Discovery of LDD-1075 as a potent FLT3 inhibitor

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Abstract. Fms-like tyrosine kinase 3 (FLT3) is a valuable pharmacological target in the treatment of acute myeloid leukemia (AML). LDD-1075 and LDD-1076 are indirubin derivatives, and LDD-1075 is the ester form of LDD-1076. LDD-1076 exhibited a potent in vitro FLT3 kinase activity inhibition with an IC₅₀ of 7.89 nM, whereas, LDD-1075 demonstrated a relatively weak activity against FLT3 (IC₅₀ of 3.19 µM). In contrast with the results of the FLT3 kinase activity inhibition assay, the LDD-1076 did not affect the growth of the MV4-11 cell line, which harbors the constitutively activated form of the FLT3 mutation. Notably, LDD-1075 exhibited a strong cytotoxic effect against the MV4-11 cells. When LDD-1075 was incubated with the MV4-11 cell lysate, the formation of LDD-1076 was observed. Treatment with LDD-1075 inhibited the FLT3 phosphorylation along with the phosphorylation of the signal transducer and activator of transcription 5 protein, which is a downstream signal transducer of FLT3. Treatment with LDD-1075 induced apoptosis and cell cycle arrest at the G1 phase. The present study demonstrated that the LDD-1076 formed by the bioconversion of LDD-1075 is a potent FLT3 inhibitor with anti-leukemic activity.

Introduction

Acute myeloid leukemia (AML) is a type of leukemia characterized by the rapid proliferation of blood-forming cells of myeloid lineage and occurs with increasing frequency in elderly patients (1). Chemotherapy, conventionally a combination of anthracyclines and cytarabine (also known as AraC), is used to treat AML patients, and the complete remission rate (CR) is ~65-85% (1). However, for patients older than 60 years of age, the CR rate is lower, and they cannot endure a high intensity therapy due to the toxicities of the chemotherapeutic agents such as myelosuppression. Thus, there are unmet medical needs for AML patients, especially for elderly patients aged over 60 years that require a low-toxicity therapy.

Novel therapeutic approaches to treat AML are under investigation such as proteasome inhibitors and DNA methyltransferase inhibitors (2). Fms-like tyrosine kinase 3 (FLT3), a member of the receptor tyrosine kinase family, is a valuable target in the AML drug development area. FLT3 is activated by the FLT3 ligand (FLT3LG) upon binding and transduces a variety of signals, including the proliferation, survival, and differentiation of hematopoietic precursor cells (3). Constitutively activating mutations of FLT3 were observed in AML patients. The most common type of activating mutation is an internal tandem duplication of the FLT3 juxtamembrane domain (FLT3-ITD) (4,5). Mutations in the kinase domain (FLT3 D835) also have resulted in the constitutive activation of FLT3 (6). FLT3-ITD mutation was reported to constitutively activate downstream signal transducer and activator of transcription 5 (STAT5) as well as induce the dysregulated cell growth (7). FLT3 mutation is found in approximately 1/3 of all AML patients, and it is associated with a poor prognosis (8,9).

The FLT3 inhibitors evaluated in clinical trials for AML therapy can be divided into three generations (10). Lestaurtinib, midostaurin, and tandutinib are first generation inhibitors with modest efficacy. Problems disclosed in first generation inhibitors (for example, maintaining an effective plasma concentration) were resolved in the second generation inhibitors, KW-2449 and quizartinib. The high potency of KW-2449 and quizartinib made it possible to achieve an effective plasma concentration in AML patients. The third generation inhibitors (crenolanib and ASP2215) try to overcome the resistance to FLT3 inhibitors observed in clinical trials. Secondary mutations of FLT3 have emerged as resistant mechanisms, and crenolanib has exhibited a potency against both FLT3-ITD and FLT3-ITD with a secondary mutation (11). Currently, midostaurin is approved by the FDA for the FLT3 mutation positive AML patients.

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Continued discovery of novel chemical and pharmacological information for FLT3 inhibitors is required. The main reason for this is due to the emergence of resistant cancer cells against the FLT3 inhibitor treatment (12). Indirubin derivatives as FLT3 inhibitors were previously reported by our group (13). Further optimization was carried out (14), and a 5-carboxy indirubin derivative (LDD-1076) exhibited the potent inhibition of FLT3 activity without any anti-proliferative activity. Corresponding 5-carboxy ester form of LDD-1076 (LDD-1075) have a strong growth inhibitory activity with a relatively weak FLT3 inhibition. In this study, LDD-1076 and LDD-1076 were characterized with an antileukemic activity, and the bioconversion of LDD-1075 to LDD-1076 was identified.

Materials and methods

Cell cultures. The MV4-11 human AML cell lines were purchased from ATCC (Rockville, MD, USA). The MV4-11 cells harbors FLT3-ITD mutation, which confers the constitutive kinase activity of FLT3. The cells were cultured in an RPMI medium (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin. The cultured cells were incubated at 37°C with 5% CO₂.

For the cytotoxicity assay, the cells were seeded in a 96-well plate (15,000 cells per well) and incubated with the indicated compound for 72 h. As a negative control, the cells were treated with the vehicle only [i.e., dimethyl sulfoxide (DMSO)]. A final concentration of 0.5% DMSO was used in all treatment groups to exclude the effects of DMSO on the cell cytotoxicity. The cell viability was measured by a tetrazolium-based assay with an EZ-Cytotox Cell Viability Assay kit (Daeil Lab Service Co., Ltd., Seoul, Korea). The half-maximal inhibitory concentration (IC₅₀) was calculated by nonlinear regression with the Prism software, version 5.01 (GraphPad, La Jolla, CA, USA).

Chemical synthesis. The LDD-1075 and LDD-1076 compounds were chemically synthesized in Professor Yong-Chul Kim's laboratory. The synthetic scheme and analytical data of LDD-1075 and LDD-1076 are previously described (14).

In vitro kinase assay. The inhibition of the FLT3 recombinant kinase activity was measured using homogeneous, time-resolved fluorescence (HTRF) assays. Recombinant proteins containing the FLT3 kinase domain were purchased from Carina Biosciences (Kobe, Japan). The optimal enzyme, ATP, and substrate concentrations were established using an HTRF KinEASE kit (Cisbio, Codolet, France) according to the manufacturer's instructions. The enzymes were mixed with serially diluted compounds and peptide substrates in a kinase reaction buffer (50 mM HEPES (pH 7.0), 500 µM ATP, 0.1 mM sodium orthovanadate, 5 mM MgCl₂, 1 µM DTT, 0.01% bovine serum albumin (BSA), and 0.02% NaN₃.). After adding the reagents for detection, the TR-FRET signal was measured with a Victor multilabel reader (Perkin Elmer, Waltham, MA, USA). The IC₅₀ was calculated by nonlinear regression with the Prism software. Met, EGFR, FAK, Jak2, and Jak3 in vitro kinase assays were also carried out using HTRF assay technology in order to determine the selectivity against kinases.

Bioconversion of LDD-1075 to LDD-1076 in MV4-11 cell lysate. To determine whether LDD-1076 can be formed from LDD-1075 in the MV4-11 cells, the whole cell lysates of MV4-11 cells were used. MV4-11 cells were suspended in PBS and disrupted by three freeze/thaw cycles followed by sonication. The LDD-1075 compound was incubated with MV4-11 cell lysates at 37°C for 0, 15, 30, 60, and 90 min. The final incubation solutions contained 10 mM LDD-1075, 1.2 mM NADPH, 1 mg/ml (total protein) cell lysates, and 100 mM phosphate buffer (pH 7.4). At each time point, the reaction was terminated by removing it from the water bath and subsequently adding100 µl of ice-cold acetonitrile containing 0.1 µg/ml of internal standard (cilostazol) to a 50 µl aliquot of the reaction mixture. The incubation solutions were then centrifuged, and the concentration of LDD-1076 in the supernatants was analyzed by LC-MS/MS.

Five microliters of the supernatant were injected into the LC-MS/MS system. The LC-MS/MS system consisted of an Agilent 1260® HPLC system (Agilent Technologies Inc., Hilden, Germany) and an Agilent 6460® triple-quadrupole mass spectrometer (Agilent Technologies Inc., Singapore). The HPLC mobile phases consisted of 0.1% formic acid (A) and 0.1% formic acid in 90% acetonitrile (B). Chromatographic separation was achieved on a reversed-phase Kinetex® C₁₈ column (50x2.6 mm, 2.6 µm; Phenomenex, Torrance, CA, USA) using a gradient elution at a flow rate of 0.3 ml/min. The total run time was 4.5 min. Multiple reaction monitoring (MRM) detection in the positive ion mode was employed using nitrogen as the collision gas with a dwell time of 50 ms for each transition; the MRM transitions monitored were m/z 322→260 for LDD-1076 and m/z 370→288 for the internal standard. The lower limit of the quantitation of LDD-1076 in the reaction mixture was 10 nM. The values of coefficients of correlation (R) were more than 0.998.

Flow cytometry analyses. MV4-11 cells were grown in 24-well plates (500,000 cells per well) and treated with LDD-1075 for 48 h. The cells were fixed with 3.7% paraformaldehyde and treated with RNase A (50 µg/ml). The cells were then stained with propidium iodide (PI; Sigma-Aldrich; Merck KGaA) and subjected to flow cytometry with an Accuri C6 flow cytometer (BD Biosciences, San Jose, CA, USA). The data were analyzed by the BD Accuri C6 software (BD Biosciences).

Table I. In vitro inhibition of LDD-1075 and LDD-1076 against activity of selected kinases.

<table>
<thead>
<tr>
<th>Kinase</th>
<th>IC₅₀, µM LDD-1075</th>
<th>LDD-1076</th>
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<tbody>
<tr>
<td>Met</td>
<td>&gt;10</td>
<td>&gt;10</td>
</tr>
<tr>
<td>EGFR</td>
<td>&gt;10</td>
<td>&gt;10</td>
</tr>
<tr>
<td>FAK</td>
<td>&gt;10</td>
<td>&gt;10</td>
</tr>
<tr>
<td>Jak2</td>
<td>&gt;10</td>
<td>0.476</td>
</tr>
<tr>
<td>Jak3</td>
<td>&gt;10</td>
<td>0.546</td>
</tr>
</tbody>
</table>

EGFR, epidermal growth factor receptor; FAK, focal adhesion kinase; Jak2, Janus kinase; IC₅₀, half maximal inhibitory concentration.
For the terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay, the APO-DIRECT kit from BD Biosciences was used (15). Cells were grown in 6-well plates and treated with LDD-1075 for 24 h. Cells were fixed with 2% formaldehyde for 30 min, washed twice in PBS, and stored in 70% ethanol at -20°C until analysis. After washing in wash buffer, cells were then incubated at 37°C for 60 min in a ratio of enzyme/FITC label solution, according to the manufacturer’s instructions. After a final wash in rinse buffer and resuspension in PI/RNase solution, FACS analysis was performed.

Immunoblotting. Cells were lysed with an SDS lysis buffer (12 mM Tris-Cl, pH 6.8, 5% glycerol, and 0.4% SDS) and 10–20 µg of protein was subjected to electrophoresis on a 10% SDS-polyacrylamide gel followed by western blotting. Antibodies against phospho-STAT5 (p-STAT5; cat. no. 9351, 1:1,000), PARP (cat. no. 9542, 1:1,000), and cyclin D1 (cat. no. 2922, 1:1,000 were purchased from Cell Signaling Technology (Danver, MA, USA). Antibody against STAT5 (cat. no. sc-835, 1:1,000) was from Santa Cruz (Santa Cruz, CA, USA), and an antibody against β-actin was obtained from Sigma-Aldrich; Merck KGaA (cat. no. A5441, 1:5,000).

Statistical analysis. The results were obtained from two or three independent experiments. Data are expressed as the mean ± standard error of the mean. Statistical significance was assessed using the Student’s t-test or one-way analysis of variance followed by Tukey’s post-hoc test using SPSS software for multiple comparisons. P<0.05 was considered to indicate a statistically significant difference.

Results

Effects of LDD-1075 and LDD-1076 on the FLT3 kinase activity and cell cytotoxicity. LDD-1075 and LDD-1076 are indirubin derivatives and LDD-1075 is the ester form of LDD-1076 (Fig. 1A). LDD-1075 and LDD-1076 were subjected to a FLT3 kinase activity assay in vitro. As shown in Fig. 1B, the FLT3 kinase activity inhibition was measured at the indicated concentrations of the LDD compounds. LDD-1076 exhibited a potent activity with an IC50 of 7.89±1.38 nM, while LDD-1075 showed a relatively weak activity against FLT3 (IC50 3.19±0.538 µM). The cytotoxic effect of the LDD compounds on the MV4-11 cells was measured (Fig. 1C). The MV4-11 cell line is AML cells with the constitutive active form of FLT3. In contrast with the results of the kinase activity assay, LDD-1076 did not affect the MV4-11 cell growth. Interestingly, LDD-1075, which exhibited a relatively low potency in the FLT3 kinase inhibition, exhibited a strong cytotoxic effect against the MV4-11 cells (54.1±2.06 nM).

Table 1 shows the kinase selectivity of the LDD-1075 and LDD-1076 compounds. LDD-1075 had an IC50 of more than 10 µM for the kinases tested. LDD-1076 also showed negligible activity against Met, EGFR, and FAK (IC50 >10 µM). Unexpectedly, Jak2 and Jak3 were relatively strongly inhibited by LDD-1076 (IC50: 0.476 and 0.546 µM, respectively).

Bioconversion of LDD-1075 to LDD-1076 in MV4-11 cell lysates. The observation in Fig. 1B and C led to a hypothesis that LDD-1075 goes inside the cells and hydrolyzes to
LDD-1076. Generally, the ester form structure compounds are more permeable to cell membranes than those of the acid form and can be easily hydrolyzed to the acid form by various enzymes. According to the hypothesis, LDD-1076 is expected to not have cytotoxicity despite the high potency of the in vitro FLT3 assay. This hypothesis also can explain the potent cytotoxic activity of the LDD-1075 with its relatively low kinase inhibitory activity. To investigate whether LDD-1076 can be formed from LDD-1075 in the MV4-11 cells, the LDD-1075 was incubated with MV4-11 cell lysates and analyzed by LC-MS/MS. The LDD-1076 was detected after incubation of the LDD-1075 with MV4-11 cell lysates (Fig. 2A), and the amounts increased with the incubation time reaching a plateau at 30 min (Fig. 2B). This result suggests that LDD-1075 can be converted to LDD-1076 inside the MV4-11 cells, and LDD-1076 may partly contribute to the high cytotoxicity of LDD-1075 in the MV4-11 cells.

Inhibition of STAT5 phosphorylation by LDD-1075. The phosphorylation levels of STAT5 were analyzed by western blotting using an antibody against p-STAT5 (Tyr694). β-actin was used as a loading control. STAT5, signal transducer and activator of transcription 5; p, phosphorylated.

Induction of apoptosis by LDD-1075. To investigate the effect of LDD-1075 on the apoptosis of MV4-11 cells, the TUNEL assay was performed. Cells were treated with 0.1, 1, and 10 µM of LDD-1075 along with the DMSO treatment as a control for 24 h. Then, the cells were subjected to the TUNEL assay and flow cytometry, as described in the Materials and Methods section. As shown in Fig. 4A, the LDD-1075 treatment...
dose-dependently increased the apoptotic population with highly FITC-labeled deoxyuridine triphosphates (FITC-dUTP) and PI stained cells.

The PARP cleavage was measured using anti-PARP and anti-cleaved PARP antibodies to confirm the apoptotic death of the MV4-11 cells. As shown in Fig. 4B, the treatment of LDD-1075 induced the PARP cleavage dose-dependently. In contrast, the PARP cleavage was not observed by the LDD-1076 treatment. These results suggest that the LDD-1075-induced growth inhibition is mediated by an apoptotic cell death.

Induction of cell cycle arrest by LDD-1075. The effect of LDD-1075 on the cell cycle was investigated. MV4-11 cells were treated with 0.01, 0.1, and 1 µM of the LDD-1075 compound along with the DMSO treatment as a control for 24 h. The LDD-1075 treatment induced a cell cycle arrest at the G1 phase dose-dependently (Fig. 5A). The cell population at the G1 phase increased from 61.6% (DMSO) to 85.4% (1 µM LDD-1075). Treatment of 10 µM LDD-1075 resulted in the increase of dead cell population extensively, which made it difficult to observe cell cycle change. Thus, results up to 1 µM LDD-1076 treatment was presented in Fig. 5A. Consistent with the G1 phase arrest, the cyclin D1 expression was reduced by the LDD-1075 treatment (Fig. 5B).

Discussion

Recognition of the antitumor effects of the indirubin derivatives goes back to the traditional Chinese medicine (TCM) used for the treatment of chronic myeloid leukemia (16). A science-based approach that can explain the anti-leukemic activity of the TCM led to the characterization of the active ingredient of the traditional Chinese preparation as indirubin (16,17). Indirubin was shown to be effective in the clinical trials of leukemia patients (18), and its mechanism of action was investigated. DNA and protein synthesis inhibition by indirubin was presented as a mechanism of the anti-tumor effects (19,20).
Later, indirubin was found to directly inhibit the activity of cyclin-dependent kinases (CDKs) and glycogen synthase kinase 3β (GSK3β) (21,22).

Many indirubin derivatives have been synthesized, to develop anti-cancer drugs based on this information (13,23-25). Depending on the substitutions of the indirubin derivatives, the compounds have been shown to inhibit kinases other than CDKs and GSK3β including, for example, the Jak family kinases (26), phosphorylase kinase (27), Aurora kinases (28), and dual-specificity tyrosine phosphorylation-regulated kinase (DYRK) (29). Our lab has been investigating indirubin derivatives as FLT3 inhibitors (13); thus, here, we report compounds with an improved FLT3 inhibitory activity.

Small molecules with the ester form are commonly used as prodrugs due to their better permeability through the membrane. Our observation in Fig. 1B and C led to a hypothesis that LDD-1075 may act like a prodrug converting to LDD-1076 inside the cells. LDD-1075, the ester form of the compound, exhibits a strong growth inhibition but a weak kinase inhibition in vitro. LDD-1076, the acid from of the compound, shows a strong kinase inhibition in vitro but almost no cytotoxicity. The bioconversion of LDD-1075 to LDD-1076 was confirmed by incubating LDD-1075 with the cell lysates (Fig. 2). This finding will be very useful when the LDD-1075 compound is developed as a prospective anti-cancer agent. LDD-1076 will be considered as the active form converted from LDD-1075.

In conclusion, this study presented the LDD-1075 compound as a potent anti-tumor agent with a mechanism for FLT3 activity inhibition by way of bioconversion to LDD-1076. These findings will extend our pharmacological understanding in the FLT3 research field.

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Availability of data and materials
The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Figure 5. Effect of LDD-1075 on cell cycle. (A) MV4-11 cells were treated with 0, 0.01, 0.1 and 1 µM of LDD-1075 for 24 h. Distribution of cell cycle was analyzed by propidium iodide staining followed by flow cytometry analysis. (B) Level of cyclin D1 expression following treatment of LDD-1075 or LDD-1076 was evaluated by western blotting. DMSO, dimethyl sulfoxide.
Authors' contributions

KBY, HJL, HJC, JL and JC conducted the experiments and analyzed the data. HJC, JDH, YCK and SYH contributed to the study design. SYH was a major contributor in writing the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

References


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