Discovery of an Indirubin Derivative as a Novel c-Met Kinase Inhibitor with *In Vitro* Anti-Tumor Effects

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Abstract

The c-Met protein is a receptor tyrosine kinase involved in cell growth, proliferation, survival, and angiogenesis of several human tumors. Overexpression of c-Met has been found in gastric cancers and correlated with a poor prognosis. Indirubin is the active component of Danggui Longhui Wan, which is a traditional Chinese antileukemic recipe. In the present study, we tested the anti-cancer effects of an indirubin derivative, LDD-1937, on human gastric cancer cells SNU-638. When we performed the *in vitro* kinase assay against the c-Met activity, LDD-1937 inhibited the activity of c-Met. This result was confirmed by immunoblot and immunofluorescence of phosphorylated c-Met. Immunoblot analysis showed that LDD-1937 decreased the expression of the Erk1/2, STAT3, STAT5, and Akt, downstream proteins of c-Met. In addition, LDD-1937 reduced the cell viability and suppressed colony formation and migration of SNU-638 cells. Furthermore, LDD-1937 induced G_2/M phase arrest in the SNU-638 cells by decreasing the expression levels of cyclin B1 and CDC2. Cleaved-PARP, an apoptosis-related protein, was up-regulated in cells treated with LDD-1937. Overall, this study suggests that LDD-1937 may be a novel small-molecule with therapeutic potential for selectively inhibiting c-Met and c-Met downstream pathways in human gastric cancers overexpressing c-Met.

Key Words: Gastric cancer, Indirubin, c-Met, LDD-1937

INTRODUCTION

Gastric cancer has been pointed out to be one of the leading causes of cancer death in the world. Although the worldwide incidence of gastric cancer has declined over the recent decades, it remains one of the most important malignant diseases (Chan and Wong, 2015). It is known that the incidence of stomach cancer varies in different parts of the world (Ervik *et al*, 2016). In South Korea, gastric tumors were the first cause of cancer death in year 2015 (Annual report of cancer statistics in Korea in 2015, 2017). Unless diagnosed in the early stage, stomach cancers are difficult to cure. Notwithstanding, researchers are attempting to develop drugs with different mechanisms of action to enhance patients' survival and to reduce mortality rates.

Indirubin is the major active component of the Chinese anti-chronic myelogenous leukemia (CML) traditional medicine recipe, Danggui Longhui Wan. Indirubin has been reported to inhibit DNA synthesis and cyclin-dependent kinases (Blazevic *et al*., 2015). Moreover, *in vitro* studies on indirubin derivatives revealed their inhibitory effects against protein kinases (Choi *et al*., 2010; Nam *et al*., 2012; Blazevic *et al*., 2015). Therefore, several indirubin derivatives were synthesized and tested as potential anti-neoplastic agents.

Currently, numerous anti-cancer agents have been developed and designed to target some specific molecular signaling pathways in cancer (Sawyers, 2004), and the receptor tyrosine kinases (RTKs) appear to be an important category of such therapeutic targets. Over 58 human RTKs have been discovered and described as having a key role in oncogenesis (Lemmon and Schlessinger, 2010). Among them, c-Met RTK has been an attractive cancer therapeutic target in recent years (You and McDonald, 2008; Zhu *et al*., 2014). The c-Met RTK and its natural ligand, hepatocyte growth factor (HGF), are involved in cell growth and proliferation, survival, angiogenesis, and metastatic progression. Upon stimulation

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by ligand binding, c-Met dimerizes and phosphorylates itself. Subsequently, multiple downstream effector pathways such as phosphoinositide-3-kinase (PI3K)/Akt, mitogen-activated protein kinase (RAS/MAPK), signal transducer and activator of transcription (STAT) are activated (Christensen *et al.*, 2005). Activation of these cascades leads to increased cell motility and invasion, angiogenesis, survival, growth and proliferation, and migration (Ma *et al.*, 2003).

The c-Met RTK overexpression and/or amplification have been detected in a number of solid tumors including gastric tumors which are correlated with a poor prognosis, depth of tumor invasion, and metastasis in patients with gastric malignancies (Rodrigues and Park, 1994). This finding provides evidence for the key role of c-Met in gastric cancers. Thus, targeting the c-Met signaling pathway has come into the spotlight and has great potential in target-based cancer therapy. Among the various strategies to inhibit c-Met, inhibition of the auto-phosphorylation of c-Met and phosphorylation of its downstream signal pathways have emerged as intriguing targets for cancer therapy (Rodrigues and Park, 1994; Liu *et al.*, 2010; Mughal *et al.*, 2013). To date, numerous anti-cancer agents that block the c-Met pathway have been or are being evaluated in clinical trials, and some of them are entering late-stage trials for cancer interventions (Kang *et al.*, 2014; Zhu *et al.*, 2014; Lee *et al.*, 2015a, 2015b). Although a number of these agents still require clinical validation, promising results have begun to emerge while the trials progress. The present study was carried out to identify and characterize an indirubin derivative, LDD-1937, as a c-Met kinase inhibitor.

MATERIALS AND METHODS

Cell culture

The human SNU-638 gastric cancer cell line was purchased from the Korean Cell Line Bank (Seoul, Korea) and grown as monolayers in RPMI-1640 medium (Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin (Hyclone, Logan, UT, USA) and maintained at 37°C in a humidified atmosphere at 5% CO_2.

In vitro kinase assay

The inhibitory activity of LDD-1937 against c-Met recombinant kinases was measured using the homogeneous time-resolved fluorescence (HTRF) assay. Concentrations of the enzyme, ATP, and substrate were optimized using an HTRF KinEASE kit following the manufacturer's instruction (Cisbio, Codolet, France). The reaction was initiated by the addition of ATP to a mixture containing c-Met enzyme, peptide substrates, and serially diluted inhibitor in a kinase reaction buffer (250 mM HEPES (pH 7.0), 100 mM ATP, 0.5 mM orthovanadate, 5 mM $MgCl_2$ 1 mM DTT, 0.05% BSA, 0.1% NaN_3). Detection reagents were added, and then, the TR-FRET signal was measured with a Victor multi-label reader (Perkin Elmer, Waltham, MA, USA). The curve was fitted by nonlinear regression, and the IC_{50} was calculated using GraphPad Prism 5.01 (GraphPad, La Jolla, CA, USA).

Immunofluorescence

Cells in the exponential phase were dispensed onto an 8-well culture slide at a density of 5,000 cells per well and treated with LDD-1937. After a 5 h incubation, cells were fixed with 4% formaldehyde solution and permeabilized with 0.5% Triton X-100 solution. Slides were blocked with 1% bovine serum albumin (BSA) in phosphate-buffered saline (0.1 M phosphate buffer, pH 7.2) containing 0.2% Tween-20 (PBST) and then incubated overnight in primary antibodies at 4°C. The following day, cells were washed with PBST, incubated with Alexa Fluor 546 labeled secondary antibody (Life Technologies, Grand Island, NY, USA) at room temperature for an hour and then washed with 1% BSA in PBST. A coverslip was then mounted on the slides with Prolong Gold Antifade reagent with DAPI (Life Technologies), and the fluorescence images of the cells were acquired with a fluorescent microscope Axiovert 200 (Carl Zeiss, Oberkochen, Germany).

Immunoblot analysis

Cells were lysed in SDS lysis buffer, and protein concentrations were measured using the SMART BCA Protein Assay kit (iNtRON Biotechnology, Seongnam, Korea). Samples were resolved on SDS-PAGE gels of 8, 10, or 15% and transferred to PVDF membranes (Millipore, Billerica, MA, USA) and then blocked with 5% nonfat milk and 5% BSA for 30 min at room temperature. Next, the membranes were incubated overnight with primary antibodies. The following day, the membranes were washed and then incubated with secondary antibodies for 30 min. at room temperature. Subsequently, the membranes were washed and immunoblots were detected using the ECL Select Western Blotting Detection Reagent (Amersham ECL select, GE Healthcare, Chicago, IL, USA). Antibodies against phosphorylated c-Met (Tyr1313), c-Met, Akt, phosphorylated Erk1/2, Erk1/2, STAT3, and STAT5 were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-phosphorylated Akt (Ser473), anti-phosphorylated STAT3 (Tyr705), anti-phosphorylated STAT5 (Tyr694), anti-cleaved PARP (Asp214) and anti-PARP were purchased from Cell Signaling Technology (Danver, MA, USA). An antibody against β-actin (Sigma-Aldrich) was used for the detection of β-actin as a loading control.

Cytotoxicity assay

Cell viability was assessed by a tetrazolium-based assay using the EZ-Cytox Cell Viability Assay kit (DaeilLab, Seoul, Korea). Cells were plated in 96-well plates (2,000 cells/well) and incubated with the LDD-1937 compound or dimethyl sulfoxide (DMSO) as a negative control. After 72 h of incubation, the viability of the SNU-638 cells was measured by the addition of 15 μL of EZ-Cytox reagent in each well. After incubation for 4 h, absorbance was measured using a Victor multi-label reader. The GI_{50} was calculated by nonlinear regression using Prism version 5.01 (GraphPad).

Anchorage-independent growth assay

The anchorage-independent growth was assessed by soft agar clonogenic assay. Cells were dispensed into a 6-well plate (40,000 cells/well), suspended in 1.5 mL of medium containing 10% fetal bovine serum and 0.3% Noble Agar (BD biosciences, San Jose, CA, USA), in the presence or absence of LDD-1937, and layered over a base prepared of medium, 10% fetal bovine serum, and 0.6% agarose. The plates were incubated (37°C, 5% CO_2), and live colonies were stained with 0.005% crystal violet solution, visualized under a microscope and photographed using the Bio-Imaging System (DNR Bio-
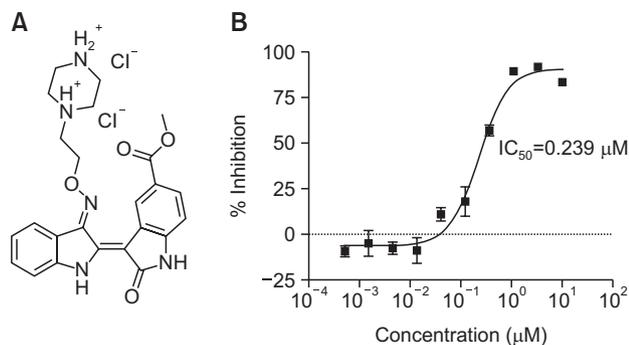
Fig. 1. Inhibition of the c-Met kinase activity by LDD-1937. (A) Chemical structure of LDD-1937. (B) The *in vitro* kinase assay using the HTRF® method was conducted, and the c-Met enzyme inhibition potency of the LDD-1937 compound was determined using recombinant human c-Met protein.

Imaging Systems Ltd, Neve Yamin, Israel).

Wound healing assay

Cells were grown and allowed to reach 90% confluency in 6 well-plates. Gaps 2 mm wide were introduced by scraping cells with a sterile 10 μL tip. The detached cells were removed, and cells were treated with varied concentrations of LDD-1937. After 24 h of incubation, the live cell images were obtained using a phase contrast microscope (Carl Zeiss).

Flow cytometric analysis

Cells were seeded in 6-well plates (20,000 cells/well) and then incubated overnight. After incubation, the cells were treated with different concentrations of LDD-1937 (0, 0.1, 1 or 10 μM) for 48 h. Then, the cells were detached, fixed, and treated with RNase A (50 μg/mL) and stained with propidium iodide (PI) (Sigma-Aldrich). Cell cycle distribution was analyzed by flow cytometry using the Accuri C6 (BD Biosciences). The percentage of cells in different phases of the cell cycle was determined with the Accuri C6 software (BD Biosciences).

RT-PCR

Total RNA was extracted using the ReliaPrep RNA cell miniprep system (Promega, Madison, WI, USA) and reverse transcribed using the cDNA reverse-transcription kit (Life Technologies). The cDNA was amplified with cyclin B1, CDC2, and EF1α primers, using polymerase chain reaction (PCR): 94°C for 2 min. followed by 30 cycles at 95°C for 30 s, 57°C for 30 s, and 74°C for 90 s. The PCR products were electrophoresed on a 1% agarose gel and stained with Redsafe (iNtRON Biotechnology). Primer sequences used and the conditions of these reactions were as follows: cyclin B1: sense 5'-AAG AGC TTT AAA CTT TGG TCT GGG-3'; antisense 5'-CTT TGT AAG TCC TTG ATT TAC CAT G-3', size 319 bp. CDC2: sense 5'-GGT TCC TAG TAC TGC AAT TCG-3'; antisense 5'-TTT GCC AGA AAT TCG TTT GG-3', size 709 bp. EF1α: sense 5'-AGG TGA TTA TCC TGA ACC ATC C-3'; antisense 5'-AAA GGT GGA TAG TCT GAG AAG C-3', size 234 bp.
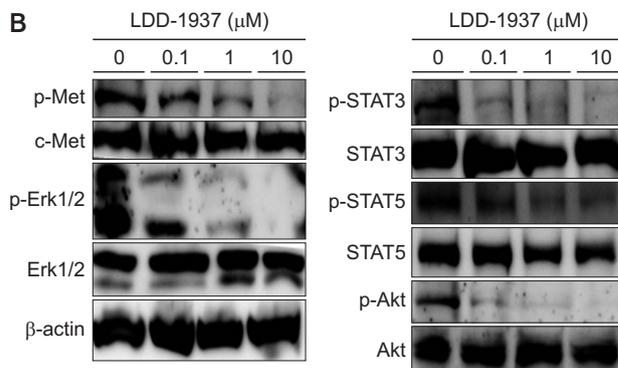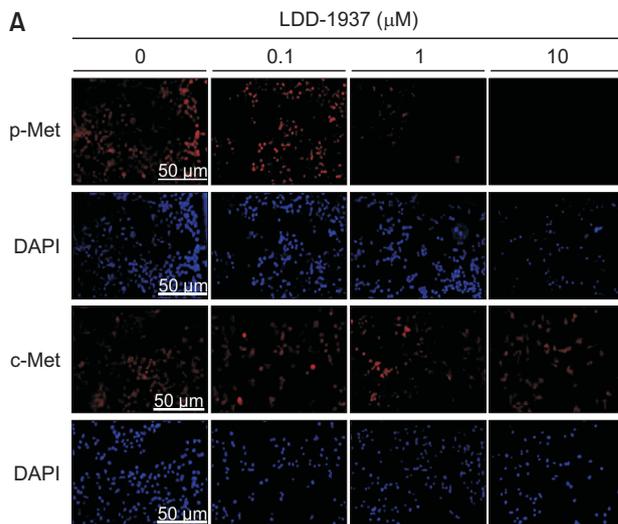
Fig. 2. Inhibitory effects of LDD-1937 on c-Met autophosphorylation and the signal transduction pathway in SNU-638 cells. (A) Cells treated with the indicated concentrations of LDD-1937 for 5 h were subjected to immunofluorescence staining using c-Met and phosphorylated c-Met antibodies (red) and DAPI (blue) (scale bars, 50 μm). Representative images from three independent experiments are shown. (B) Cells were incubated in the absence or presence of increasing concentrations of LDD-1937 for 5 h. Western blot using specific antibodies was performed as described. β-Actin western blot was used as a loading control.

RESULTS

LDD-1937 inhibits the kinase activity of c-Met *in vitro*

The indirubin derivative LDD-1937 compound (Fig. 1A) was reported as potent FLT3 inhibitor *in vitro* and *in vivo*, potentially useful for the treatment of acute myeloid leukemic patients (Lee *et al*., 2018). LDD-1937 was tested against the c-Met kinase activity using the purified recombinant c-Met protein as described in Materials and Methods. LDD-1937 potently inhibited the c-Met kinase activity in a concentration-dependent manner with an IC_{50} value of 0.239 μM (Fig. 1B).

LDD-1937 inhibits c-Met phosphorylation and downstream signaling pathways

The SNU-638 human gastric cancer cell line was used to assess the effect of LDD-1937 on c-Met phosphorylation and c-Met-dependent signaling pathways, including Erk1/2,

A

GI$_{50}$=2.25 μM

B

LDD-1937 (μM)

| 0 | 0.1 | 1 | 10 |

C

LDD-1937 (μM)

| 0 | 0.1 | 1 | 10 |
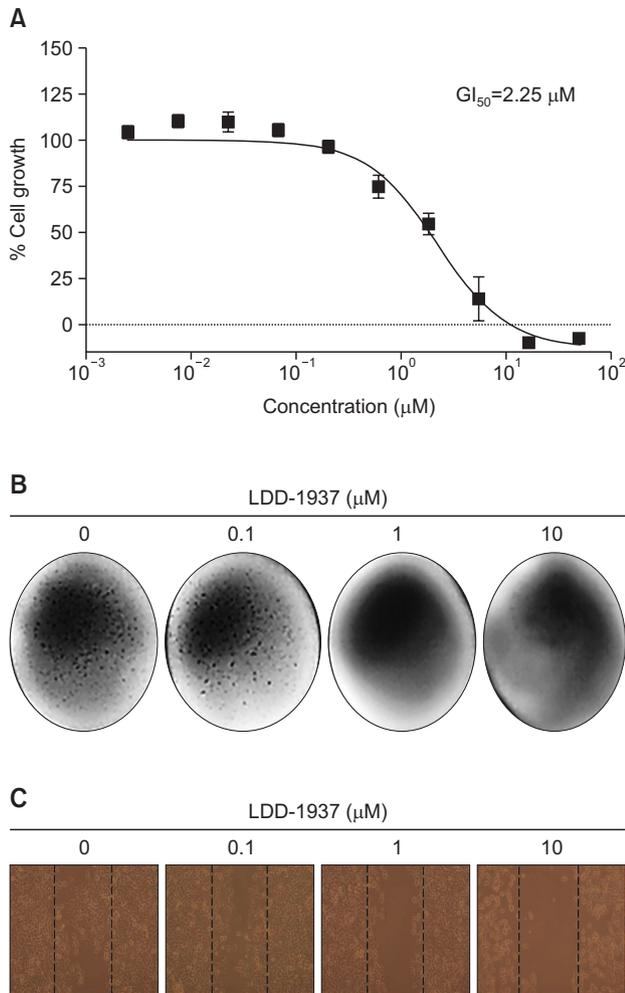
Fig. 3. Effects of LDD-1937 on SNU-638 cell growth. (A) Cells at a density of 2,000 cells/well were seeded into 96-well plates and treated with the LDD-1937 compound. After incubation for 72 h, the viability of the SNU-638 cells was measured using the EZ-Cytox Cell Viability Assay kit. The percentage of cell growth was evaluated with 0.5% DMSO treatment as a negative control. (B) Cells were grown in soft agar in the presence or absence of LDD-1937. After 4 weeks of incubation, live colonies were stained with 0.005% crystal violet solution and photographed under a microscope. (C) SNU-638 cells were seeded into 6-well plates and allowed to reach 90% confluency. A wound was made on the monolayer, and cells were then washed and treated with varied concentrations of LDD-1937 for 24 h. The migration of cells was observed under a microscope at 24 h post-incubation.

STAT3, STAT5, and Akt. The expression and phosphorylation of c-Met and its signaling proteins were evaluated by immunoblot analyses and immunofluorescence with antibodies listed in the Materials and Methods.

Immunofluorescence staining results are shown in Fig. 2A. When the SNU-638 cells were treated with the LDD-1937 compound at the indicated concentration, the number of phosphorylated c-Met decreased dose-dependently. Consistent with the immunofluorescence results, the immunoblot assay data showed that exposure of the SNU-638 cells to the LDD-1937 compound inhibited the auto-phosphorylation of c-Met in a dose-dependent manner, while the total expression level of

c-Met remained unchanged (Fig. 2B). To examine the downstream signaling pathway by which LDD-1937 blocked the cell proliferation, the cellular phosphorylation of Erk1/2, STAT3, STAT5 and Akt was checked because the inhibition of the c-Met activity may cause the suppression of downstream signaling pathways, which are important for cell survival and proliferation (Christensen *et al.*, 2005). Indeed, the data showed that phosphorylation of the signaling molecules was also inhibited by LDD-1937 in a dose-dependent manner (Fig. 2B).

LDD-1937 inhibits the growth of the SNU-638 cells

A cytotoxicity assay was undertaken to assess the effects of LDD-1937 on cell proliferation using SNU-638 cells. As shown in Fig. 3A, LDD-1937 exposure resulted in the inhibition of tumor cell growth with a GI$_{50}$ value of 2.25 μM.

To examine the ability of LDD-1937 to cause a modulation in SNU-638 gastric cancer cell growth in soft agar, an anchorage-independent growth assay was carried out. SNU-638 cells were grown in soft agar containing various concentrations of LDD-1937 for 4 weeks. The number of colonies of SNU-638 cells decreased after the treatment of LDD-1937 at 1 and 10 μM concentration (Fig. 3B). These results show the inhibition of the anchorage-independent growth of the SNU-638 cells by LDD-1937.

Cell migration was assessed by the wound healing assay under treatments of increasing concentrations of LDD-1937. A wound 2 mm wide was made in the monolayer, and the cells were treated with LDD-1937. Migration of the SNU-638 cells was checked after 24 h, revealing that the exposure to LDD-1937 interrupted the migration of the SNU-638 cells in a dose-dependent manner when compared with the untreated cells, in which the cells moved from the edge of the wound towards the center of the wound (Fig. 3C). Overall, these results correlated well with the inhibition of the c-Met activity.

LDD-1937 inhibits cell proliferation through G$_2$/M arrest

The effect of LDD-1937 on the SNU-638 cell cycle progression was examined to understand its anticancer mechanism. As shown in Fig. 4A, exposure to LDD-1937 increased the cell population in the G$_2$/M phase of the cell cycle, indicating cell cycle arrest in the G$_2$/M phase. The G$_2$/M population of the SNU-638 cells was increased from 22.7% in the control group to 45.6% in the 10 μM LDD-1937 treatment group (Fig. 4A).

The activation of the cyclin B1/CDC2 complex is essential for the G$_2$/M transition and regulates the entry into mitosis during the cell cycle (Chen *et al.*, 2008). Thus, the expression level of cyclin B1 and CDC2 mRNA was investigated to confirm the effect of LDD-1937 on the cell cycle through c-Met inhibition. The SNU-638 cells were treated with LDD-1937 for 24 h and subjected to RT-PCR. LDD-1937 decreased the mRNA levels of cyclin B1 and CDC2 dose-dependently (Fig. 4B). The results showed that the reduced c-Met activity by LDD-1937 results in decreased cyclin B1 and CDC2 expression, followed by cell cycle arrest at the G$_2$/M phase. Inhibition of cell proliferation can also result from apoptotic cell death. The apoptotic cell death of the SNU-638 cells by LDD-1937 was confirmed by PARP cleavage. As shown in Fig. 4C, an increased level of cleaved PARP was observed after the LDD-1937 treatment, while the level of PARP remained unchanged.

A

LDD-1937 (µM)

Phase	% Gated
G$_1$	59.9
S	15.8
G$_2$/M	21.6

Phase	% Gated
G$_1$	67.0
S	11.0
G$_2$/M	21.6

Phase	% Gated
G$_1$	69.4
S	6.80
G$_2$/M	23.9

Phase	% Gated
G$_1$	47.5
S	6.40
G$_2$/M	45.6

B

LDD-1937 (µM)

Cyclin B1
CDC2
EF1α

C

LDD-1937 (µM)

PARP
Cleaved PARP
β-actin
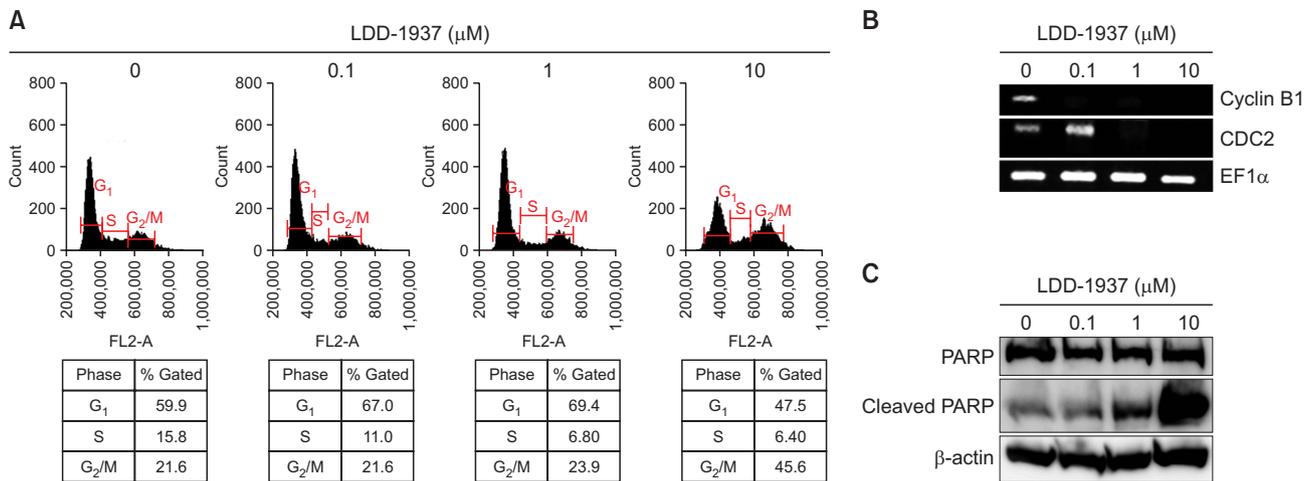
Fig. 4. Effects of LDD-1937 on SNU-638 cell cycle and apoptosis. (A) SNU-638 cells were treated with the indicated concentration of LDD-1937 for 48 h. After staining with PI, the DNA content was measured by FACS analysis. (B) Cells were treated with LDD-1937 for 24 h at the indicated concentration, and total RNA extracted from the cells was subjected to RT-PCR. The cyclin B1 and CDC2 mRNA expression level was measured using specific primers. EF1α mRNA level was used as loading control. (C) SNU-638 cells were treated with various concentrations of LDD-1937 for 24 h and subjected to immunoblot analysis for measuring PARP and cleaved PARP levels.

DISCUSSION

Over the past decade, target-based cancer therapy has emerged as a promising approach for the management of cancer that may enhance the survival of cancer patients. There are approximately 40 FDA-approved small molecule inhibitors targeting kinases (Wilson *et al*., 2018). The c-Met RTK, as a target, appears to hold promise for future therapeutic applications.

To date, natural indirubin as well as its derivatives has widely been studied for molecular targeted therapy of cancer (Blazevic *et al*., 2015). Indirubin toxicity was reported to be low with mild side effects in the treatment of chronic myeloid leukemia, and researchers were stimulated by these facts to explore the use of indirubin and its derivatives also in other types of cancers.

In this study, we demonstrated that LDD-1937, an indirubin derivative, possessed a prominent inhibitory activity against c-Met kinase and its downstream signaling pathways, resulting in the inhibition of cell proliferation, migration, and induction of the G$_2$/M phase arrest of the cell cycle in the human gastric cancer cell SNU-638. We first tested the inhibitory effect of LDD-1937 against c-Met kinase (Fig. 1B). Furthermore, the anti-cancer effect of this compound was characterized. To evaluate the ability of the inhibition of c-Met by LDD-1937, the auto-phosphorylation of c-Met and phosphorylation of its downstream signaling pathways in SNU-638 cancer cells were investigated. We found that LDD-1937 effectively inhibited c-Met phosphorylation in gastric cancer cells (Fig. 2). It is well established that activation and phosphorylation of c-Met involve numerous downstream targets (Rodrigues and Park, 1994). Based on our results, when we evaluated several potential targets of LDD-1937, we found that phosphorylation of Akt, Erk1/2, STAT3, and STAT5 is involved. Overall, through the inhibition of the c-Met kinase activity, LDD-1937 exhibited the ability to block effectively c-Met-related signaling cascades (Fig. 2B).

There have been several clinical trials of c-Met pathway

modulators for gastric cancer patients. Rilotumumab, onartuzumab and ABT-700 are antibody-based therapeutics targeting HGF or c-Met receptor. Foretinib, tivantinib, and AMG-337 are small molecule c-Met inhibitors that have been underwent gastric cancer clinical studies. Clinical testing results for the c-Met modulators are disappointing in terms of efficacy (Bradley *et al*., 2017). However, subgroup of patients with c-Met amplification exhibited improvements in clinical trials of ABT-700 and AMG-337. From these results, patient selection with c-Met overerepression appears to be important in evaluation of clinical efficacy.

It has been reported that the deregulated HGF/c-Met signaling pathway has an important role in angiogenesis, tumor growth, proliferation, invasion, and metastasis (Ma *et al*., 2003). On the other hand, several studies have shown that downregulation of the c-Met activity leads to the inhibition of cell proliferation and invasion as well as the induction of apoptosis (Puri *et al*., 2007; Que and Chen, 2011). Similarly, when LDD-1937 was applied to the SNU-638 cells, it inhibited growth, proliferation and migration of the SNU-638 cells (Fig. 3).

Treatments with LDD-1937 appeared to induce cell cycle arrest at the G$_2$/M phase as shown by the entrapment of cells in the G$_2$/M phase, indicating a delay into the entry of mitosis which leads to the retardation of cell division (Fig. 4A). Moreover, LDD-1937 inhibited the expression of cyclin B1 and CDC2 mRNA levels (Fig. 4B). The cyclin B/CDC2 complex is activated during the late G$_2$ phase of the cell cycle, thereby triggering the initiation of mitosis (Smits and Medema, 2001). These results were consistent with the previously reported inhibition of CDKs and arrest of the G$_2$/M phase of the cell cycle by indirubin and its derivatives (Hoessel *et al*., 1999; Moon *et al*., 2006; Blazevic *et al*., 2015).

Taken together, these observations have provided evidence that LDD-1937 possesses significant *in vitro* anti-cancer activity in gastric tumors. LDD-1937 inhibits cancer cell growth, proliferation, migration and cell cycle at G$_2$/M phase and induces apoptosis. Although additional studies are yet needed,

LDD-1937 may be a potential anti-tumor agent targeting the c-Met pathway in gastric cancers expressing c-Met.

CONFLICT OF INTEREST

The authors declare no conflict of interests.

ACKNOWLEDGMENTS

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