PLK1 Expression and BI 2536 Effects in Childhood Acute Lymphoblastic Leukemia

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Background. Polo-like kinase 1 (PLK1) is a conserved kinase that mediates various mitotic events. Compelling data have repeatedly demonstrated its upregulation in different neoplasia, being frequently associated with poor prognosis. However, in childhood acute lymphoblastic leukemia (ALL), no studies have yet been conducted.

Procedure. PLK1 expression and association with biological features were evaluated in 65 consecutively diagnosed childhood ALL samples by quantitative real-time PCR. Moreover, the effects of a specific PLK1 inhibitor, BI 2536, was tested against a panel of nine ALL cell lines at nanomolar concentrations (10, 50, 100 nM). Results. The mRNA expression of PLK1 showed great variability in pediatric ALL, but no difference was evidenced compared to normal bone marrow. Additionally, no association was found between PLK1 mRNA expression with any clinical or biological features. Alternatively, high mRNA expression of PLK1 was present in ALL cell lines. In vitro treatment with BI 2536 strongly diminished growth, while presenting significant reduction in colony formation capacity and increased apoptosis rates. Moreover, strong G2/M arrest was detected suggesting important impaired proliferation after treatment.

Conclusions. PLK1 mRNA expression level is not associated with prognosis in childhood ALL; however, considering the great variability observed in the sample and the in vitro experiments presented herein, BI 2536 treatment might serve as a promising therapeutic to enhance the efficacy of conventional treatment modalities in some childhood ALL cases.

Key words: BI 2536; cell cycle; childhood acute lymphoblastic leukemia; PLK1 expression

INTRODUCTION

Acute lymphoblastic leukemia (ALL) represents the most common type of childhood malignant neoplasia and, despite the significant progress in current treatment, 20–30% of affected children relapse [1]. Post-relapse survival for most of these patients remains dismal. Consequently, novel therapeutic approaches are still needed to improve the outcome in this cohort.

Polo-like kinase 1 (PLK1) is a conserved serine/threonine-protein kinase that mediates various mitotic events, including mitotic entry, spindle formation and chromosome segregation [2]. A compelling body of evidence supports PLK1 upregulation in different neoplasia which is generally associated with poor prognosis [3–7].

In malignant hematopoietic cells, aberrantly PLK1 mRNA expression (compared with normal bone marrow mononuclear cells) was previously confirmed in human acute myelogenous leukemia (AML) and in a minor sample of adult patients with ALL (n = 15) [8]. Further validation of growth arrest and apoptosis after in vitro PLK1 inhibition by GW843682X in NB4 cells (acute promyelocytic leukemia) was also corroborated [8], suggesting a possible role of PLK1 in leukemia, though no studies have been conducted for pediatric ALL.

Based on such findings, the aim of this study was to evaluate the PLK1 gene expression in bone marrow samples from children with ALL and to correlate its expression levels with biological and prognostic features along with the evaluation of the effects of a specific PLK1 inhibitor, BI 2536, in a panel of childhood ALL cell lines.

METHODS

Patients

Bone marrow (BM) samples were obtained at diagnosis from 65 consecutive patients with childhood ALL classified and treated from 2002 to 2011 according to the Brazilian Childhood Leukemia Treatment Group (GBTLLI ALL-99) protocol [9,10]. In addition, seven normal BM samples from children without hematological diseases (aged 1 month to 13 years) were analyzed. The study was approved by the National Research Ethics Committee (CONEP, n°. 9373/2003), and was based on the Helsinki convention criteria. Samples were collected after written informed consent was obtained from parents or legal guardians.

The diagnosis was made by standard morphological analysis and flow cytometry in BM at diagnosis, with all patients presenting more than 70% of blast cells. Among the 65-pediatric patients with ALL studied, 58 (89.2%) presented B-derived ALL and 7 (10.8%) T-ALL. Three patients presented t(9;22), four t(4;11), and 10 t (12;21), as detected by reverse transcription (RT)-PCR [11]. Patients were characterized as poor responders, regardless of the initial risk group, if they met one or more of the following criteria during the phase of remission induction: WBC > 5,000/mm3 on day 7 (D7) as a proxy for peripheral blast count, M3 bone marrow (>25% blasts) on day 14 (D14), or M2/M3 bone marrow (>5 and 25% blasts, respectively) on day 28 (D28) [9]. Thirty-seven patients were classified as high risk (56.9%) and seven (10.8%) as poor responders. Unfavorable events (death due to any cause or relapse) occurred in 17 patients (26.1%).

RNA Extraction, cDNA Synthesis, and Quantitative Real-Time PCR

BM mononucleated cells were separated by a Ficoll-Hypaque® centrifugation gradient and the total cellular RNA was extracted using Trizol Reagent (Invitrogen, Carlsbad, CA). RNA from each
sample was stored in DEPC-treated water at −80°C and, before use the quantity and quality of samples were evaluated with ND-1000 NanoDrop spectrophotometer (NanoDrop Technologies, Wilmington, DE). Then, total RNA (1 μg) was retrotranscribed with the High Capacity Kit (Applied Biosystems, Foster City, CA), and qRT-PCR was performed using Taqman™ assays according to the manufacturer’s protocol.

The levels of PLK1 (Hs00153444_m1) were measured using the ABI 7500 real-time PCR System (PE Applied Biosystems, Foster City, CA). The relative expression was calculated using the 2−ΔΔCT method [12] with two internal controls, GUS (Hs00999908_m1) and ABL1 (Hs01104728_m1), used to normalize the cDNA levels. The JURKAT cell line sample was used as calibrator in all reactions. Real-time PCR was performed in duplicate and a standard deviation (SD) of <0.5 between duplicates was accepted. A blank control was run in parallel to determine the absence of contamination within each experiment.

Cell Culture

The human childhood ALL cell lines: JURKAT, MOLT 4, REH, NALM-6, 697, ALL-SIL, CCRF-CEM, P12/Ichikawa, NALM-16 and the adult ALL cell line: RS4:11, were purchased from the American Type Culture Collection. Cell cultures were maintained in RPMI (Gibco Invitrogen, Grand Island, NY) supplemented with 10% fetal calf serum, 100 U/ml penicillin and 100 μg/ml streptomycin (Sigma-Aldrich, St. Louis, MO). Cells were maintained in a humidified atmosphere of 5% CO2 at 37°C. Cell counting and trypsinization was performed using a BD FACSCalibur flow cytometer (BD Biosciences Pharmigen). Assays were performed in triplicate.

Drug and Treatment

BI 2536 was purchased from Axon Medchem (Groningen, The Netherlands) and diluted in dimethyl sulfoxide (DMSO) according to the manufacturer’s instructions. For all experiments, cells were treated with nanomolar concentrations (10, 50, and 100 nmol/L). Corresponding control cultures received an equal volume of solvent.

Measurement of Cell Growth by the XTT Cell Proliferation Assay

In brief, cells were seeded in 96-well flat-bottom plates (10⁴ cells/well) and incubated overnight. After this period, BI 2536 was added in varying concentrations to each well and incubated for 24, 48, and 72 hours. After treatment, the XTT dye (3 mg/ml) was added (XTT II; Roche Molecular Biochemicals, Indianapolis, IN) in each well. The plates were incubated for 2 hours at 37°C and the formazan product was measured at 455 and 650 nm by using an iMark microplate reader (Bio-Rad Laboratories Inc., Benicia, CA). Each experiment was performed in triplicate wells and repeated in three sets of tests.

Colony Formation Assay

Cell lines were plated in triplicate in MethoCult GF H4534 methylcellulose medium (Stem Cell Technologies, Vancouver, BC, Canada) and incubated with three different concentrations of BI 2536 and untreated control samples for 48 hours. The number of plated cells was 1,000 cells/ml. The colonies (>50 cells) were visualized and counted on day 14 after plating. Assays were performed in triplicate.

Detection of Apoptotic Cells

Apoptotic cell death was determined by labeling with Annexin V—fluorescein isothiocyanate (FITC) (BD Biosciences Pharmigen, San Jose, CA). Briefly, after drug treatment, 1.5 × 10⁵ cells were centrifuged at 1,000 rpm for 5 minutes at 4°C, washed with ice-cold PBS, and then resuspended in 300 μl of 1X Annexin V Binding Buffer (BD Biosciences Pharmigen). Cells were stained with 5 μl of annexin V-FITC and 50 μl of propidium iodide (PI) (50 μM) (Sigma-Aldrich), and incubated at room temperature in the dark. The samples were analyzed by using a BD FACSCalibur flow cytometer (BD Biosciences Pharmigen). Assays were performed in triplicate.

Cell Cycle Analysis

After drug treatment, cells were fixed in 70% ethanol, stained with PI, and analyzed on a Guava Personal Cell Analysis system (Guava Technologies, Hayward, CA) according to the standard protocol provided by the manufacturer. Percentages of cells in G0/ G1, S, or G2/M phase were collected and processed using the Guava CytoSoft 4.2.1 version software.

Statistical Analysis

Data were analyzed using the Statistical Package for the Social Sciences (SPSS) software for Windows, version 15.0 (SPSS Inc., Chicago, IL). The association between the variables analyzed (age, WBC count at diagnosis, immunophenotype, risk group, molecular cytogenetics, and bone marrow status at day 28) and mRNA expression levels were determined by the Mann–Whitney test.

For survival analysis, patients were stratified into values below and above the median, the quartiles (P75) and the average more than 2 standard deviation (SD) of expression levels in normal bone marrow. The Kaplan–Meier curves and log rank test were used to estimate event-free survival (5-year EFS), which was calculated from the date of complete continuous remission (CCR) to the last follow-up, or unfavorable event (induction failure, relapse, and/or death due to any cause). Patients who did not attain CCR or died during induction were considered as an unfavorable event at time 0.

Statistical analysis for BI 2536 effect was performed using one-way or two-way ANOVA followed by Bonferroni’s test, as appropriate. P < 0.05 was considered to be statistically significant. Effective concentrations (IC₅₀) were analyzed using the CalcuSyn software v2.0 (Biosoft, Ferguson, MO).

RESULTS

PLK1 Is Not Differentially Expressed in Childhood ALL

To verify the mRNA expression of PLK1 in children with ALL, a panel of BM samples from 65 patients with ALL and nine leukemia cell lines were examined and compared to normal BM samples by real-time PCR. PLK1 mRNA presented a wide variability of expression in patients (range difference between lower and higher value of 100 times). Twenty-two patients (34%) were regarded to have high PLK1 expression, considering more than 2 SD above normal bone marrow average. However, no difference was evidenced when PLK1 mRNA expression in childhood ALL was
compared to normal BM. On the other hand, high PLK1 mRNA expression was found in ALL cell lines compared to patients with ALL (5.7/C2) and control (9.5/C2), suggesting a hyper-activation of this protein in leukemia cells in vitro (Fig. 1).

Clinical or biological features such as WBC count at diagnosis, age, immunophenotype, presence of translocations [t(12;21), t(4;11), or t(9;22)], response, and 5 years event-free survival were further investigated in order to examine any possible association with PLK1 mRNA expression levels. No significant correlations were detected between any of these aspects and PLK1 expression levels in our sample.

**BI 2536 Inhibits Cell Proliferation In Vitro**

BI 2536 significantly inhibited growth of all the nine ALL cell lines tested when compared to control (DMSO 0.1%) in dose- and time-dependent manner (P < 0.05) (Fig. 2). The drug presented a maximum effect after 72 hours at 100 nM reducing proliferation in about 66%, ranging from 54% in NALM16 to 79% in MOLT4 (Fig. 2). The median-effect dose (IC50) of BI 2536 after 24 hours of treatment was 208.87 nmol/L. At longer periods, the IC50 was strongly reduced to 38.54 and 9.84 nmol/L for 48 and 72 hours, respectively (Table I). Based on PLK1 expression and BI 2536 cell proliferation inhibition, we selected NALM6 (non-T non-B ALL) and REH (precursor B-cell) cell lines to evaluate other cell effects caused by this drug.

**BI 2536 Induces Cell Cycle Arrest**

The analysis of the cell cycle progression of BI 2536-treated ALL cells showed strong induction of G2/M arrest when compared with untreated controls after 24 hours treatment at all doses analyzed, achieving more than 70% or arrested cells even at the lowest dose tested (10 nmol/L). Correspondingly, the percentage of the cells in G1 and S phases decreased as a consequence of treatment (Table II).

**BI 2536 Abrogates the Clonogenic Capacity of ALL Cells**

PLK1 inhibition by BI 2536 significantly reduced the colony formation capacity for both cell lines when compared with the control at all concentrations analyzed (P < 0.05) (Fig. 3A). The clonogenic capacity after treatment was reduced approximately in 86% and 79% for REH and NALM6 cells respectively, although such effect was not dose-dependent.

**BI2536 Increases Apoptosis**

Compared to control, BI 2536 treatment induced a significant increase in the percentage of apoptotic cells for REH and NALM6 cell lines after 48 hours (P < 0.05). However, even at 100 nmol/L, the increase of cells positively marked with annexin was modest, at maximum of 11% for both cell lines (Fig. 3B). Necrotic cell death was not observed suggesting that reduced cell numbers in vitro may result from impaired proliferation rather than cell death, at least, at 48 hours.
when these cells are grown in vitro, marrow and ALL samples, suggesting different expression profiles in childhood ALL exists in the literature, though our results present upregulation of PLK1 mRNA compared to normal bone marrow and ALL samples, suggesting different expression profiles when these cells are grown in vitro. It is widely accepted that culture conditions may significantly alter the expression profiles of cell lines, and their utility depends on how closely they equate to the progenitor tumor [20,21]. PLK1 mRNA hyper-expression, being an important stimulator of cell proliferation, could be introduced by selection during the establishment of cell lines in vitro, and consequently on this case, the expediency of PLK1 inhibition in cell line models should be interpreted with caution.

Despite the expression analyses, our in vitro results with the potent adenosine triphosphate (ATP)-competitive kinase inhibitor BI 2536, showed that patients with PLK1 levels above the mean might benefit with this strategy. BI 2536 strongly inhibited growth in all the nine ALL cell lines tested in a dose- and time-dependent manner and prompted a significant reduction in colony formation capacity in the two cell lines tested (REH and NALM6). Additionally a significant increase in apoptotic cell death was detected after treatment.

Similar growth impairing effects after treatment with BI 2536 have also been found by our group in various cancer types such as glioblastoma, bladder carcinoma, melanoma and osteosarcoma, where the PLK1 inhibitor induces cell cycle arrest after 24 hours of treatment and mitotic catastrophe leads to cell death [22–25]. Herein BI 2536-treated ALL cells showed strong G2/M arrest (~80% of cells are in G2/M after 24 hours) when compared to untreated cells at all doses analyzed, though differentially from our preceding reports, the growth inhibition in ALL cells might result from impaired proliferation rather than cell death. Nonetheless, this important proliferation arrest still gives an indication of future therapeutic achievements.

Considering PLK1 inhibition in ALL cell lines, Spaniol et al. [26] evaluated the effect of the PLK1 inhibitor GW843682X in three childhood ALL cell lines among a panel of 18 pediatric tumor cell lines. These cell lines (CCRF-CEM, MOLT-4, REH) were also tested in the present study and, in concordance; CCRF-CEM and MOLT-4 were sensitive to the growth inhibitory effect. Nevertheless, the authors were unable to show reduction of cell viability for the REH cell line, even at the highest test concentration (LC50 > 100 nmol/L) [26]. Our results show great antiproliferative effects by BI 2536 in all ALL tested. Likewise, a recent study by Gorlick et al. [27], evaluated the effect of BI 6727 against the PPTP (Pediatric Preclinical Testing Program) in vitro cell line panel that includes six ALL cell lines, among which NALM-6, RS4;11, MOLT-4 and CCRF-CEM. This study had an indicative of a

TABLE II. Doses Required to Induce 50% Inhibition of Cell Growth (IC50) in Leukemia Cell Lines After 24, 48, and 72 hours of Treatment With BI 2536

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>IC50 (nmol/L)</th>
<th>24 hours</th>
<th>48 hours</th>
<th>72 hours</th>
</tr>
</thead>
<tbody>
<tr>
<td>JURKAT</td>
<td>0.94</td>
<td>13.3</td>
<td>20.0</td>
<td>17.4</td>
</tr>
<tr>
<td>MOLT-4</td>
<td>2.33</td>
<td>20.0</td>
<td>17.4</td>
<td>15.0</td>
</tr>
<tr>
<td>REH</td>
<td>1.17</td>
<td>15.0</td>
<td>17.4</td>
<td>20.0</td>
</tr>
<tr>
<td>NALM-6</td>
<td>0.94</td>
<td>13.3</td>
<td>20.0</td>
<td>17.4</td>
</tr>
<tr>
<td>RS 4;11</td>
<td>2.33</td>
<td>20.0</td>
<td>17.4</td>
<td>15.0</td>
</tr>
<tr>
<td>697.00</td>
<td>1.17</td>
<td>15.0</td>
<td>17.4</td>
<td>20.0</td>
</tr>
<tr>
<td>S1L-ALL</td>
<td>0.94</td>
<td>13.3</td>
<td>20.0</td>
<td>17.4</td>
</tr>
<tr>
<td>CEM</td>
<td>2.33</td>
<td>20.0</td>
<td>17.4</td>
<td>15.0</td>
</tr>
<tr>
<td>P12</td>
<td>1.17</td>
<td>15.0</td>
<td>17.4</td>
<td>20.0</td>
</tr>
<tr>
<td>NALM-16</td>
<td>0.94</td>
<td>13.3</td>
<td>20.0</td>
<td>17.4</td>
</tr>
<tr>
<td>Mean</td>
<td>1.17</td>
<td>15.0</td>
<td>17.4</td>
<td>20.0</td>
</tr>
</tbody>
</table>

**DISCUSSION**

PLK1 is an important serine/threonine kinase with central role in mitosis progression regulation [13]. Cumulative evidence demonstrates overexpression of PLK1 mRNA in a variety of human tumors, such as colorectal, pancreatic, prostate and skin cancer, among many others [14–17]. Particularly, its overexpression has regularly been correlated with poor prognosis, evoking PLK1 as an attractive target for cancer treatment [5,18,19].

In acute lymphoblastic leukemia, on the other hand, aberrant *PLK1* overexpression was reported by Ikezoe et al. [8] in a small number of adults with ALL (n = 15) when compared with normal bone marrow. So far, no evidence concerning *PLK1* expression profiles in childhood ALL exists in the literature, though our results (n = 65) were unable to corroborate previous data, on adult samples, nor to elucidate any correlation between PLK1 mRNA levels and biological parameters or prognosis.

Nonetheless, all the 9 cell lines (8 pediatric and 1 adult) analyzed presented upregulation of PLK1 mRNA compared to normal bone marrow and ALL samples, suggesting different expression profiles when these cells are grown in vitro. It is widely accepted that culture
complete cytotoxic response in all ALL cell lines evaluated, furthermore, BI 6727 induced a great toxicity rate in tumor xenografts models in the ALL [27], reinforcing the potent growth interference of PLK1 inhibition in childhood ALL cells.

In phase I/II clinical trials, BI 2536 has shown well-tolerated effective doses although hematological side effects such as neutropenia were described in most patients and thrombocytopenia, with anemia as minor adverse effects [28–30]. Other specific PLK1 inhibitors, such as BI 6727 and GSK 461364, are also being evaluated in clinical trials showing, in lower extent, hematotoxicity as the dominant adverse event. This result may suggest a mechanism-based inhibition of bone marrow cellular proliferation, nonetheless, this side effect is treatable, reversible, and noncumulative [28,31].

In conclusion, PLK1 mRNA expression levels are not associated with prognosis in childhood ALL. However, the great variability observed in our samples (100× between the lowest and highest), along with the in vitro data, evokes that individual cases with high levels of PLK1 might benefit from BI 2536 treatment which may serve as a promising tool for adjuvant therapy in childhood ALL. Nonetheless, further investigation of PLK1 inhibitors in these patients is warranted.

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