Abstract. Increasing resistance of imatinib, a BCR-ABL tyrosine kinase inhibitor, hinders its use in the therapy of chronic myeloid leukemia (CML). The PI3K pathway is known to be closely involved in BCR-ABL transformation and the tumorigenesis of CML, suggesting that PI3K may be a potential target for CML therapy. Idelalisib, a specific inhibitor of PI3K p110δ, has been approved for the treatment of chronic lymphocytic leukemia (CLL). However, the antileukemia effect of idelalisib on CML remains unknown. In the present study, the antileukemia activity of idelalisib alone or in combination with imatinib was investigated by use of K562 cells. Idelalisib inhibited K562 proliferation in a dose-dependent manner. G1 arrest was induced, in which upregulation of p27 and p21, as well as downregulation of cyclin D1 and p-pRb, may be involved. Furthermore, idelalisib induced apoptosis in the K562 cells, with increased expression of pro-apoptotic molecules such as Bad and Bax, cleavage of caspase-9, -8 and -3, and PARP, in contrast to downregulation of anti-apoptotic protein Bcl-2. Combination of idelalisib with imatinib led to a synergistic antiproliferative effect on K562 cells, together with enhanced activity of G1 arrest and apoptosis induction. In conclusion, idelalisib exhibited in vitro antitumor activity on CML K562 cells alone or in combination with imatinib, suggesting potential application in CML therapy.

Introduction

Chronic myeloid leukemia (CML), a clonal myeloproliferative disorder, is characterized by the Philadelphia (Ph) chromosome which originates from the t(9;22)(q34;q11) reciprocal translocation and leads to the BCR-ABL chimeric oncoprotein (1-3). This BCR-ABL oncoprotein bears constitutive tyrosine kinase activity and therefore promotes uncontrolled growth and proliferation of leukemia cells (4). Development of inhibitors targeting BCR-ABL has been generally successful in the past 15 years, with highly decreased mortality for CML patients. However, resistance to these drugs has been increasingly reported. CML remains an incurable disease (5,6). Therefore, novel drugs with targets other than BCR-ABL are expected.

Idelalisib, also named CAL101, was approved by the US Food and Drug Administration in July, 2014. It is a first-in-class oral PI3K inhibitor that has shown substantial and sustained antileukemia efficacy in patients with relapsed/refractory chronic lymphocytic leukemia (CLL) (7,8). Compared with other chemotherapy regimens, idelalisib showed advantages of long-term efficacy and reduced toxicity (9).

It has been reported that PI3K signaling contributes to BCR-ABL transformation and is essential for in vivo leukemogenesis of CML (10). Furthermore, the PI3K p110δ isoform is preferentially expressed in hematopoietic cells (11), suggesting that idelalisib which targets p110δ shows favorable antitumor efficacy against CML.

Therefore, in the present study, we investigated the antileukemia activities of idelalisib in CML K562 cells.

Materials and methods

Reagents. Idelalisib and imatinib were purchased from Selleck (London, ON, Canada). 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) reagent was purchased from Amresco (Solon, OH, USA). Antibodies against Akt, phospho-Akt (Ser473), phospho-GSK-3β (Ser9), caspase-3, -8 and -9, poly(ADP-ribose) polymerase (PARP), β-actin, and anti-mouse and anti-rabbit HRP-conjugated secondary antibodies were obtained from Cell Signaling Technology (Danvers, MA, USA). Antibodies against phospho-pRb (pS780), cyclin D1 and p27 were obtained from...
Cell culture. The human CML K562 cell line was purchased from the Cell Resource Center, Peking Union Medical College (Beijing, China). The cells were routinely maintained in RPMI-1640 medium supplemented with 10% fetal bovine serum, 1% kanamycin (100 µg/ml), and 1% glutamine (0.44 µg/ml) at 37°C in a humidified atmosphere containing 5% CO₂.

**MTT assay.** The MTT assay was performed to assess cell viability as previously described (12,13). Briefly, cells (2x10⁴ cells/ml) were cultured in a 96-well plate for 48 h in the presence of 0, 1, 5, 10, 20, 50, 100, 150, and 200 µM of idelalisib. After addition of MTT (5 mg/ml) to each well, the cells were further incubated for 4 h. The produced formazan blue was dissolved with dimethyl sulfoxide (DMSO), and the absorbance was measured at 490 nm using the microplate reader iMark (Bio-Rad, Hercules, CA, USA).

**Soft agar assay.** The soft agar assay was carried out as previously described (14) with a small modification. K562 cells were treated with 0, 20, 50, and 100 µM of idelalisib for 48 h. Then, the treated cells were seeded on solidified agarose in 60-mm dishes (1.2x10⁴ cells/dish). After incubation for 10 days at 37°C, the cells were fixed with 4% paraformaldehyde and stained with 0.5% crystal violet for 30 min. Colonies were counted under a microscope. Each assay was performed 3 times.

**Flow cytometric analysis of cell cycle distribution.** Cell cycle analysis was carried out as previously described (15). The cell suspension (4x10⁴ cells/2 ml/well) of K562 cells was planted in a 6-well plate and exposed to various concentrations of idelalisib for 48 h. The cells were harvested, washed with ice-cold phosphate-buffered saline (PBS), and fixed with 75% ethanol. After centrifugation, the fixed cells were resuspended in propidium iodide (PI) solution (25 µg/ml), and incubated in the dark for 30 min at 4°C to be available for analysis by BD Accuri C6 flow cytometer (BD Biosciences, San Jose, CA, USA). Data were analyzed using FlowJo 7.6 software.

**Flow cytometric analysis of apoptosis with Annexin V-FITC/PI staining.** Analysis of apoptosis was carried out by Annexin V-FITC/PI double staining as previously described (16). K562 cells treated with or without idelalisib in a 6-well plate for 48 h were collected, washed with ice-cold PBS, and then stained with 2.5 µl of Annexin V-FITC and 2.5 µl of PI (5 µg/ml) in binding buffer for 15 min at room temperature in the dark. Flow cytometric analysis was conducted using BD FACSVerse flow cytometer (BD Biosciences).
Phosphorylation of Akt and GSK-3β was dose-dependently inhibited by idelalisib, suggesting that the cell cycle arrest effect of idelalisib may be attributed to its blockade of the PI3K/Akt pathway.

*idelalisib induces apoptosis in the K562 cells.* Since apoptosis may contribute to a decrease in cell viability, we also examined whether idelalisib induces apoptosis in the K562 cells. Flow cytometric analysis was carried out after Annexin V-FITC/
PI staining of K562 cells with or without idelalisib treatment. As indicated in Fig. 3A and B, the cell population in the upper- and lower-right quadrants was increased in a dose-dependent manner after idelalisib treatment, with 22.94% for cells treated with 100 µM idelalisib vs. 7.55% for control cells, suggesting that idelalisib induced apoptosis in the K562 cells. Notably, the increased apoptotic cells were mainly in the early-stage (lower-right quadrant; 19.8% for cells treated with 100 µM idelalisib vs. 5.69% for control cells).

Then, we investigated the effect on apoptosis-related proteins by western blot analysis. As shown in Fig. 3C, idelalisib treatment increased the level of cleaved caspase-9, -8 and -3, and PARP, as well as the expression of Bad and Bax. In contrast, the expression of anti-apoptotic protein Bcl-2 was reduced. These results suggest that the apoptosis induction by idelalisib in K562 cells may be related to the Bad/Bcl-2/Bax family and the cleavage of caspases and PARP.

Synergistic effect of idelalisib and imatinib in K562 cells. A well-designed drug combination may enhance efficacy while reducing toxicity. To investigate whether idelalisib can enhance the antileukemia activity of the first-line drug imatinib, we carried out a combination study using Chou and Talalay method. K562 cells were treated with idelalisib and imatinib alone or in combination for 48 h, respectively. MTT assay was conducted to determine the inhibitory activities of each drug and the combination. The IC_{50} of imatinib was calculated to be 0.183 µM (Fig. 4A). A synergism study was performed using a series of drug combinations (20, 40, 60, 80 and 100% of IC_{50} value of each drug) with a fixed ratio of IC_{50} idelalisib to IC_{50} imatinib (390:1). As shown in Fig. 4B, co-treatment with the two drugs led to an enhanced cell growth inhibition compared to either treatment alone. Analysis of the data by CalcuSyn software indicated a synergistic effect for the combination, since CI values were <1 at all fraction affected (Fa) levels (Fig. 4C). All of the 3 data points (ED_{50}, ED_{75} and ED_{90}) were far below the additivity line (Fig. 4D), with the respective CI values as 0.62, 0.47 and 0.35 (Table I), suggesting strong synergy of the combination in regards to the growth inhibition of K562 cells.

Then, we further confirmed the combinational effect of idelalisib and imatinib on K562 cells using various assay methods. Soft agar assay showed that co-treatment with idelalisib (50 µM) and imatinib (0.128 µM) decreased cell proliferation more potently than either drug alone (Fig. 4E and F). Cell cycle distribution analysis indicated an increased G1 arrest (Fig. 5A and B), accompanied by further reduction in the level of p-pRb and cyclin D1, and further enhancement in p21 and p27 expression (Fig. 5C). In addition,
Figure 4. Combination effect of idelalisib and imatinib on K562 cell proliferation. (A) Imatinib inhibited the proliferation of K562 cells. Cells were treated with imatinib (0, 0.01, 0.03, 0.09, 0.27, 0.81, 2.43, 7.29 and 21.87 µM). Cell viability was determined by MTT assay. IC50 value of imatinib was calculated to be 0.183 µM. Data are mean ± SD, representative of 3 independent experiments. (B) Combination with idelalisib enhanced the inhibitory activity of imatinib. K562 cells were exposed to a series of concentrations of idelalisib and imatinib (20, 40, 60, 80 and 100% IC50 of each drug), alone or in combination. Cell viability was determined by MTT assay. Data are mean ± SD, representative of 3 independent experiments. (C) Analysis of the combinational effect using CalcuSyn software. Combinational index (CI) values of drug combinations below the horizontal line (CI=1) represent synergy. Fa, fraction affected. (D) Isobologram of idelalisib and imatinib combination. Data points of growth inhibition at 50% (ED50), 75% (ED75) and 90% (ED90) are on the left side of the respective lines, indicating a synergistic effect. (E and F) Combination of idelalisib and imatinib enhanced the inhibitory activity on K562 proliferation which was determined by soft agar assay. The cells treated with idelalisib (50 µM) and imatinib (0.128 µM) alone or in combination for 48 h, were grown in soft agar for 10 days at 37°C. Colonies were counted under a microscope. Data are mean ± SD, representative of 3 independent experiments; **p<0.01, ***p<0.001, compared with mixture (combination).

Table I. Combination indices (CI) for idelalisib and imatinib.

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Drug(s)</th>
<th>IC50 (µM)</th>
<th>r</th>
<th>ED50</th>
<th>ED75</th>
<th>ED90</th>
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</thead>
<tbody>
<tr>
<td>K562</td>
<td>Idelalisib</td>
<td>71.4</td>
<td>0.982</td>
<td>0.62±0.09</td>
<td>0.47±0.05</td>
<td>0.35±0.09</td>
</tr>
<tr>
<td></td>
<td>Imatinib</td>
<td>0.183</td>
<td>0.998</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Idelalisib + imatinib</td>
<td>-</td>
<td>0.987</td>
<td></td>
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</table>
Combined treatment with idelalisib (50 µM) and imatinib (0.128 µM) led to increased cell apoptosis than that induced by either drug alone (Fig. 6A and B). Notably, co-treatment with the two drugs induced highly increased cell population...
in both the upper- and lower-right quadrants, suggesting that the combination of idelalisib and imatinib treatment induced apoptosis in both late and early stages. Consistently, the levels of cleaved caspase-3 and PARP were significantly increased following the combination treatment, as compared with each drug alone (Fig. 6C).

Discussion

Phosphatidylinositol 3-kinases (PI3Ks), consisting of 3 classes (class I, II and III), are closely involved in cell growth and survival (23,24). Among these 3 classes, class I PI3K is the most studied and is closely related to signaling in hematopoietic cells (25). Class I PI3K is further divided into 2 subtypes as class IA and class IB. Class IA PI3K comprises a regulatory subunit and a catalytic subunit (p110α, p110β or p110δ), whereas class IB PI3K comprises a p101 regulatory subunit and a p110α catalytic subunit. Catalytic isoform p110δ is preferentially expressed in hematopoietic cells (26), suggesting that targeting p110δ may be a promising strategy for leukemia therapy. Furthermore, it has been reported that PI3K signaling contributes to BCR-ABL transformation and is essential for leukemogenesis of chronic myeloid leukemia (CML) (10). In addition, sustained activation of the PI3K/Akt signaling pathway may contribute to drug resistance due to enhanced drug efflux by ATP-binding cassette transporters such as P-gp (27,28). Occurrence of resistance has become a big challenge in the chemotherapy of CML in recent years. Therefore, targeting PI3K p110δ may be an alternative approach for CML treatment.

In the present study, the antileukemia activity of idelalisib, a specific inhibitor of PI3K p110δ, on CML K562 cells was investigated. Our results demonstrated that idelalisib dose-dependently inhibited K562 cell proliferation, which were supported by MTT and soft agar assays. Both MTT and soft agar assays are well known assays which are used to evaluate cell proliferation in vitro, whereas the latter one can additionally predict tumorigenic ability which is correlated with anchorage-independent growth in vivo (14). G1 cell cycle arrest was induced by idelalisib treatment, accompanied by the decreased expression of cyclin D1 and phosphorylation of pRb in contrast to the increased expression of cyclin-dependent kinase (CDK)-inhibitors p27 and p21. Meanwhile, idelalisib treatment blocked the phosphorylation of Akt and GSK-3β in a dose-dependent manner. Since Akt is known to promote cell cycle progression by upregulating GSK-3β and cyclin D1 (29), and downregulating CDK inhibitors p27 and p21 (30), the cell cycle arrest effect of idelalisib may be attributed to the blockade of the PI3K/Akt pathway.

Apoptotic cell death is triggered either by the mitochondrial pathway or the death receptor pathway. The former is mainly regulated by the Bcl-2 family which comprises pro-apoptotic proteins such as Bax and Bak, anti-apoptotic proteins such as Bcl-2, and the BH3-only proteins such as Bad, while the latter is controlled by cell surface death receptors such as Fas (31). In the mitochondrial pathway, Bax and Bak are known to disrupt mitochondrial outer membrane integrity through multimerization and therefore release cytochrome c into the cytosol, which in turn activates caspases including caspase-9 and -3, finally leading to apoptosis (32). Bcl-2 exhibits an anti-apoptotic effect by inhibiting formation of the Bax/Bak complex, while Bad promotes apoptosis via neutralizing the inhibitory activity of Bcl-2 on Bax/Bak (32). For the death receptor pathway, Fas induces activation of caspase-8, which cleaves downstream caspases such as caspase-3, promoting apoptosis (31). In the present study, after treatment with idelalisib, expression of Bad and Bax was increased while that of Bcl-2 was reduced; the levels of cleaved caspase-9, -8 and -3 and PARP were enhanced. Akt is known to upregulate Bcl-2 via inhibition of the antagonist Bad in the mitochondrial pathway (33), and to mediate Fas via regulation of FoxO in the cell death receptor pathway (34). Therefore, idelalisib promoted cell apoptosis by activating both the mitochondrial and death receptor pathways, in which targeting PI3K p110δ and the downstream effectors may be involved.

Development of imatinib for CML treatment has met with great success. However, the efficacy has been challenged by the increasing occurrence of acquired resistance, and the original insensitivity of a population of patients. The present study indicates that idelalisib could highly enhance the anti-leukemia activity of imatinib on K562 cells, suggesting the possibility for the combinational use of the two drugs in the future.

In conclusion, idelalisib, a novel PI3Kδ-specific inhibitor, alone or in combination with imatinib, exhibited potential antileukemia activity against CML K562 cells, suggesting the possible future application in the treatment of CML patients.

Acknowledgements

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References


