

## ORIGINAL ARTICLE

## Aurora kinases in childhood acute leukemia: the promise of aurora B as therapeutic target

SA Hartsink-Segers<sup>1,2</sup>, CM Zwaan<sup>1</sup>, C Exalto<sup>1</sup>, MWJ Luijendijk<sup>1</sup>, VS Calvert<sup>3</sup>, EF Petricoin<sup>3</sup>, WE Evans<sup>4</sup>, D Reinhardt<sup>5</sup>, V de Haas<sup>6</sup>, M Hedtjörn<sup>7</sup>, BR Hansen<sup>7</sup>, T Koch<sup>7</sup>, HN Caron<sup>2,8</sup>, R Pieters<sup>1,6</sup> and ML Den Boer<sup>1,2,6</sup>

We investigated the effects of targeting the mitotic regulators aurora kinase A and B in pediatric acute lymphoblastic leukemia (ALL) and acute myeloid leukemia (AML). Aurora protein expression levels in pediatric ALL and AML patient samples were determined by western blot and reverse phase protein array. Both kinases were overexpressed in ALL and AML patients ( $P < 0.0002$ ), especially in E2A-PBX1-translocated ALL cases ( $P < 0.002$ ), compared with normal bone-marrow mononuclear cells. Aurora kinase expression was silenced in leukemic cell lines using short hairpin RNAs and locked nucleic acid-based mRNA antagonists. Aurora B knockdown resulted in proliferation arrest and apoptosis, whereas aurora A knockdown caused no or only minor growth delay. Most tested cell lines were highly sensitive to the AURKB-selective inhibitor barasertib-hydroxyquinazoline-pyrazol-anilide (AZD1152-HQPA) in the nanomolar range, as tested with an MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) assay. But most importantly, primary ALL cells with a high aurora B protein expression, especially E2A-PBX1-positive cases, were sensitive as well. In adult AML early clinical trials, clear responses are observed with barasertib. Here we show that inhibition of aurora B, more than aurora A, has an antiproliferative and pro-apoptotic effect on acute leukemia cells, indicating that particularly targeting aurora B may offer a new strategy to treat pediatric ALL and AML.

Leukemia (2013) 27, 560–568; doi:10.1038/leu.2012.256

**Keywords:** aurora kinase; pediatric acute leukemia; AZD1152; LNA-oligonucleotide; gene silencing; targeted therapy

## INTRODUCTION

Acute leukemia accounts for approximately 30% of all pediatric cancers and is therefore the most common type of cancer in children. In approximately 85% of the cases, acute lymphoblastic leukemia (ALL) is diagnosed, in 15% acute myeloid leukemia (AML). Over the past decades, clinical outcome of pediatric acute leukemia has improved toward 5-year event-free survival rates of 80% for ALL and 60–70% for AML by the use of multiagent chemotherapy.<sup>1,2</sup> Targeting leukemia-specific molecular abnormalities may help further improve outcome and reduce adverse side effects of therapy.

Promising targets in this respect may be the members of the aurora kinase (AURK) family, which consists of three serine/threonine kinases: aurora A, B and C, encoded by the *AURKA*, *AURKB* and *AURKC* genes, respectively. Although aurora C is mainly expressed in the testis and associated with male fertility and meiosis, aurora A and B are both important regulators of mitosis. Despite sharing 71% identity in their C-terminal catalytic domains, aurora A and B have a different subcellular localization and perform essential and distinct functions during different phases of the cell cycle. Aurora A is active during late S and early G2 phase, localizing to the centrosomes and spindle poles, and ensuring proper spindle assembly and chromosome alignment during mitosis. Throughout G2, aurora B functions in a protein complex, mainly associating with microtubules near the kinetochores, and is responsible for bipolar attachment of the spindle to the

centromeres and correct segregation of the daughter chromatids. Subsequently, aurora B redistributes to the midbody where it has a key role in cytokinesis.<sup>3,4</sup>

Aberrant expression of aurora A and B is associated with chromosomal instability and dysfunctional cell division. Not surprisingly, amplification and overexpression of aurora kinases A and B are associated with tumorigenesis. Both aurora A and B are overexpressed in a large number of human solid tumors as well as leukemia cell lines and adult AML patients,<sup>5</sup> also showing a correlation with a poor prognosis in these patients.<sup>6,7</sup> The clinical value of aurora A and B expression in adult ALL and in pediatric leukemias is still unknown.

Several small-molecule inhibitors of aurora kinases induce apoptosis in cancer cell lines *in vitro* and *in vivo*. Hesperadin, ZM447439 and VX-680 (also known as MK-0457 or tozasertib)<sup>8–10</sup> were among the first aurora kinase inhibitors to be described. VX-680, a pan-aurora inhibitor, was the first to show antitumor activity *in vivo*,<sup>10</sup> but clinical trials were terminated because of side effects. Trials are currently ongoing for several aurora A- and B-selective inhibitors, among which are the second-generation aurora A-selective inhibitor MLN8237 (alisertib) and the aurora B-selective compound barasertib (AZD1152) (www.clinicaltrials.gov). Barasertib induces apoptosis in xenograft models of several cancer types, including lung, colon and AML.<sup>11</sup> Phase I/II clinical trials in adult AML show that barasertib has the ability to produce complete responses in relapsed/refractory and treatment-naive newly diagnosed elderly patients.<sup>12–14</sup>

<sup>1</sup>Department of Pediatric Oncology/Hematology, Erasmus MC/Sophia Children's Hospital, Rotterdam, The Netherlands; <sup>2</sup>Kids Cancer Kinome Consortium, Villejuif, France; <sup>3</sup>Center for Applied Proteomics and Molecular Medicine, George Mason University, Manassas, VA, USA; <sup>4</sup>St Jude Children's Research Hospital, Memphis, TN, USA; <sup>5</sup>Department of Pediatric Hematology/Oncology, Hannover Medical School, Hannover, Germany; <sup>6</sup>Dutch Childhood Oncology Group, The Hague, The Netherlands; <sup>7</sup>Santaris Pharma A/S, Hørsholm, Denmark and <sup>8</sup>Department of Pediatric Oncology, Academic Medical Center/Emma Children's Hospital, Amsterdam, The Netherlands. Correspondence: Dr ML Den Boer, Department of Pediatric Oncology/Hematology, Erasmus MC/Sophia Children's Hospital, Room Sp-2456, Dr Molewaterplein 60, Rotterdam 3015 GJ, The Netherlands. E-mail: m.l.denboer@erasmusmc.nl

Received 8 June 2012; revised 24 August 2012; accepted 27 August 2012; accepted article preview online 3 September 2012; advance online publication, 19 October 2012

Although adult studies are numerous, only few studies address the efficacy of aurora kinase inhibition in the treatment of childhood acute leukemia and no inhibitor has entered the clinic yet. To determine the potential of aurora A and B as a target for therapy, we investigated the expression of both kinases in large series of childhood ALL and AML patients and studied the effects of silencing these genes in these malignancies. Our study demonstrates that inhibition of aurora B in particular may be effective for the treatment of childhood acute leukemia.

## MATERIALS AND METHODS

More detailed descriptions of the Materials and Methods used are included as Supplementary Information on the *Leukemia* website.

### Cell-line culture

Cell lines were purchased from DSMZ (Braunschweig, Germany) and cultured in Roswell Park Memorial Institute medium + glutamax (Gibco BRL, Life Technologies, Bleiswijk, The Netherlands) (leukemia cell lines) or Dulbecco's Modified Eagle's medium + glutamax (Gibco BRL, Life Technologies) (HEK293T) supplemented with pen-strep (Gibco BRL, Life Technologies), fungizone (Gibco BRL, Life Technologies) and 10 or 20% fetal calf serum (Integro, Zaandam, The Netherlands). Cells were cultured in a humidified 37 °C incubator, with 5% CO<sub>2</sub>. RNA was isolated with an RNeasy minikit (Qiagen, Venlo, The Netherlands) according to the manufacturer's protocol.

### Patient samples

Bone marrow (BM) or peripheral blood samples from children with newly diagnosed ALL ( $n=521$ ) and AML ( $n=237$ ), and from children with no hematological disorder (normal BM (nBM)) ( $n=18$ ), were processed as described in the Supplementary Information. Written informed consent was obtained from patients and/or parents/guardians to use excess of diagnostic material for research purpose, as approved by the institutional review boards.

### Gene-expression arrays and normalization

RNA was extracted from patient samples with TRIzol reagent (Invitrogen, Life Technologies, Breda, The Netherlands) according to the manufacturer's protocol and gene expression analyzed by Affymetrix U133 GeneChips version A or plus 2.0 (Santa Clara, CA, USA). The original data files have been deposited in the Gene Expression Omnibus database (accession no. GSE13351, GSE13425 and GSE17855). Using the probe sets common to both chips, the data sets of ALL ( $n=297$ ), AML ( $n=237$ ) and nBM ( $n=8$ ) were normalized together using robust multichip average and variance stabilizing normalization 2. Batch effects were corrected for by applying the ComBat procedure. *AURKA*, *B* and *C* were represented by probe sets 204092\_s\_at, 209464\_at, and 211107\_s\_at, respectively. There was a good correlation between results obtained by gene-expression array and reverse transcription quantitative real-time PCR ( $r_s=0.77$  and  $P<0.0001$ ; Supplementary Figure 1).

### Lentiviral infections

pLKO.1 Mission short hairpin RNA (shRNA) vectors SHC002 (nonsilencing control vector), SHC005 (against eGFP), TRCN0000000655 (against *AURKA*) and TRCN0000000776 (against *AURKB*), containing a puromycin selection marker, were purchased from Sigma-Aldrich (Zwijndrecht, The Netherlands). Leukemia cell lines were spin-infected with a multiplicity of infection of 2.5 in the presence of polybrene (Sigma-Aldrich) and cultured for 24 h before selection on puromycin (Sigma-Aldrich). Cell viability was determined with a trypan blue exclusion assay.

### Locked nucleic acid (LNA)-oligonucleotide (LNA-oligo) transfections

Cell lines were cultured in the presence of 10  $\mu$ M LNA-oligos against *AURKA* (SPC5587), *AURKB* (SPC5549), a scrambled control oligo (SPC3088) or control medium only. Unassisted uptake of oligos ('gymnosis') after 48 h was assessed using a 6-carboxyfluorescein (FAM)-labeled LNA-oligo (SPC5059). Cytospins of transfected cells were stained with vectashield 4',6-diamidino-2-phenylindole (DAPI) and analyzed with fluorescent microscopy. Cell viability was determined with a trypan blue exclusion test.

### *In vitro* MTS assay for drug sensitivity

Cells were plated at a concentration of  $0.2-1 \times 10^6$  cells per ml in triplicate (cell lines) or  $1.6 \times 10^6$  per ml in duplicate (primary cells) and exposed to doses of barasertib-hydroxyquinazoline-pyrazol-anilide (Barasertib-HQPA; AstraZeneca, Cheshire, UK), PHA-739358 (danusertib; Selleck Chemicals, Munich, Germany), MLN8237 (Alisertib; Selleck Chemicals) or VX-680 (tozasertib; LC Laboratories, Woburn, MA, USA) ranging  $1 \text{ nM}-20 \mu\text{M}$ . Cells were incubated with MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulphophenyl)-2H-tetrazolium; Promega, Madison, WI, USA) and PMS (phenazine methosulfate; Sigma-Aldrich) after 72 h for 3 h (cell lines) or after 96 h for 4 h (primary cells), and absorbance was quantified at  $\lambda=490 \text{ nm}$  on a VersaMax microplate reader (Molecular Devices, Sunnyvale, CA, USA).

### Protein electrophoresis and western blot

A total of 20  $\mu$ g of protein was loaded onto gel and blotted onto nitrocellulose (Schleicher & Schuell, Dassel, Germany). Primary antibodies were from AbD Serotec (Kidlington, UK) (aurora A, no. MCA2249), Cell Signaling Technology (Danvers, MA, USA) (aurora B, no. 3094; cleaved poly (ADP-ribose) polymerase (PARP), no. 9541; glyceraldehyde 3-phosphate dehydrogenase (GAPDH), no. 2118) and Abcam (Cambridge, UK) ( $\beta$ -actin, ab6276). Proteins were detected with either an horseradish peroxidase-tagged secondary antibody (Cell Signaling Technology) and scanned with a Syngene Chemigenius (Cambridge, UK) or detected with a fluorescently labeled secondary antibody (LI-COR Biosciences, Lincoln, NE, USA) and scanned with an Odyssey Infrared Imaging System (LI-COR Biosciences). Signals were digitally quantified with subtraction of background.

### Reverse phase protein array

Lysates of 172 pediatric ALL patient samples and 10 nBM samples were spotted twice in triplicate on glass-backed nitrocellulose-coated array slides (FAST slides; Whatman Plc, Kent, UK). The slides were stained with an aurora A (AbD Serotec; no. MCA2249) or aurora B (Cell Signaling Technology; no. 3094) antibody, followed by incubation with a biotinylated secondary antibody. Slides were scanned using a NovaRay CCD fluorescent scanner (Alpha Innotech, San Leandro, CA, USA). Protein levels were calculated relative to the total amount of protein per sample.

### Statistical analyses

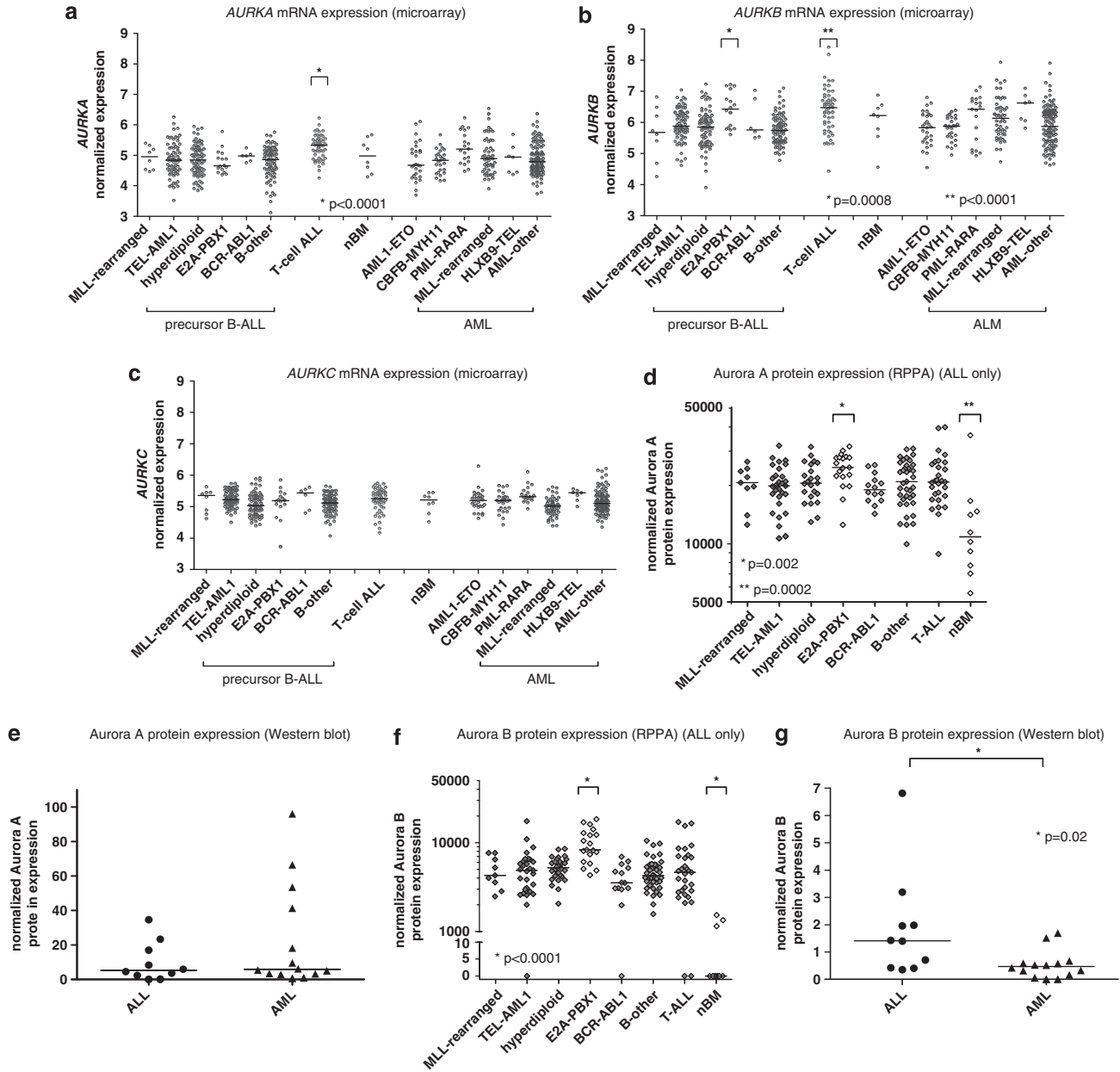
The nonparametric Mann-Whitney *U*-test was applied to compare expression levels between patient groups. Significance of correlation between the gene-expression array data and reverse transcription quantitative real-time PCR data, and between expression levels and drug sensitivity, was determined with a Spearman's rank correlation test. Cell-cycle distribution proportions of replicate experiments were compared with a paired Student's *t*-test.  $P<0.05$  was considered statistically significant.

## RESULTS

Aurora A and B proteins are overexpressed in pediatric ALL and AML cases

At the mRNA level, *AURKA*, *AURKB* and *AURKC* levels did not significantly differ between leukemic cells of patients and normal BM cells of healthy children (Figures 1a-c), but *AURKA* was differentially expressed in T-ALL patients ( $P<0.0001$ ) and *AURKB* both in T-ALL ( $P<0.0001$ ) and E2A-PBX1 patients ( $P=0.0008$ ) compared with (other) precursor B-ALL patients (Figures 1a and b). Because aurora C is believed to have a role mainly in meiosis in the testis, we further focused on the role of aurora A and B in leukemia.

At the protein level, aurora A was 1.9-fold more highly expressed in ALL than in nBM ( $P=0.0002$ ; Figure 1d) and expression levels in ALL and AML cases were comparable (Figure 1e; see Supplementary Figure 2 for original blots). Elevated protein expression of aurora B in leukemic cells was even more striking because seven out of ten nBM samples did not express aurora B protein at all ( $P<0.0001$ ; Figure 1f) and both ALL and AML cases had detectable protein levels more often than nBM (Figures 1f and g). Remarkably, protein expression levels of aurora A and especially aurora B were higher in precursor B-ALL cases with an E2A-PBX1 translocation than in cases without this translocation: 1.2 fold ( $P=0.002$ ; Figure 1d) and 1.8 fold ( $P<0.0001$ ; Figure 1f), respectively. No difference was seen between other genetic subtypes of ALL.



**Figure 1.** Expression patterns of aurora A, B and C in ALL and AML patients and nBM mononuclear cells. Gene expression of *AURKA* (a), *AURKB* (b) and *AURKC* (c) in genetic subtypes of ALL cases ( $n = 297$ ), AML cases ( $n = 237$ ) and nBM samples ( $n = 8$ ) was analyzed by gene-expression array. Asterisks indicate statistical significance with  $P < 0.01$  of nBM vs ALL or AML, T-ALL vs precursor B-ALL, and cytogenetic subtypes of precursor B-ALL or AML vs all other subtypes of the B-lymphoid or myeloid lineage. Reverse phase protein arrays (RPPAs) were performed for ALL ( $n = 172$ ) and nBM ( $n = 10$ ) samples to measure aurora A (d) and aurora B (f) protein levels. Values were corrected for total protein and background signals. Statistical significance was determined by comparing groups to all (other) ALL patients. Additionally, protein levels of aurora A (e) and aurora B (g) in ALL ( $n = 10$ ) and AML ( $n = 14$ ) samples were quantified with western blot analysis and corrected for total protein loading by GAPDH expression. Horizontal bars indicate median values. nBM, normal BM mononuclear cells.

The differential expression of aurora B in ALL was not likely caused by amplification or deletion of the *AURKB* gene, as array comparative genomic hybridization analysis of 49 precursor B-ALL and 87 T-ALL patients did not reveal any aberrations specific for any of the three aurora genes (Supplementary Information and Supplementary Figure 3).

shRNA-mediated silencing of *AURKB* in ALL and AML results in inhibition of cell growth and apoptosis

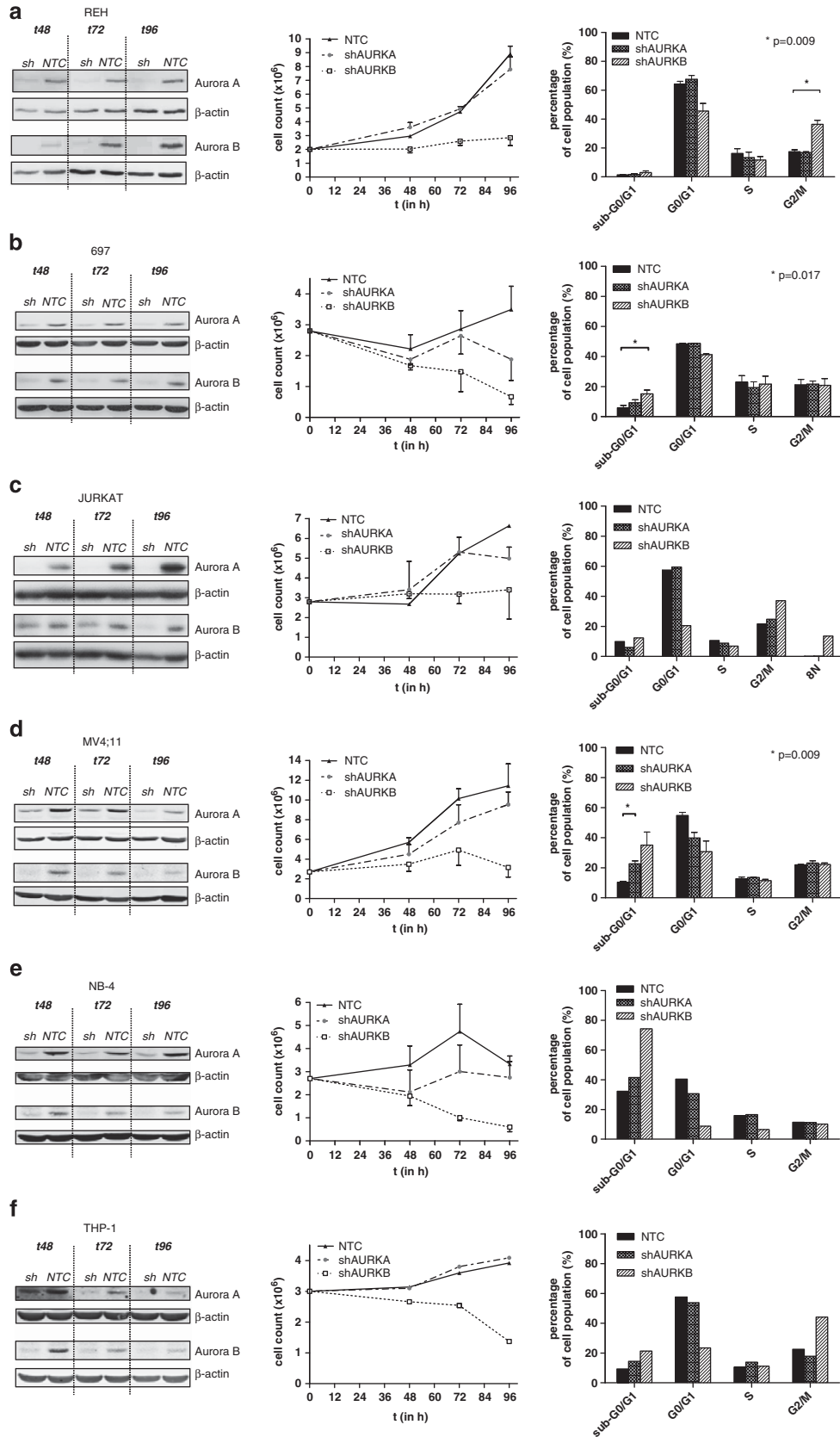
The expression of *AURKA* and *AURKB* was silenced in five pediatric and one adolescent leukemic cell line (NB-4) using shRNAs.

Nontargeting control shRNAs were used as a control for infection and off-target RNA interference effects.

Aurora A protein levels were reduced within 96 h by at least 47% (THP-1) and up to 97% (JURKAT), and aurora B protein levels by 69% (THP-1) up to 96% (JURKAT) (Figure 2; left panels). In all cell lines, knockdown of aurora B clearly affected cell growth by growth arrest and/or induction of apoptosis (Figure 2; middle panels). Cleaved PARP was detected in all cell lines 72 h after aurora B silencing (Supplementary Figure 4), indicating that apoptosis was initiated. Aurora A silencing did not affect or only moderately affected cell growth (Figure 2; middle panels).

In line with the modest phenotypic effects of aurora A knockdown on cell proliferation, no profound effect on cell-cycle distribution was observed after 72 h of aurora A silencing. In

contrast, we observed various cell-cycle effects of aurora B knockdown (Figure 2; right panels). In JURKAT, a tetraploid cell line, silencing of aurora B resulted in a small population of 8N cells,





containing a double amount of DNA. In the other cell lines, cells tended to accumulate in the apoptotic sub-G0/G1 phase (697, MV4;11, NB-4 and THP-1) and/or in the G2/M phase (REH, JURKAT and THP-1), but not in the G1 or S phase. This is in correspondence with the function of aurora B in the spindle checkpoint during the metaphase of the cell cycle and suggests that some cell lines accumulate in G2/M upon loss of aurora B, whereas others become apoptotic and may leave the cell cycle at any stage.

Effects of LNA-based mRNA antagonists of *AURKA* or *B* are comparable to silencing by shRNAs

Next, we silenced *AURKA* or *AURKB* expression using target-specific LNA-based mRNA antagonists (LNA-oligos). Transfection of a fluorescently labeled LNA-oligo showed that these oligonucleotides efficiently accumulate in cell lines within 48 h (Figure 3a). LNA-oligos against *AURKA* and *AURKB* decreased their target mRNA and protein expression levels within 96 h of exposure by at least 75% compared with a scrambled control LNA-oligo, whereas the *AURKA* LNA-oligo did not decrease *AURKB* mRNA levels and vice versa, confirming specificity (Figure 3b). Phosphorylation of histone H3 (Ser10), a known downstream target of aurora B, was reduced upon *AURKB* but not *AURKA* knockdown (Supplementary Figure 5).

ALL and AML cell lines were far more sensitive to the *AURKB* LNA-oligo than to the *AURKA* LNA-oligo. Targeting aurora B resulted in proliferation arrest and cell death, whereas aurora A knockdown only led to a slight growth delay in most cell lines (Figure 3c). This is in line with the effects observed with *AURK*-specific shRNAs (Figure 2, middle panels). Knocking down both aurora A and B simultaneously did not additively or synergistically reduce growth compared with silencing of aurora B only (Figure 3d). These findings suggest that aurora B may be a more relevant target in pediatric ALL and AML than aurora A.

Cell lines and patients with a high aurora B expression tend to be sensitive to barasertib-HQPA

We compared the efficacy of the pan-aurora kinase inhibitors VX-680 and danusertib, aurora A-selective inhibitor MLN8237 and aurora B-selective inhibitor barasertib-HQPA (the more active metabolite of barasertib) in REH, 697 and NALM-6 (ALL) and MV4;11 (AML). All compounds inhibited cell growth when used in concentrations in the low to mid-nanomolar range, with cell lines being comparably sensitive to VX-680, MLN8237 and barasertib-HQPA, and relatively resistant to danusertib (Supplementary Figure 6). Because silencing experiments with shRNA and LNA mRNA antagonists identified aurora B as suitable target, the efficacy of aurora B-selective barasertib-HQPA was tested in a range of ALL and AML cell lines. Exposure to barasertib-HQPA induced a phenotype typically associated with aurora B inhibition: cells accumulated in G2/M phase or sub-G0/G1 phase similarly to observations made upon silencing with shAURKB (Supplementary Figure 7a). In addition, phosphorylation of histone H3 (Ser10) was reduced (Supplementary Figure 7B). Eighteen out of twenty cell lines tested were equally sensitive to growth inhibition by barasertib-HQPA in the nanomolar range (GI50 = 19–233 nM; median 65 nM) (Figure 4a). Only JURKAT (T-ALL) and THP-1 (MLL-rearranged AML) were highly resistant to this drug, with GI50

values in the micromolar range (8.4 and 16  $\mu$ M, respectively). There was no correlation between sensitivity to barasertib-HQPA and aurora B mRNA or protein levels.

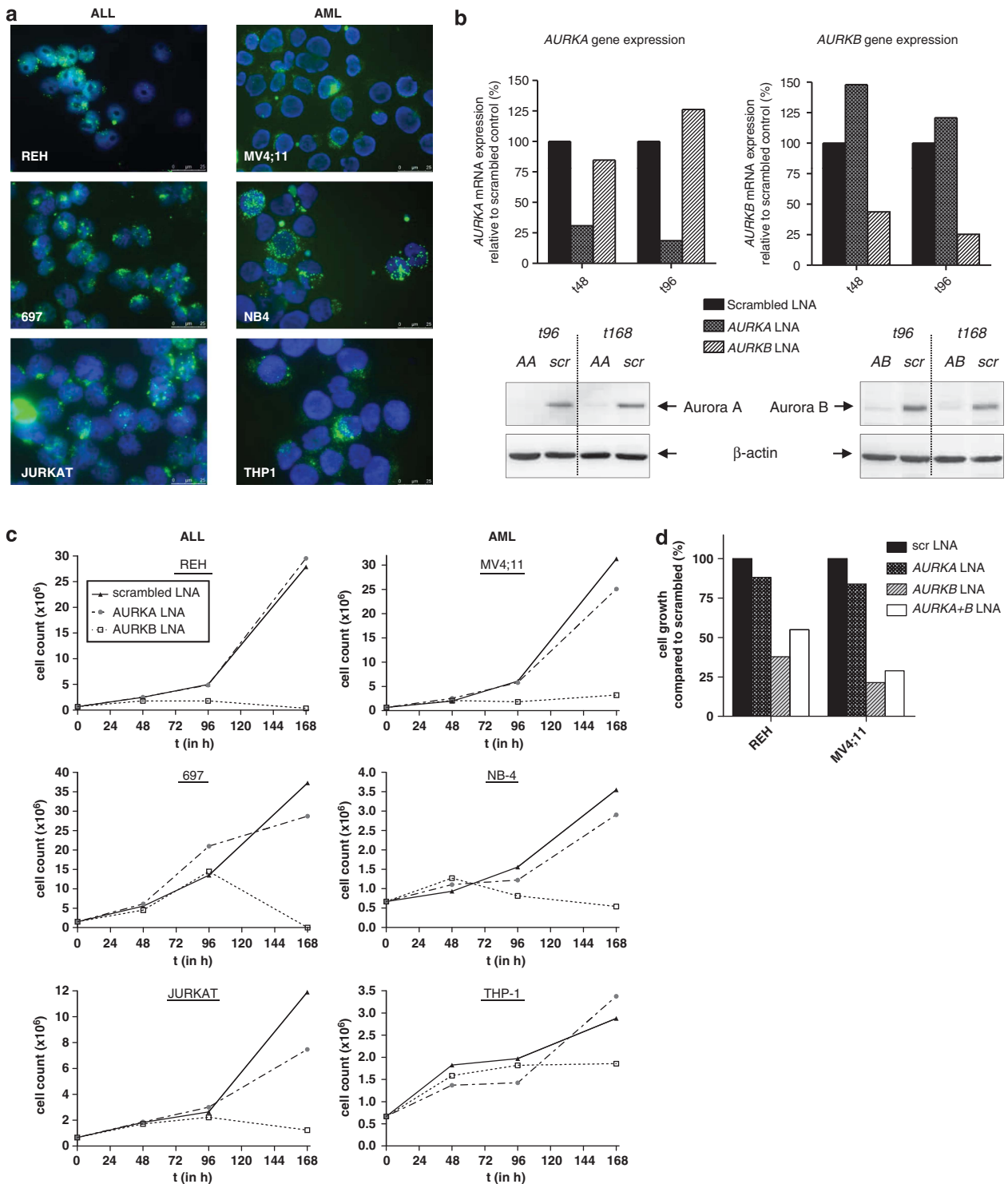
We next exposed eight primary ALL patient samples with a high aurora B protein expression and eight with a low expression to barasertib-HQPA *in vitro*. Cell survival reversely correlated with aurora B protein expression level ( $r_s = -0.532$  and  $P = 0.034$ ; Figure 4b); patient samples with a high aurora B expression were more sensitive to barasertib-HQPA than those with a low expression ( $P = 0.046$ ; Figure 4c). The most sensitive patient samples comprised two E2A-PBX1-translocated cases, a hyperdiploid and a karyotypically normal case, but E2A-PBX1-positive cells appeared to be more sensitive ( $P = 0.037$ ) than samples without this translocation (Figure 4c and Supplementary Figure 8).

## DISCUSSION

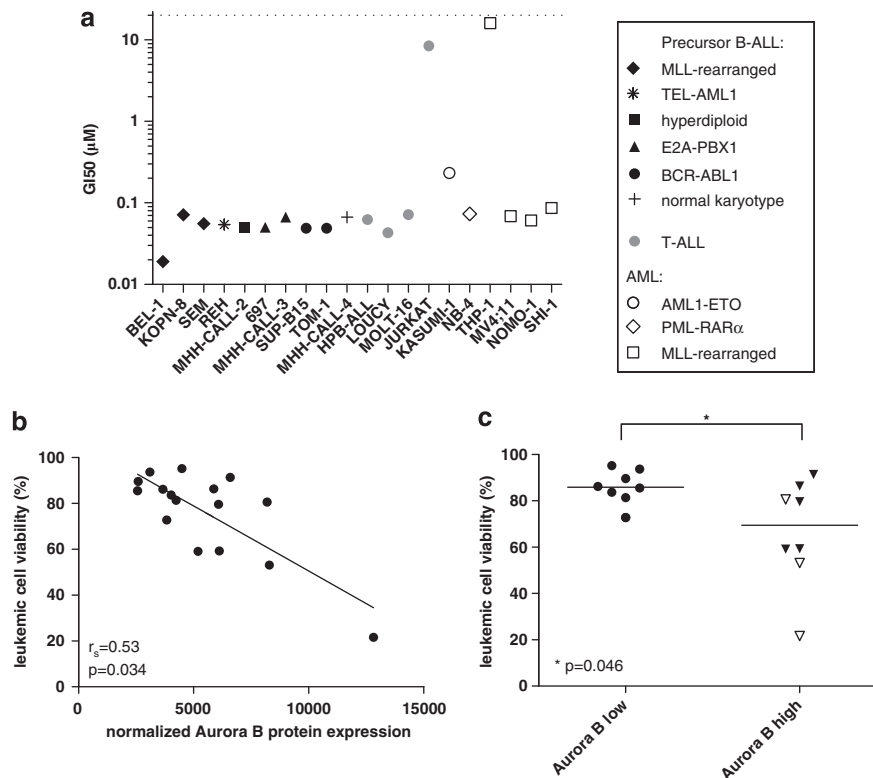
With leukemia being the most prevalent of cancers among children, and the medical world striving to improve clinical outcome achieved with the current treatment strategies and reduce long-term side effects among cancer survivors, there is a great medical need to improve therapy options for children with ALL or AML. This study aimed to investigate aurora kinases A and B as potential therapeutic targets for the treatment of childhood ALL and AML, and has shown that (1) aurora B, more so than aurora A, is an essential protein for proliferation and survival of acute leukemia cells, (2) both ALL and AML cell lines are sensitive to *AURKB* LNA-oligos and the aurora B-selective inhibitor barasertib-HQPA *in vitro* and (3) primary ALL patient samples with high protein levels of aurora B are more sensitive to barasertib-HQPA than those with low expression of aurora B.

Of the three aurora kinase family members A, B and C, most studies have focused their attention on aurora A, showing its relevance to oncogenesis. Surprisingly, our findings indicate that aurora A is of less importance in childhood ALL and AML than aurora B. First, the difference in aurora A protein expression levels between acute leukemia patients and healthy controls was not as profound as that of aurora B. This is also the case in adult ALL, where only some patients express aurora A and only at a low level compared with adult AML.<sup>5</sup> Second, compared with aurora B inhibition, we observed no or a delayed phenotypic effect of aurora A inhibition in acute leukemia cell lines, as shown by normal cell-cycle distribution and modest effects on cell proliferation. Taken together, these results suggest that aurora A is functionally less significant for pediatric acute leukemia cells and may therefore be a less likely choice as target for therapy. This is in contrast to many adult and pediatric solid tumors as well as adult AML,<sup>5,15</sup> in which aurora A is aberrantly expressed and a promising target for treatment as shown by many *in vitro* and *in vivo* inhibitor studies (reviewed by Dar *et al.*<sup>16</sup>). Interestingly though, our cell lines seem equally sensitive to growth-inhibitory effects of the aurora A-inhibitor MLN8237 and aurora B-inhibitor barasertib-HQPA, and Maris *et al.*<sup>17</sup> recently achieved tumor reduction with MLN8237 in murine xenograft models for pediatric ALL. It is known that the methodological differences between a small-molecule kinase inhibitor and RNA interference can cause different phenotypic effects. The former is usually directed against the kinase function of the protein, whereas the latter also

**Figure 2.** Effect of short hairpin-mediated aurora A or B silencing in ALL and AML cell lines. Left panels show western blot analysis of aurora A or B knockdown in three ALL and three AML cell lines, respectively: REH (a), 697 (b), JURKAT (c), MV4;11 (d), NB-4 (e) and THP-1 (f). Brightness and contrast of images was optimized and dotted lines indicate where separated samples from the same gel were grouped. Blots are representative of multiple independent experiments. Middle panels show the effect of aurora A and B silencing on cell growth. For a–e, graphs present the mean of multiple experiments and error bars indicate one side of the s.e. of the mean. Right panels present effects on cell-cycle distribution measured by flow-cytometry analysis of propidium iodide incorporation after 72 h of aurora kinase knockdown. NTC, nontargeting control short hairpin; sh/AURK, short hairpin against *AURK*.



**Figure 3.** The *in vitro* efficacy of *AURK*-targeting LNA-oligos in ALL and AML cell lines. Cells were incubated with  $10\ \mu\text{M}$  of LNA-oligos complementary to *AURKA* or *AURKB* or a scrambled control. **(a)** Microscopic fluorescent imaging of the uptake of a FAM-labeled LNA-oligo by ALL (left) and AML cell lines (right) after 48 h of exposure. Nuclei were stained blue with DAPI. FAM is depicted in green. Bars represent  $25\ \mu\text{m}$ . **(b)** Target-specific reduction of aurora A (left) and aurora B (right) mRNA (upper panels) and protein (lower panels) after exposure of REH cells to an *AURKA*, *AURKB* or scrambled control LNA-oligo. Gene expression was determined relative to housekeeping gene RPS20 and was set at 100% for scrambled controls. Brightness and contrast of protein images was optimized and dotted lines indicate where separated samples from the same gel were grouped. **(c)** Effects of LNA-mediated aurora A or B knockdown on growth of ALL (left) and AML cell lines (right). **(d)** Effect on cell growth of simultaneous aurora A and B knockdown by LNA-oligos. REH (ALL) and MV4;11 (AML) were incubated for 96 h with  $5\ \mu\text{M}$  LNA-oligos targeting *AURKA* and/or  $5\ \mu\text{M}$  LNA-oligos targeting *AURKB*. Cell growth was determined by trypan blue exclusion and expressed as a percentage of the cell count upon incubation with an equal concentration of a scrambled nontargeting LNA-oligo. AA, LNA-oligo targeting *AURKA*; AB, LNA-oligo targeting *AURKB*; scr, scrambled nontargeting LNA-oligo.



**Figure 4.** Sensitivity of ALL and AML cell lines and primary patient cells to barasertib-HQPA. Cell lines were exposed in triplicate for 72 h and primary cells in duplicate for 96 h to 0.001–20  $\mu\text{M}$  of barasertib-HQPA. Growth or viability was determined with an MTS assay and calculated as the percentage of live cells compared with unexposed cells. **(a)** Drug concentrations necessary to inhibit growth of leukemic cell lines by 50% (GI50) within 72 h, calculated from the average of at least three experiments. Symbols represent different leukemic subtypes. The dashed horizontal line indicates the maximum concentration tested. **(b)** Correlation plot between aurora B protein expression and viability of 16 ALL patient samples when exposed to 78 nM barasertib-HQPA for 96 h. **(c)** Cell viability after exposure of primary leukemic samples, including E2A-PBX1-positive cases (open triangles), with low vs high aurora B protein expression. The median aurora B protein expression of 172 patients evaluated by RPPA was taken as a cut off for selecting cases with high and low expression for the MTS assay. Horizontal bars indicate the median of the presented data.

interferes with the architecture of protein complexes where the target takes part in.<sup>18</sup> This functional difference may explain why aurora A-targeting drugs do seem to have clinical potential in childhood leukemia, whereas our study shows that silencing of *AURKA* expression has only a minor impact on leukemic cell lines compared with *AURKB* silencing.

A nonredundant role for aurora B in maintenance of the leukemic cell is supported by our findings that knocking down *AURKB* results in inhibition of cell growth and apoptosis in our cell-line models. Although some cell lines accumulate in mitotic state, polyploidy is not observed for most. Indeed, it has been described that, perhaps among other contributing factors, the p53 and p73 status of cells may determine whether aurora B inhibition leads to polyploidy or cell-cycle arrest and apoptosis.<sup>18,19</sup> We showed that most leukemic cell lines in this study are sensitive to low concentrations of barasertib-HQPA and that this sensitivity does not seem to correlate with aurora B expression. This is consistent with previous studies in AML cell lines.<sup>20–22</sup> Two out of twenty tested cell lines, JURKAT and THP-1, are relatively resistant to barasertib-HQPA, although functional studies with shRNAs and LNA-oligos in these cell lines clearly show an antiproliferative effect of *AURKB* silencing. These two discordant cell lines have in common that they are tetraploid, in contrast to the other cell lines tested. This might render the barasertib-HQPA compound less toxic in these two cell lines. In addition, barasertib-HQPA may induce senescence rather than being toxic to these cell lines, as recently being shown for THP-1 by Oke *et al*.<sup>22</sup> This again

illustrates the potentially different phenotypic effects of small-molecule inhibitors and RNA interference.

Similarly to adult solid tumors<sup>23–27</sup> and adult acute leukemias,<sup>5</sup> we show that aurora B protein is overexpressed in pediatric acute leukemia and that primary pediatric leukemia samples display a differential sensitivity to barasertib-HQPA *in vitro*, which correlates with aurora B expression levels. In this respect, the high aurora B protein expression in precursor B-ALL cells with an E2A-PBX1 translocation is of special interest, for it may indicate that E2A-PBX1-positive patients may benefit even more from aurora B inhibition. Indeed, silencing of aurora B induced cell death most rapidly in 697 cells, carrying the E2A-PBX1 translocation (Figure 2b), and E2A-PBX1-positive primary cells, which highly expressed aurora B protein, seemed to be more sensitive to barasertib-HQPA (Figure 4c). A direct relation between the presence of the E2A-PBX1 fusion protein and elevated expression of aurora B has not been established at this point but definitely warrants further investigation. Nonetheless, extrapolating the differential sensitivity of primary patient samples to barasertib-HQPA to the *in vivo* situation would suggest that barasertib could effectively target leukemic cells of a group of patients including but not limited to E2A-PBX1-translocated ALL cases.

The differential aurora B protein expression between leukemic cells and normal BM cells that we show in this study may offer a therapeutic advantage when a targeted drug is aurora B-specific. Phase I and II clinical trials currently address the toxicity and efficacy of the aurora B-selective inhibitor barasertib in adult solid

tumors and adult AML. Studies in adult patients with advanced solid tumors were terminated because of a lack of objective tumor response and dose-limiting toxicities like neutropenia and leukopenia at relatively low doses.<sup>28</sup> In contrast, studies in relapsed and newly diagnosed adult AML patients with a very poor prognosis reported well-tolerated doses up to 1200 mg and remarkable clinical response rates of 19–25%,<sup>12,14</sup> or 43% in the combination of barasertib with cytosine arabinoside.<sup>13</sup> At the moment, other pivotal studies in AML are underway.

Small-molecule inhibitors in general often interfere with proteins other than their main target, increasing the chance of side effects in clinical practice. LNA-based mRNA antagonists may offer an alternative and more specific way to silence a gene. Several studies have reported a clinical potential for LNA antagonists in cancer, such as those targeting BCL-2, ERBB-3 and HIF-1A.<sup>29–31</sup> Three LNA-based mRNA antagonists are currently being tested in phase I early clinical trials, one of them being EZN-3042, an inhibitor of the aurora B-binding partner Survivin, in children with relapsed ALL (NCT01186328). We have in this study shown that LNA-oligos accumulate and actively silence targeted genes in ALL and AML cells without further formulation, a process called gymnosis.<sup>32</sup> The phenotype obtained *in vitro* with gymnotic delivery more closely mimics the situation *in vivo* where LNA-based oligonucleotides have to enter cells without delivery vehicles. Our study indicated that targeting *AURKB* by LNA-based antagonists effectively induced growth arrest and apoptosis in acute leukemia cell lines similarly to the clinically less applicable lentiviral delivery of shRNAs. Together with the emerging clinical opportunities of LNA-based antagonists, these data imply a second option besides conventional small-molecule inhibitors for addressing the therapeutic value of aurora B silencing in children with ALL and AML.

In conclusion, there is a great therapeutic potential for aurora kinase inhibition in childhood acute leukemia. Moreover, we have shown that downregulation of *AURKB* expression targets pediatric ALL and AML more effectively than downregulation of *AURKA*. Contributing to the constant search for alternative therapies that improve long-term clinical outcome and reduce side effects of treatment, these findings offer a rationale for focusing on aurora B-targeting strategies in the treatment of children with acute leukemia.

## CONFLICT OF INTEREST

MH, BRH and TK are employed at Santaris Pharma A/S, Hørsholm, Denmark. The other authors declare no conflict of interest.

## ACKNOWLEDGEMENTS

We thank Santaris (Hørsholm, Denmark) for developing the LNA-oligos and AstraZeneca (London, UK) for providing the barasertib-HQPA compound. We thank all the members of the Kids Cancer Kinome consortium for their fruitful discussions and exchange of technical skills. This project was partly funded by the European Union FP6–2005-call (project LIFESCIHEALTH-6037390, KidsCancerKinome) and the KIKa foundation 'Kinderen Kankervrij'.

## REFERENCES

- Pui CH, Carroll WL, Meshinchi S, Arceci RJ. Biology, risk stratification, and therapy of pediatric acute leukemias: an update. *J Clin Oncol* 2011; **29**: 551–565.
- Kaspers GJL, Zwaan CM. Pediatric acute myeloid leukemia: towards high-quality cure of all patients. *Haematologica* 2007; **92**: 1519–1532.
- Fu J, Bian M, Jiang Q, Zhang C. Roles of aurora kinases in mitosis and tumorigenesis. *Mol Cancer Res* 2007; **5**: 1–10.
- Lens SMA, Voest EE, Medema RH. Shared and separate functions of polo-like kinases and aurora kinases in cancer. *Nat Rev Cancer* 2010; **10**: 825–841.
- Ikezoe T, Yang J, Nishioka C, Tasaka T, Taniguchi A, Kuwayama Y *et al*. A novel treatment strategy targeting aurora kinases in acute myelogenous leukemia. *Mol Cancer Ther* 2007; **6**: 1851–1857.

- Ye D, Garcia-Manero G, Kantarjian HM, Xiao L, Vadhan-Raj S, Fernandez MH *et al*. Analysis of aurora kinase A expression in CD34(+) blast cells isolated from patients with myelodysplastic syndromes and acute myeloid leukemia. *J Hematop* 2009; **2**: 2–8.
- Lucena-Araujo AR, de Oliveira FM, Leite-Cueva SD, dos Santos GA, Falcao RP, Rego EM. High expression of AURKA and AURKB is associated with unfavorable cytogenetic abnormalities and high white blood cell count in patients with acute myeloid leukemia. *Leuk Res* 2011; **35**: 260–264.
- Hauf S, Cole RW, LaTerra S, Zimmer C, Schnapp G, Walter R *et al*. The small molecule Hesperadin reveals a role for aurora B in correcting kinetochore-microtubule attachment and in maintaining the spindle assembly checkpoint. *J Cell Biol* 2003; **161**: 281–294.
- Ditchfield C, Johnson VL, Tighe A, Ellston R, Haworth C, Johnson T *et al*. Aurora B couples chromosome alignment with anaphase by targeting BubR1, Mad2, and Cenp-E to kinetochores. *J Cell Biol* 2003; **161**: 267–280.
- Harrington EA, Bebbington D, Moore J, Rasmussen RK, Ajose-Adeogun AO, Nakayama T *et al*. VX-680, a potent and selective small-molecule inhibitor of the Aurora kinases, suppresses tumor growth *in vivo*. *Nat Med* 2004; **10**: 262–267.
- Wilkinson RW, Odedra R, Heaton SP, Wedge SR, Keen NJ, Crafter C *et al*. AZD1152, a selective inhibitor of aurora B kinase, inhibits human tumor xenograft growth by inducing apoptosis. *Clin Cancer Res* 2007; **13**: 3682–3688.
- Löwenberg B, Muus P, Ossenkoppele G, Rousselot P, Cahn JY, Ifrah N *et al*. Phase I/II study to assess the safety, efficacy, and pharmacokinetics of barasertib (AZD1152) in patients with advanced acute myeloid leukemia. *Blood* 2011; **118**: 6030–6036.
- Kantarjian HM, Sekeres MA, Ribrag V, Rousselot P, Garcia-Manero G, Jabbour E *et al*. Phase I study to assess the safety and tolerability of AZD1152 in combination with low dose cytosine arabinoside in patients with acute myeloid leukemia (AML). *Blood (ASH Annual Meeting Abstracts)* 2010; **116**: Abstract 656.
- Tsuboi K, Yokozawa T, Sakura T, Watanabe T, Fujisawa S, Yamauchi T *et al*. A phase I study to assess the safety, pharmacokinetics and efficacy of barasertib (AZD1152), an aurora B kinase inhibitor, in Japanese patients with advanced acute myeloid leukemia. *Leuk Res* 2011; **35**: 1384–1389.
- Huang XF, Luo SK, Xu J, Li J, Xu DR, Wang LH *et al*. Aurora kinase inhibitory VX-680 increases Bax/Bcl-2 ratio and induces apoptosis in Aurora-A-high acute myeloid leukemia. *Blood* 2008; **111**: 2854–2865.
- Dar AA, Goff LW, Majid S, Berlin J, El-Rifai W. Aurora kinase inhibitors—rising stars in cancer therapeutics? *Mol Cancer Ther* 2010; **9**: 268–278.
- Maris JM, Morton CL, Gorlick R, Kolb EA, Lock R, Carol H *et al*. Initial testing of the aurora kinase A inhibitor MLN8237 by the Pediatric Preclinical Testing Program (PPTP). *Pediatr Blood Cancer* 2010; **55**: 26–34.
- Keen N, Taylor S. Aurora-kinase inhibitors as anticancer agents. *Nat Rev Cancer* 2004; **4**: 927–936.
- Ikezoe T, Yang J, Nishioka C, Yokoyama A. p53 is critical for the aurora B kinase inhibitor-mediated apoptosis in acute myelogenous leukemia cells. *Int J Hematol* 2010; **91**: 69–77.
- Walsby E, Walsh V, Pepper C, Burnett A, Mills K. Effects of the aurora kinase inhibitors AZD1152-HQPA and ZM447439 on growth arrest and polyploidy in acute myeloid leukemia cell lines and primary blasts. *Haematologica* 2008; **93**: 662–669.
- Yang J, Ikezoe T, Nishioka C, Tasaka T, Taniguchi A, Kuwayama Y *et al*. AZD1152, a novel and selective aurora B kinase inhibitor, induces growth arrest, apoptosis, and sensitization for tubulin depolymerizing agent or topoisomerase II inhibitor in human acute leukemia cells *in vitro* and *in vivo*. *Blood* 2007; **110**: 2034–2040.
- Oke A, Pearce D, Wilkinson RW, Crafter C, Odedra R, Cavenagh J *et al*. AZD1152 rapidly and negatively affects the growth and survival of human acute myeloid leukemia cells *in vitro* and *in vivo*. *Cancer Res* 2009; **69**: 4150–4158.
- Ikezoe T, Takeuchi T, Yang J, Adachi Y, Nishioka C, Furihata M *et al*. Analysis of aurora B kinase in non-Hodgkin lymphoma. *Lab Invest* 2009; **89**: 1364–1373.
- Chen YJ, Chen CM, Twu NF, Yen MS, Lai CR, Wu HH *et al*. Overexpression of aurora B is associated with poor prognosis in epithelial ovarian cancer patients. *Virchows Arch* 2009; **455**: 431–440.
- Vischioni B, Oudejans JJ, Vos W, Rodriguez JA, Giaccone G. Frequent overexpression of aurora B kinase, a novel drug target, in non-small cell lung carcinoma patients. *Mol Cancer Ther* 2006; **5**: 2905–2913.
- Tatsuka M, Katayama H, Ota T, Tanaka T, Odashima S, Suzuki F *et al*. Multi-nuclearity and increased ploidy caused by overexpression of the aurora- and Ipl1-like midbody-associated protein mitotic kinase in human cancer cells. *Cancer Res* 1998; **58**: 4811–4816.
- Chieffi P, Cozzolino L, Kisslinger A, Libertini S, Staibano S, Mansueto G *et al*. Aurora B expression directly correlates with prostate cancer malignancy and influence prostate cell proliferation. *Prostate* 2006; **66**: 326–333.



- 28 Boss DS, Witteveen PO, van der Sar J, Lolkema MP, Voest EE, Stockman PK *et al*. Clinical evaluation of AZD1152, an i.v. inhibitor of aurora B kinase, in patients with solid malignant tumors. *Ann Oncol* 2011; **22**: 431–437.
- 29 Dürig J, Dührsen U, Klein-Hitpass L, Worm J, Hansen JBR, Orum H *et al*. The novel antisense Bcl-2 inhibitor SPC2996 causes rapid leukemic cell clearance and immune activation in chronic lymphocytic leukemia. *Leukemia* 2011; **25**: 638–647.
- 30 Zhang Y, Qu Z, Kim S, Shi V, Liao B, Kraft P *et al*. Down-modulation of cancer targets using locked nucleic acid (LNA)-based antisense oligonucleotides without transfection. *Gene Ther* 2011; **18**: 326–333.
- 31 Greenberger LM, Horak ID, Filipula D, Sapra P, Westergaard M, Frydenlund HF *et al*. A RNA antagonist of hypoxia-inducible factor-1alpha, EZN-2968, inhibits tumor cell growth. *Mol Cancer Ther* 2008; **7**: 3598–3608.
- 32 Stein CA, Hansen JB, Lai J, Wu S, Voskresenskiy A, Høg A *et al*. Efficient gene silencing by delivery of locked nucleic acid antisense oligonucleotides, unassisted by transfection reagents. *Nucleic Acids Res* 2010; **38**: e3.



This work is licensed under the Creative Commons Attribution-NonCommercial-No Derivative Works 3.0 Unported License. To view a copy of this license, visit <http://creativecommons.org/licenses/by-nc-nd/3.0/>

Supplementary Information accompanies the paper on the Leukemia website (<http://www.nature.com/leu>)