5	2.8	78	85	85	84
G304	F/60	B/I	V1-69 (100%)	pos.	21%	del11q22	0.1	3.8	2.8	1.3	89	94	95	95
G302	M/53	B/I	n.d.	neg.	1%	n.d.	2.50	40.0	29.0	8.0	n.d.	n.d.	n.d.	n.d.

Pos.: positive; neg.: negative; n.d.: not determined.



Online Supplementary Figure S1. The Aurora kinase inhibitor VX-680 blocks proliferation of EHEB and MEC1 cells and induces apoptosis. (A) Unsynchronized MEC1 and EHEB cells were incubated with the indicated concentrations of VX-680 for 48 h. The percentage of dividing cells was determined by BrdU incorporation analysis. (B) Summary of the results of five different BrdU incorporation experiments with MEC1 and five experiments with EHEB cells. Mean values ± SD are shown. Significant differences ($P<0.05$) with respect to cells cultured in the absence of VX-680 (medium) are indicated by asterisks. (C) Analysis of cell-cycle distribution in MEC1 and EHEB cells incubated with the indicated concentrations of VX-680. Cells were synchronized with thymidine and analyzed 24 h after release from thymidine block by propidium iodide staining and flow cytometry. 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 cultured in the absence of VX-680 are indicated by asterisks. (D) Analysis of Aurora A and Aurora B inhibition by VX-680. MEC1 and EHEB cells were incubated for 48 h with the indicated concentrations of VX-680 before lysis. Cellular extracts were analyzed by immunoblotting with phospho-AURKA^{T288} and phospho-Histone H3S10 antibodies to evaluate Aurora A and Aurora B kinase activity, respectively. Aurora A was used as a loading control. (E) Analysis of apoptosis induction by VX-680 in MEC1 and EHEB cells. Cells were analyzed by annexin V/propidium iodide staining and flow cytometry after 48 h in culture with the indicated concentrations of VX-680. The mean percentages (± SD) of viable, annexin V/propidium iodide-double negative cells are indicated. Data represent the results of five different experiments with MEC1 and five different experiments with EHEB cells. Significant differences ($P<0.05$) with respect to cells cultured in the absence of VX-680 are indicated by asterisks.