Essential role for the p110δ isoform in phosphoinositide 3-kinase activation and cell proliferation in acute myeloid leukemia

Running title: Control of AML blast proliferation by p110δ PI3K

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Abstract

The phosphoinositide 3-kinase (PI3K)/Akt signaling pathway has been shown to be frequently activated in blast cells from patients with acute myeloid leukemia (AML) and to contribute to survival and proliferation of these cells. Of the 8 distinct mammalian isoforms of PI3K, it is the class I PI3Ks (p110alpha, p110beta, p110gamma and p110delta) that are responsible for Akt activation. It is not known which PI3K isoform is critical in AML. Here we show that the p110delta isoform of PI3K is consistently expressed at high level in blast cells from AML in contrast to the other class I isoforms, the expression of which was very variable among patients. IC87114, a p110delta-selective inhibitor, suppressed both constitutive and Flt-3-stimulated Akt activation in blasts to the same extent as LY294002, an inhibitor of all PI3K isoforms. Moreover, IC87114 inhibited AML cell proliferation without affecting the proliferation of normal hematopoietic progenitor cells. These observations identify p110delta as a potential therapeutic target in AML.
AML is a clonal hematological disease and is due to acquired mutations in immature progenitors, resulting in a block of differentiation leading to an accumulation of myeloid blasts. Two classes of mutations, one impairing cell differentiation and the other conferring survival and proliferative benefits, are known to cooperate to cause acute leukemia. Phosphoinositide 3-kinase (PI3K) and its downstream target Akt have been reported to be frequently constitutively activated in leukemic blasts from AML patients and to contribute to cell survival and proliferation. Moreover, constitutive phosphorylation of Akt or of forkhead in rhabdomyosarcoma (FKHR), one of its substrates, correlates with decreased survival in AML patients. Among the 8 isoforms of PI3K, the class I PI3Ks are responsible for Akt activation in cells. These PI3Ks are heterodimers composed of a catalytic and a regulatory subunit. The class IA PI3K catalytic subunits (p110alpha, p110beta and p110delta) associate with SH2-containing regulatory subunits and signal downstream of cytokine and tyrosine kinase receptors. P110gamma is the only class IB PI3K, and functions in the context of heterotrimeric G-protein-coupled receptor signaling. P110alpha and beta are widely distributed in mammalian tissues whereas p110delta and gamma show a more restricted distribution and are mainly but not exclusively expressed in blood cells and their precursors.

In this study, we examined which of the class I PI3K isoforms is responsible for PI3K activation in AML blasts.

**Materials and Methods**

**Patients**

All patients were included in the AML2001 trial of chemotherapy initiated by the French Multicenter Group GOELAMS. All biological studies performed in this report were approved by the institutional review board of the GOELAMS, and signed informed consent provided according to the declaration of Helsinki. Classification of AML was based on the FAB criteria. Patients who presented with acute promyelocytic leukemia (AML3) or AML6 and AML7 FAB subtypes were excluded from the study. Blasts from 64 patients with AML were tested for constitutive activation of PI3K by analysis of Akt phosphorylation on Ser473 and constitutive activation of PI3K was detected in 37 patients (58%). The expression of the four class I isoforms of PI3K p110 subunit was tested in 21 patients with primary AML that presented a constitutive activation of PI3K. Due to limitation of available amount of material, the full set of experiments depicted in Figure 1 and 2 was performed in only ten of these patients. Their characteristics are presented in Table 1.
Cells

Bone marrow cells from newly diagnosed AML patients were obtained before induction of chemotherapy. Bone marrow samples were subjected to Ficoll-Hypaque density gradient separation to isolate mononuclear cells (BMMCs). CD34+ cells from cord blood were isolated as previously reported 9. The OPM2 cell line was established from the peripheral blood of a patient with multiple myeloma 10.

Reagents

The p110delta inhibitor IC87114 was from ICOS Corporation, Bothell, WA, USA 11.

Western blot analysis

BMMCs of AML patients were starved in serum-free medium for 4 h. Cells were incubated with or without inhibitor for 30 min at 37°C. Cells were then boiled in Laemmli sample buffer and proteins were analyzed by Western blot. ECL (Amersham Pharmacia Biotech®) or SuperSignal® West Femto (Pierce) chemiluminescence kits were used for detection. Western blots were quantified using the ImageJ 1.32 software (NIH) after densitometric scanning of the films.

Cell proliferation assays

BMMCs were cultured in α–medium with 5% FCS with or without FLT-3 ligand (10ng/ml) for 48 h and with or without 10 μM IC87114. [3H]-thymidine (1μCi) was added for a final 6 h and the amount of radioactivity incorporated was determined by trichloracetic acid precipitation. CD34+ cells from cord blood were cultured in SCF (20 ng/ml), FLT-3 ligand (10 ng/ml) and Tpo (20 nM) for 48 h with or without 10 μM IC87114 and pulsed for 12 h with [3H]-thymidine.

Results and discussion

p110delta is the only class I PI3K isoform consistently present in AML blasts

Expression of the 4 catalytic subunits of class I PI3K was tested by Western blot of AML blast extracts in 21 patients with constitutive activation of PI3K. Expression of p110alpha, beta and gamma was highly variable between patients and did not correlate with the FAB subtype. In contrast, p110delta expression was consistently detected at comparable levels in all samples. Representative expression patterns are presented in Figure 1 for the 10 patients listed in Table 1. We have used the OPM2 cell line as a negative control in our
experiments. These cells only express p110alpha and p110beta but no detectable p110delta or p110gamma (Figure 1).

**Inhibition of Akt phosphorylation by IC87114, a p110delta-selective inhibitor**

Recently, IC87114, an inhibitor with selectivity for p110delta over the other class I PI3K isoforms, has been developed. In AML blast cells, IC87114 decreased Akt phosphorylation in a dose-dependent manner. In three patient samples, maximal PI3K inhibition was observed at 10 μM IC87114 (Figure 2A). Constitutive Akt phosphorylation in p110δ-negative OMP2 cells was fully inhibited by 25 μM LY294002 but not by IC87114 (Figure 2A, right panel). We next tested the sensitivity of Akt phosphorylation to 10 μM IC87114 and 25 μM LY294002 in the 10 patient samples shown in Table 1 and Figure 1. The results of a typical experiment are presented in Figure 2B (insert). Blots were analyzed by densitometric scanning for Akt phosphorylation in the presence of either IC87114 or LY294002 and results are presented as a percentage of that seen in control cells treated with vehicle only (Figure 2B, graph). From these data, it is clear that IC87114 was as effective as the pan-PI3K inhibitor LY294002 to inhibit Akt phosphorylation in AML blasts. This indicates that p110delta is the main contributor of PI3K activity in AML blasts.

Akt phosphorylation in AML blasts increases in response to FLT-3 ligand. We tested the impact of p110delta inhibition on Akt phosphorylation. Figure 2C shows that FLT-3 ligand-stimulated Akt phosphorylation was inhibited by IC87114 to the same extent as by LY294002, showing that p110delta can be responsible for PI3K activation after FLT-3 ligand stimulation. At present, it is not clear why p110delta is also the main contributor of PI3K activity in cells that also express p110alpha and p110beta. One possible explanation would be that the expression level of p110delta is significantly higher than that of the other isoforms.

**Inhibition of AML cell proliferation by IC87114**

Next, we tested the effect of IC87114 on cell proliferation on the blast samples described above. AML proliferation was found to be almost completely blocked by 10 μM IC87114. IC87114 also strongly reduced the proliferation of cells stimulated with FLT-3 ligand (Figure 2D). We observed that FLT-3 ligand was still able to stimulate both PI3K activity and proliferation of blast cells from patient#102 that presented an activating mutation (ITD) of FLT-3 (Figure 2C and 2D), confirming the observations previously reported by
Bruserud et al. In contrast, cell proliferation of OMP2 and cord blood CD34+ cells was not decreased by IC87114 (data not shown).

AML is associated with poor long-term survival. The development of new therapeutic strategies directed against specific targets is an area of intense interest and may prove effective as adjunct treatments in combination with traditional chemotherapy. The PI3K/Akt pathway is often activated in AML blast cells, contributing to their survival and their proliferation (results described in this manuscript). Blockade of all PI3K isoforms in the organism, using non-selective PI3K inhibitors such as LY294002 or wortmannin is very toxic in vivo, possibly due to a general requirement of PI3K for many cellular functions. Mice lacking functional p110delta are viable and fertile, in contrast to mice lacking p110alpha or p110beta which are embryonic lethal. This suggests that specifically blocking p110delta might be less toxic than inhibiting all PI3K activities. Our data suggest that in AML patients, pharmacological inhibition of p110delta may offer clinical benefit.

Acknowledgments

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References


Table 1: Characteristics of patient samples

<table>
<thead>
<tr>
<th>Patient #</th>
<th>Sex</th>
<th>Age (Y)</th>
<th>FAB subtype</th>
<th>WBC (10^9/L)</th>
<th>Blasts (%)</th>
<th>Cytogenetics</th>
<th>FLT-3 ITD</th>
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<tr>
<td>51</td>
<td>M</td>
<td>26</td>
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<td>6.6</td>
<td>88</td>
<td>46XY</td>
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<tr>
<td>62</td>
<td>F</td>
<td>48</td>
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<td>91XXXX,-17</td>
<td>-</td>
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<tr>
<td>19</td>
<td>F</td>
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<td>200</td>
<td>72</td>
<td>46XX</td>
<td>-</td>
</tr>
<tr>
<td>27</td>
<td>M</td>
<td>66</td>
<td>AML2</td>
<td>200</td>
<td>72</td>
<td>46XX</td>
<td>-</td>
</tr>
<tr>
<td>18</td>
<td>M</td>
<td>25</td>
<td>AML4</td>
<td>150</td>
<td>66</td>
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<tr>
<td>24</td>
<td>M</td>
<td>44</td>
<td>AML4Eo</td>
<td>42</td>
<td>73</td>
<td>inv(16)</td>
<td>-</td>
</tr>
<tr>
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<td>M</td>
<td>59</td>
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<td>-</td>
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<td>95</td>
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</table>

All patients listed showed constitutive PI3K activation as defined by Akt phosphorylation on Ser473 after starvation in serum free medium for 4h. Blast percentages were determined based on differential of bone marrow aspirations prior to Ficoll sedimentation. WBC: white blood cells; ITD: internal tandem duplication; nd: not done. None of the samples listed was positive for D835 mutation in FLT-3 (data not shown).
Figure legends

Figure 1: Expression of p110 isoforms in leukemic blasts from AML patients

Expression of class IA (p110alpha, beta and delta) and class IB (p110gamma) catalytic subunits of PI3K was tested in leukemic blasts of 21 patients showing a constitutive activation of PI3K. Results from the 10 patients described in table 1 are presented. Blast analysis of the other patients gave similar results. Proteins from 10^6 cells were separated by SDS-PAGE and analyzed by Western blot using antibodies specific for the various isoforms of p110. P110alpha and p110beta antibodies were from Cell Signaling Technology. P110delta antibodies have been described previously and p110gamma antibodies were provided by Dr R Wetzker. Purified recombinant p110 proteins and cell lysates from 10^6 OPM2 cells were used as controls. Western blot using anti-actin antibodies (Sigma, cat number A5441) was used to assess equal loading of the samples.

Figure 2: P110 delta activity is required for Akt phosphorylation and proliferation of AML blasts

Part A: IC87114 dose-response relationship and specificity. BMMCs from patient #89 (90% leukemic blasts) or OPM2 cells were incubated for 30 min with the indicated concentrations of IC87114 or 25 μM LY294002. Cell extracts from 10^6 cells were analyzed by Western blot using antibodies against phosphoSer473 of Akt (Cell Signaling Technology). After stripping, the blots were re-probed with anti-actin antibodies to ascertain equal loading of the samples.

Part B: IC87114 inhibits constitutive PI3K activity. BMMCs were incubated for 30 min with 10 μM IC87114 or 25 μM LY294002 or with solvent (DMSO) alone. Cell extracts from 10^6 cells were analyzed by Western blot using anti-p-Akt (Ser473) and anti-actin antibodies. Insert shows the results of a typical experiment using blasts from patient #51. Similar experiments were performed with the blasts of each patient presented in Table 1 and Figure 1. The blots were scanned and analyzed using ImageJ software. For each sample, Akt phosphorylation observed in the absence of inhibitor was set at 100% and Akt phosphorylation in the presence of inhibitors was expressed relative to this value (main graph). Error bars represent standard deviations of the 10 patient samples. Significance determined by Student’s t-test: Control/LY294002: p<0.001, Control/IC87114: p<0.001, LY294002/IC87114: p>0.2 (not significant).
Part C: IC87114 inhibits Flt-3 ligand-stimulated PI3K activity. BMMCs from patient #102 were incubated for 30 min with or without 10 μM IC87114 or 25 μM LY294002, followed by stimulation for 15 min with 50 ng/ml FLT-3 ligand, and Ser473 phosphorylation of Akt was analyzed by western blotting.

Part D: IC87114 inhibits AML cell proliferation. BMMCs from each patient presented in Table 1 were incubated for 48 h with or without 10 μM IC87114 and pulsed for 6 h with [3H]-thymidine. Moreover, BMMCs from patients 89, 102 and 110 were also incubated for 48 h with 10 ng/ml FLT-3 ligand in the presence or absence of IC87114. For each BMMC sample, [3H]-thymidine incorporation in the absence of FLT-3 ligand and IC87114 (control) was set at 100% and [3H]-thymidine incorporation in the presence of FLT-3 ligand and/or IC87114 was expressed relative to this value. Significance determined by Student’s t-test: Control/IC87114 : p<0.001, FLT-3L/FLT-3L + IC87114 p<0.01.
Figure 1

Figure 2
Essential role for the p110δ isoform in phosphoinositide 3-kinase activation and cell proliferation in acute myeloid leukemia

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